MOLECULAR AND CELLULAR BIOLOGY, Oct. 1995, p. 5740–5749 Vol. 15, No. 10
0270-7306/95/$04.00+0


# Yeast *RLM1* Encodes a Serum Response Factor-Like Protein That May Function Downstream of the Mpk1 (Slt2) Mitogen-Activated Protein Kinase Pathway

YASUYUKI WATANABE, KENJI IRIE,* AND KUNIHIRO MATSUMOTO

*Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan*

Received 26 May 1995/Returned for modification 27 June 1995/Accepted 17 July 1995

**The *MPK1* (*SLT2*) gene of *Saccharomyces cerevisiae* encodes a mitogen-activated protein kinase that is regulated by a kinase cascade whose known elements are Pkc1 (a homolog of protein kinase C), Bck1 (Slk1) (a homolog of MEK kinase), and the functionally redundant Mpk1 activators Mkk1 and Mkk2 (homologs of MEK). An activated mutation of *MKK1*, *MKK1$^{P386}$*, inhibits growth when overexpressed. This growth-inhibitory effect was suppressed by the *mpk1*Δ mutation, suggesting that hyperactivation of the Mpk1 pathway is toxic to cells. To search for genes that interact with the Mpk1 pathway, we isolated both chromosomal mutations and dosage suppressor genes that ameliorate the growth-inhibitory effect of overexpressed Mkk1$^{P386}$. One of the genes identified by the analysis of chromosomal mutations is *RLM1* (resistance to lethality of *MKK1$^{P386}$* overexpression), which encodes a protein homologous to a conserved domain of the MADS (Mcm1, Agamous, Deficiens, and serum response factor) box family of transcription factors. Although *rlm1*Δ cells grow normally at any temperature, they display a caffeine-sensitive phenotype similar to that observed in mutants defective in *BCK1*, *MKK1*/*MKK2*, or *MPK1*. A gene fusion that provides Rlm1 with a transcriptional activation domain of Gal4 suppresses *bck1*Δ and *mpk1*Δ. A screening for dosage suppressors yielded the *MSG5* genes, which encode a dual-specificity protein phosphatase. Our results suggest that Rlm1 functions as a transcription factor downstream of Mpk1 that is subject to activation by the Mpk1 mitogen-activated protein kinase pathway.**

Extracellular molecules that regulate cell proliferation and differentiation in eukaryotes depend on pathways that detect signals at the cell surface and transmit them through the cytoplasm to the nucleus, where transcription factors that are among the ultimate targets of such signaling pathways elicit alterations in gene expression that in turn regulate cellular events. One mechanism for transmitting these signals involves a protein phosphorylation cascade leading to activation of mitogen-activated protein kinases (MAPKs) or extracellular signal-regulated kinases (ERKs) (4, 35, 36). Thus, these enzymes are thought to function as intermediaries between membrane-associated signaling molecules and the nucleus. MAPKs/ERKs are activated by tyrosine and threonine phosphorylation; both phosphorylation events are catalyzed by the dual-specificity protein kinase called MEK (MAPK kinase or ERK kinase). MEK itself is phosphorylated and activated by at least three upstream kinases, Raf, MEK kinase, and Mos (35). Each of these upstream components also functions in multiple cell signaling processes.

Elements of the MAPK activation pathways appear to have been conserved across evolution, as the kinases that comprise MAPK pathways have been identified in organisms ranging from mammals to *Saccharomyces cerevisiae* (3, 21). In *S. cerevisiae*, there is presently evidence for at least four separate but structurally related MAPK activation pathways that mediate distinct responses to different extracellular or cell autonomous signals. First, the Mpk1 (Slt2) pathway is required for the control of cell wall construction. Second, the Fus3/Kss1 pathway is activated by exposure of haploid yeast cells to mating pheromones. Third, the Hog1 pathway is activated by exposure of yeast cells to hyperosmolar environments and leads to increased glycerol biosynthesis. Fourth, the Smk1 pathway has been shown to be required for control of sporulation.

Recently, extensive effort has been devoted to identifying the downstream targets of MAPKs. Among the direct targets of MAPKs that have been identified in mammalian systems are several transcription factors, including c-Fos, NF-IL-6, c-Jun, c-Myc, and p62$^{TCF}$ (1, 7, 23, 40, 48, 55). p62$^{TCF}$ is a ternary complex factor (TCF) and is recruited to the c-*fos* serum response element by interaction with the serum response factor (SRF) (59, 60). Recent findings indicate that phosphorylation of TCF occurs in response to activation of the MAPK pathway and that regulation of TCF activity is an important mechanism by which the serum response element responds to growth factor signals (24, 27, 28). However, although progress in identifying possible targets of MAPKs has been made, much remains to be learned about the downstream events caused by MAPK activation in vivo.

*S. cerevisiae* is an attractive organism in which to identify and characterize MAPK targets because it is readily manipulated by genetic techniques. One of the MAPK pathways in *S. cerevisiae* the Mpk1 signaling cascade, is mediated by the yeast protein kinase C homolog, Pkc1, and consists of Bck1 (Slk1) and Mkk1/Mkk2, which are proposed to catalyze a protein phosphorylation cascade culminating in the activation of the Mpk1 MAPK homolog (9, 11, 26, 30, 31, 37, 38). The upstream activators of Mpk1 are the functionally redundant kinases Mkk1 and Mkk2, which share high sequence identity with MEK (26). Furthermore, the Bck1 kinase, which has been

* Corresponding author. Mailing address: Department of Molecular Biology, Faculty of Science, Nagoya University, Chikusa-ku, Nagoya 464-01, Japan. Phone: 81-52-789-3000. Fax: 81-52-789-3001. Electronic mail address: g44177a@nucc.cc.nagoya-u.ac.jp.

demonstrated to function upstream of Mkk1/Mkk2, is closely related MEK kinase. Finally, a dominant allele of *BCK1* can suppress *pkc1*Δ disruption, suggesting that Pkc1 is an upstream regulator of Bck1 function in this pathway, similar to protein kinase C regulation of MAPK pathways in other eukaryotes (31). Thus, genetic approaches identified these components and established that Bck1, Mkk1/Mkk2, and Mpk1 kinases act in that order in the Pkc1-mediated pathway (26, 30). This pathway is essential for integrity of the cell wall and also participates in nutrient sensing, growth control, and actin organization. The signal for activation of Pkc1 is unknown, and targets of Mpk1 have not yet been identified. Identification of such targets would contribute to an understanding of how the Pkc1-Mpk1 pathway directs downstream events. Mpk1 is suspected to phosphorylate a variety of substrates, including transcription factors, in a manner analogous to the Fus3/Kss1-dependent activation of Ste12 (17).

Previous attempts to identify downstream effectors of the Mpk1 pathway have exploited gene dosage (high-copy-number) suppressor analyses. Lee et al. (29) have identified the *PPZ1*, *PPZ2*, and *BCK2* genes as multicopy suppressors of *mpk1*Δ deletion mutants. *PPZ1* and *PPZ2* encode a pair of type 1-related protein phosphatases, and *BCK2* is a gene of unknown function (29, 47). Genetic and phenotypic analysis suggests that they do not function as components downstream of the Mpk1 pathway but rather appear to coordinately regulate components that function downstream of Mpk1. Similarly, Costigan et al. (10) have identified the *NHP6A* and *NHP6B* genes, which exhibit significant homology to each other, as suppressors of defects in the Mpk1 pathway. They encode proteins with DNA-binding domains similar to those of mammalian high-mobility-group proteins (10). Although Nhp6A and Nhp6B proteins do not appear to be direct targets of Mpk1 kinase, they are candidates for downstream components of Mpk1.

We have recently identified a hyperactive mutation of *MKK1*, *MKK1*$^{P386}$, resulting from the change of serine to proline at position 386 (65). This mutation site lies between protein kinase subdomains VII and VIII close to the putative phosphate acceptor residue(s), which is thought to be phosphorylated by Bck1. The *MKK1*$^{P386}$ mutation is able to suppress both the *pkc1*Δ and *bck1*Δ deletion, indicating that the mutationally activated Mkk1 kinase transmits a signal even in the absence of Pkc1 and Bck1 upstream kinases. On the other hand, the *MKK1*$^{P386}$ mutation fails to suppress defects associated with *mpk1*Δ mutations, consistent with a model in which Mkk1 acts upstream of Mpk1. In this study, we found that *MKK1*$^{P386}$ inhibited growth when overexpressed by fusion to the strong *GAL1* promoter. This growth-inhibitory effect was suppressed by the *mpk1*Δ deletion, indicating that hyperactivation of the Mpk1 pathway is toxic to yeast cells. We took advantage of this system to identify downstream components of the Mpk1 MAPK pathway. We isolated yeast strains harboring mutations in the *RLM1* gene that were resistant to the growth inhibition caused by overexpression of *MKK1*$^{P386}$. The *RLM1* gene product belongs to the MADS (MCM1, Agamous, Deficiens, and SRF) family of transcription factors. The molecular and genetic analyses presented here suggest that Rlm1 functions downstream of Mpk1.

## MATERIALS AND METHODS

**Strains, media, and general methods.** *Escherichia coli* DH5α was used for DNA manipulation (20). The yeast strains used in this study are listed in Table 1. Standard procedure for yeast manipulations (50) were followed. The media used include rich medium (YEPD), synthetic complete medium (SC), synthetic minimal medium (SD), and sporulation medium (50). SC lacking amino acids or other nutrients (e.g., SC-Ura lacks uracil) was used to score auxotrophies and to select transformants. YEPG and SG were identical to YEPD and SC, respectively, except that they contained 2% galactose instead of 2% glucose. YEPR was identical to YEPD except that it contained 2% raffinose instead of 2% glucose. Yeast cells were transformed by the lithium acetate method, using single-stranded DNA as the carrier (19). Other recombinant DNA procedures were carried out as described by Sambrook et al. (52).

TABLE 1. Strains used

| Strain | Genotype |
|---|---|
| 15Du | *MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his2/his2 ade1/ade1* |
| 15Dau | *MAT***a** *ura3 leu2 trp1 his2 ade1* |
| 15Dαu | *MAT*α *ura3 leu2 trp1 his2 ade1* |
| 1783 | *MAT***a** *ura3 leu2 trp1 his4 can1* |
| 1788 | *MAT***a**/*MAT*α *ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1* |
| DL251 | *MAT*/*MAT*α *bck1*Δ::*URA3*/*bck1*Δ::*URA3 ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1* |
| DL456 | *MAT***a**/*MAT*α *mpk1*Δ::*TRP1*/*mpk1*Δ::*TRP1 ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1* |
| DL456-3B | *MAT***a** *mpk1*Δ::*TRP1 ura3 leu2 trp1 his4 can1* |
| GMI1783 | *MAT***a** *GAL1p-MKK1*$^{P386}$::*TRP1 ura3 leu2 trp1 his4 can1* |
| GMR14 | *MAT***a** *rlm1-1 rlt1-1 ura3 leu2 trp1 his4 can1* |
| GMR52 | *MAT***a** *rlm1-2 rlt1-2 ura3 leu2 trp1 his4 can1* |
| GMY61-1A | *MAT*α *rlm1-1 rlt1-1 ura3 leu2 trp1* |
| GMY62-3C | *MAT***a** *rlm1-2 rlt1-2 ura3 leu2 trp1 his2 his4* |
| GMY63 | *MAT***a**/*MAT*α *RLM1*/*rlm1*Δ::*LEU2 ura3/ura3 leu2/leu2 trp1/trp1 his4/his4 can1/can1* |
| GMY63-5B | *MAT***a** *ura3 leu2 trp1 his4 can1* |
| GMY63-5C | *MAT*α *rlm1*Δ::*LEU2 ura3 leu2 trp1 his4 can1* |
| GMY63-5D | *MAT***a** *rlm1*Δ::*LEU2 ura3 leu2 trp1 his4 can1* |
| GMY64 | *MAT***a**/*MAT*α *RSP5*/*rsp5*Δ::*URA3 ura3/ura3 leu2/leu2 trp1/trp1 his2/his2 ade1/ade1* |
| GMY67-5A | *MAT*α *rlm1*Δ::*LEU2 rlt1-1 ura3 leu2 trp1 his4 can1* |
| GMY68-3A | *MAT***a** *rlm1*Δ::*LEU2 rsp5*Δ::*URA3 ura3 leu2 trp1 his2 ade1* |
| GMY70-10B | *MAT***a** *rlm1*Δ::*TRP1 rlt1-1 ura3 leu2 trp1 his4 ade1* |
| GMY72-1B | *MAT*α *rlm1*Δ::*LEU2 ssd1*Δ::*URA3 ura3 leu2 trp1 his3* |
| KA31-2A-2 | *MAT***a** *ssd1*Δ::*URA3 ura3 leu2 trp1 his3* |
| L40 | *MAT***a** *leu2 trp1 his3 LYS2*::*lexA-HIS3 URA3*::*lexA-lacZ* |
| W303-1A | *MAT***a** *ura3 leu2 trp1 his3 ade2 can1* |

**Plasmids.** Plasmids pYS91 and pYS91W are YEp-based *URA3* and *TRP1* plasmids, respectively, containing the *ACT1* gene under the control of *GAL1* promoter (57). Plasmid YCpMPK1 is YCplac33 carrying the 2.3-kb *Eco*RI-*Sal*I fragment of the *MPK1* gene (30). Plasmids pYW52-3 and pYW52-4 were constructed by inserting the 4.9-kb *Sal*I-*Sph*I fragment of the *RSP5* gene into YCplac33 and YCplac22, respectively. Plasmids pYW14-6, YEp181-RLM1, and YEp195-RLM1 were constructed by inserting the 3.5-kb *Pst*I-*Sna*BI fragment of the *RLM1* gene into YCplac33, YEp181, and YEp195, respectively. The N-terminal portion of the *RLM1* coding sequence was amplified by PCR using a 5′ primer (5′-AAAAGATCTTTATGGGTAGACGGAAGATTGAAAATCC-3′) incorporating a *Bgl*II site and a 3′ primer (5′-AAAGGATCCGGTTTCAATC TTT-3′). A 590-bp *Bgl*II-*Bam*HI fragment generated by PCR and a 2.0-kbp *Bam*HI-*Pst*I fragment containing the C-terminal portion of *RLM1* were inserted into the *Bam*HI-*Pst*I gap of YCpG33 (13) and the *Bam*HI-*Sal*I gap of pACTII (32) to generate YCpG33-RLM1 and pACT-RLM1, respectively. In pACT-RLM1, the transcriptional activation domain of Gal4 is fused at the $NH_2$ terminus of Rlm1. The *MKK1* and *MKK1*$^{P386}$ coding sequences were amplified by PCR using a 5′ primer (5′-TTCTCGAGTATGGCTTCACTGTTCAGAC-3′) incorporating an *Xho*I site and a 3′ primer (5′-CCTCTAGATTAATCTTTCCAGC ACTT-3′) incorporating an *Xba*I site. PCR amplification generated 1.5-kb fragments that were digested with *Xho*I and *Xba*I and inserted into the *Xho*I-*Xba*I gap of pNV7s (41) to generate pNV7-MKK1 and pNV7-MKK1$^{P386}$. The 3.0-kb *Sal*I-*Eco*RI fragment containing the *GAL1p-MKK1*$^{P386}$ construct of pNV7-MKK1$^{P386}$ was cloned into the *Sal*I-*Eco*RI gap of YEplac112 and YIplac204 to generate pNV7W-MKK1$^{P386}$ and YIpGAL-MKK1$^{P386}$, respectively. A *TRP1*-based integration plasmid, YIpGAL-MKK1$^{P386}$, was used for its integration by homologous recombination at the *trp1* locus in wild-type strain 1783.

**Isolation of *rlm* mutations.** To isolate mutations that suppress the toxicity of *MKK1*$^{P386}$ overexpression and simultaneously confer sorbitol-dependent growth

at a higher temperature (38°C), cells of wild-type strain 1783 carrying pNV7-MKK1$^{P386}$ were streaked out on SG-Ura plates containing 1 M sorbitol and incubated at 25°C. A total of 25 colonies grew in the presence of galactose, and these candidates were screened for temperature sensitivity ($Ts^-$) by replica plating to YEPD and YEPD containing 1 M sorbitol then incubation at 38°C. Nine of them did not grow at 38°C on YEPD alone but grew on YEPD containing 1 M sorbitol. These nine mutants were then each transformed with YCpMPK1 to test whether the $Ts^-$ phenotype was rescued by the *MPK1* gene.

**Cloning of *RLM1* and *RSP5*.** Strains GMY61-1A and GMY62-3C were transformed with a YCp50 yeast genomic library (49) at 25°C and then replica plated to YEPD medium at 38°C. After 2 days of incubation, two transformant clones gave confluent growth. Two plasmids (pYW14 and pYW52) that rescue the $Ts^-$ phenotype were recovered from *S. cerevisiae* by transforming *E. coli*. They contained different genomic DNA inserts, as determined by restriction site mapping (see Fig. 4). The nucleotide sequences of the 4.4-kb *Pst*I-*Hin*dIII region of pYW14 and the 1.0-kb *Bam*HI fragment of pYW52 were determined for both strands by the dideoxy-chain termination technique (53).

**Construction of yeast strains with *RLM1* or *RSP5* deletions.** Deletion alleles of *RLM1* and *RSP5* were constructed by the one-step gene replacement method (see Fig. 4) (51). The 2.1-kb *Acc*I-*Sna*BI fragment of *RLM1* was replaced with the 1.6-kb *Sma*I-*Sal*I fragment of *LEU2* or the 0.9-kb *Sal*I-*Sma*I fragment of *TRP1*, and the 1.0-kb *Bam*HI fragment of *RSP5* was replaced with the 1.1-kb *Bam*HI fragment of *URA3*, after appropriate conversion of restriction sites. These constructions were made separately in the vector pBluescript SK+ carrying the 5.8-kb *Pst*I fragment of *RLM1* or the 4.9-kb *Sal*I-*Sph*I fragment of *RSP5*, respectively. The DNAs containing the entire *rlm1*Δ::*LEU2*, *rlm1*Δ::*TRP1*, and *rsp5*Δ::*URA3* constructions were used to transform diploid strains 1788 and 15Du by selection for $Leu^+$, $Trp^+$, or $Ura^+$. Restriction mapping and Southern analysis of genomic DNAs from the resulting transformants were conducted to confirm that transplacement had occurred at each locus. In the case of *rlm1*Δ::*LEU2*, we confirmed that the caffeine-sensitive phenotype caused by the *rlm1*Δ::*LEU2* mutation was suppressed by YCpG33-RLM1 in a galactose-dependent manner.

**Assessment of cell lysis.** Qualitative assessment of lysis of the cells in colonies was done by an alkaline phosphatase assay as described by Cabib and Duran (5). Cells were spotted onto YEPD agar plates, incubated at 25°C, and then shifted overnight to 38°C. The plates were then overlaid with an alkaline phosphatase assay solution containing 0.05 M glycine hydrochloride (pH 9.5), 1% agar, and 10 mM chromogenic substrate 5-bromo-4-chloro-3-indolylphosphate. Colonies which contained lysed cells stained blue, whereas intact colonies remained white.

**RNA (Northern) blot analysis.** RNAs were prepared from exponentially growing wild-type (GMY63-5B) and *rlm1*Δ::*LEU2* (GMY63-5D) cells, separated in an agarose gel containing formaldehyde, and blotted as described previously (13). RNA samples were split into two aliquots and probed for *MPK1* and *ACT1*. RNA was quantitated by densitometric analysis. *MPK1* RNA levels were normalized by determining the ratio of *MPK1* RNA to *ACT1* RNA.

**Two-hybrid and β-galactosidase assays.** To create an in-frame protein fusion between LexA and Rlm1, the 2.6-kb *Bgl*II-*Pst*I fragment of pACT-RLM1 containing the full-length coding sequence was cloned into the *Bam*HI-*Sal*I gap of pBTM116 (62) to generate pYW61. The LexA-Rlm1 fusion protein strongly activated transcription of *HIS3* and *lacZ* reporter genes containing LexA operators in the reporter strain L40 (62). Then, plasmid pYW62 was constructed by removing the 1-kb *Cla*I-*Cla*I fragment from pYW61. pYW62 expresses LexA-Rlm1ΔC, a LexA-Rlm1 fusion protein lacking the COOH-terminal 151 amino acids. The LexA-Rlm1ΔC fusion protein moderately activated expression of reporter genes, but histidine auxotrophy of transformants with pYW62 could be obtained by growing cells in the presence of 100 mM 3-aminotriazole, a chemical inhibitor of the *HIS3* gene product, imidazole glycerol phosphate dehydrogenase. pB331 (kindly provided by M. Snyder) (10) expresses a fusion protein between an activation domain of Gal4 and the full-length coding sequence of the *SLT2*/*MPK1* gene. Two-hybrid and β-galactosidase assays were performed as described previously (13, 18, 62).

**Nucleotide sequence accession number.** The GenBank/EMBL accession number for *RLM1* is D63340.

## RESULTS

**Effect of *MKK1$^{P386}$* overexpression.** A gain-of-function mutation of *MKK1*, *MKK1$^{P386}$*, was previously identified by a screen for spontaneous *MKK1* mutations that suppress the cell lysis phenotype associated with loss of *PKC1* function (65). This mutation also suppresses the *bck1*Δ mutation, indicating that *MKK1$^{P386}$* constitutively activates the pathway even in the absence of upstream components. We constructed a plasmid, pNV7-MKK1$^{P386}$, containing *GAL1p-MKK1$^{P386}$*, in which the expression of *MKK1$^{P386}$* is under the control of the strong *GAL1* promoter. Thus, overexpression of *MKK1$^{P386}$* can be achieved by inducing *GAL1p-MKK1$^{P386}$* with the addition of galactose. When this plasmid was introduced into a variety of strains, including 15Dau, 1783, and W303-1A, cell growth was strongly inhibited in the presence of galactose (Fig. 1A), indicating that induced overproduction of the *MKK1$^{P386}$* gene was toxic. This growth-inhibitory effect was more severe when cells were grown at a higher temperature or in the presence of sorbitol.

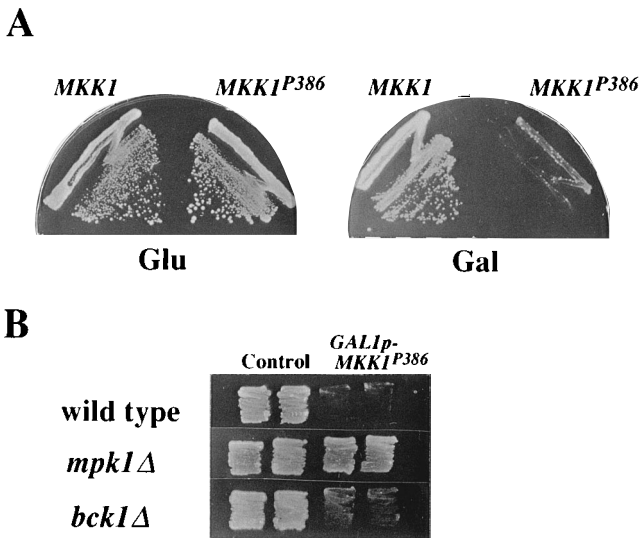


FIG. 1. Effect of overexpression of *MKK1$^{P386}$* on cellular growth. (A) Wild-type cells (strain 1788) carrying pNV7-MKK1 (*MKK1*) or pNV7-MKK1$^{P386}$ (*MKK1$^{P386}$*) were streaked onto SD-Ura (Glu) or SG-Ura containing 2% galactose (Gal). Plates were incubated for 3 days at 30°C. (B) Wild-type cells and *mpk1*Δ cells transformed with pNV7s (Control) or pNV7-MKK1$^{P386}$ (*GAL1p-MKK1$^{P386}$*) were patched onto SG-Ura supplemented with 1 M sorbitol. *bck1*Δ cells transformed with YCplac22 (Control) or pNV7W-MKK1$^{P386}$ (*GAL1p-MKK1$^{P386}$*) were patched onto SG-Trp supplemented with 1 M sorbitol. Plates were incubated for 3 days at 35°C. Each patch represents an independent transformant. Strains: wild type, 1788; *mpk1*Δ, DL456; *bck1*Δ, DL251.

If a growth-inhibitory effect of the overexpressed *MKK1$^{P386}$* gene by the inducible *GAL1* promoter is caused by hyperactivation of the Mpk1 pathway, this toxicity might be rescued by mutations in *MPK1* but not in *BCK1*. To examine this possibility, we tested the effects of mutations in *MPK1* and *BCK1*. Deletion of *MPK1* or *BCK1* results in failure to form colonies at an elevated temperature, a phenotype that can be suppressed by the addition of 1 M sorbitol to the medium. Plasmids pNV7-MKK1$^{P386}$ and pNV7W-MKK1$^{P386}$ were transformed into *mpk1*Δ (DL456) and *bck1*Δ (DL251) strains, respectively, and transformants were tested for growth on galactose medium containing 1 M sorbitol to allow *mpk1* and *bck1* mutants to grow. The *mpk1*Δ mutation relieved toxicity, whereas the *bck1*Δ mutation did not (Fig. 1B). These results indicate that inhibition of cell growth by *MKK1$^{P386}$* overexpression requires Mpk1 activity. In other words, hyperactivation of the Mpk1 pathway is toxic to yeast cells. The *bck1*Δ mutant allowed a greater degree of residual growth upon *MKK1$^{P386}$* overexpression than did the wild-type strain (Fig. 1B). One explanation for this result is that the level of Mkk1$^{P386}$ activity may be lower in *bck1*Δ strains than in *BCK1$^+$* strains.

To examine the effects of *MKK1$^{P386}$* overexpression on growth, we constructed strain GMI1783, which carries a *GAL1p-MKK1$^{P386}$* construct integrated at the *trp1* locus of the wild-type strain, 1783. Strains 1783 and GMI1783 were grown in raffinose-containing medium (YEPR) and then shifted to galactose-containing medium (YEPG). The induced expression of *MKK1$^{P386}$* caused growth arrest after 6 h in galactose. While cells did not exhibit cell cycle stage-specific growth ar-

rest, *MKK1$^{P386}$* overexpression did induce morphological changes such as elongated buds and multiple buds (data not shown).

**Isolation of *MKK1$^{P386}$* overexpression-resistant mutants.** To search for genes that interact with the Mpk1 pathway, we carried out a screen to isolate mutations which suppress the growth-inhibitory effect of *MKK1$^{P386}$* overexpression and simultaneously confer sorbitol-dependent growth at a higher temperature (38°C), because mutations in the Mpk1 pathway were expected to result in a temperature-dependent cell lysis defect. It seemed probable that mutations identified by this screen might affect components acting downstream of Mkk1. Cells of strain 1783 carrying plasmid pNV7-MKK1$^{P386}$ (*GAL1p-MKK1$^{P386}$*) were streaked on galactose-containing plates and incubated at 25°C. Colonies that grew in the presence of galactose were screened for temperature sensitivity as described in Materials and Methods. We isolated nine suppressor mutants that were Ts$^-$ for growth at 38°C but were rescued by 1 M sorbitol (see Materials and Methods). We expected to find *mpk1* mutants among them. Indeed, the Ts$^-$ phenotype of seven mutants was efficiently rescued by the *MPK1* gene on a low-copy-number plasmid, suggesting that they may represent mutations in *MPK1*. We further characterized the remaining two mutants.

We carried out several experiments to address the possibility that the *GAL1p-MKK1$^{P386}$* suppression was due to mutations affecting galactose induction per se. Plasmid-free descendants of the primary yeast mutant isolates were retransformed with pNV7-MKK1$^{P386}$ and with pYS91 carrying *ACT1* fused to the *GAL1* promoter. Overexpression of *ACT1* is known to cause lethality (33). Transformants of each primary isolate with each plasmid were tested for growth on galactose-containing medium. They once again suppressed the toxic phenotype of *MKK1$^{P386}$* overexpression but did not suppress that of *ACT1* (data not shown). Furthermore, transformation of a plasmid carrying *lacZ* fused to the *GAL7* promoter into mutants showed no effect on expression of β-galactosidase in the presence of galactose (data not shown). Thus, these strains bore host mutations that resulted in resistance to *MKK1$^{P386}$* overexpression, and their effects were not related to control of transcription from the *GAL1* promoter.

The two mutants, GMR14 and GMR52, carrying pNV7-MKK1$^{P386}$ were crossed with the wild-type strain 15Dαu, and the resulting diploid cells were examined. Each of the heterozygous diploids failed to grow in the presence of galactose and grew on YEPD at 38°C. Thus, both mutants were recessive for both phenotypes. We analyzed segregants from a cross between strains GMR14 carrying pNV7-MKK1$^{P386}$ and 15Dαu (Fig. 2A). In this cross, analysis of the tetrad sets in which all four segregants carried the *GAL1p-MKK1$^{P386}$* plasmid revealed a 2+:2− segregation for growth on galactose-containing medium in at least 10 tetrads, suggesting that a single mutation was responsible for the resistance to growth inhibition of *MKK1$^{P386}$* overexpression. The suppressor locus was designated *rlm1* (for resistance to lethality of *MKK1$^{P386}$* overexpression). The *rlm1* single mutation suppressed not only growth inhibition but also morphological changes caused by overexpression of *MKK1$^{P386}$* (data not shown). In contrast, Ts$^+$:Ts$^-$ segregated as a mixture of 4+:0− 3+:1−, and 2+:2− tetrads, indicating that two mutations were required for the Ts$^-$ phenotype (Fig. 2A). All Ts$^-$ segregants were suppressed by the addition of sorbitol to the media and were resistant to the toxicity by *MKK1$^{P386}$* overexpression, indicating that a combination of the *rlm1* mutation with another mutation is necessary to produce the Ts$^-$ phenotype. In other words, the *rlm1* mutation is lethal at 38°C only in combination with a second mutation, which was tentatively designated *rlt1* (for *rlm1*-associated Ts$^-$). Complementation data indicated that the GMR52 mutant also contained the mutations in the *RLM1* and *RLT1* genes.

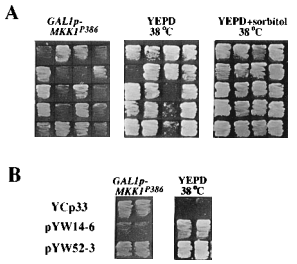


FIG. 2. Growth properties of *rlm1 rlt1* double mutants. (A) GMR14 (*rlm1-1 rlt1-1*) carrying pNV7-MKK1$^{P386}$ (*GAL1p-MKK1$^{P386}$*) was crossed with the wild-type strain 15Dαu; the resulting diploid was sporulated, and then tetrads were dissected. The spores were allowed to germinate at 25°C and transferred to SG-Ura containing 1 M sorbitol at 35°C (left), YEPD at 38°C (middle), or YEPD supplemented with 1 M sorbitol at 38°C (right). Each row of cells was derived from one tetrad. (B) GMY61-1A (*rlm1-1 rlt1-1*) carrying pNV7W-MKK1$^{P386}$ was transformed with YCp33, pYW14-6 (*RLM1*), or pYW52-3 (*RSP5*). Transformants were patched onto SG-Ura containing 1 M sorbitol at 35°C (left) or YEPD at 38°C (right).

**Isolation of genes by complementation.** In an attempt to clone the *RLM1* and *RLT1* genes, we transformed the *rlm1 rlt1* mutants GMY61-1A and GMY62-3C with plasmid DNA from a YCp50-based yeast genomic library. Approximately 10,000 independent transformants were screened for complementation of the Ts$^-$ phenotype of *rlm1 rlt1* cells, and two plasmids (pYW14 and pYW52) were obtained (see Materials and Methods). Restriction fragment analysis of the plasmids indicated that they contained different inserts (Fig. 3). Consistent with the physical differences between the two plasmids, pYW14 restored the growth inhibition of *MKK1$^{P386}$* overexpression

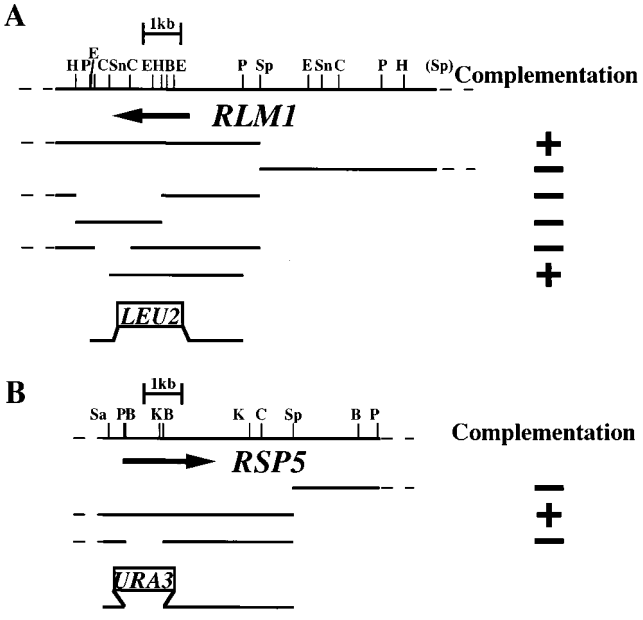


FIG. 3. Restriction maps and disruptions of *RLM1* (A) and *RSP5* (B) The arrows represent the open reading frames and direction of transcription of each gene. The ability of each plasmid to complement the growth defect of the mutant GMY61-1A (*rlm1-1 rlt1-1*) at 38°C is indicated on the right: + complementation; −, no complementation. Restriction enzymes: B, *Bam*HI; C, *Cla*I; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sn, *Sna*BI; Sp, *Sph*I.

FIG. 4. Nucleotide and predicted amino acid sequences of the *RLM1* gene. The nucleotide sequence was determined as described in Materials and Methods. The MADS domain is boxed. The acidic region is underlined with a dashed line. The asparagine-rich regions are underlined with solid lines.

and complemented the Ts$^-$ growth defect, whereas pYW52 complemented the Ts$^-$ phenotype but failed to restore the toxicity of *MKK1$^{P386}$* overexpression (Fig. 2B). Thus, by both physical and phenotypic analyses, these two plasmids defined two genes. pYW14 and pYW52 plasmids contain *RLM1* itself and a suppressor of *rlm1 rlt1*, respectively (see below).

***RLM1* encodes an SRF-like protein.** The essential region of plasmid pYW14 was determined by analysis of subclones (Fig. 3A). The smallest restriction endonuclease fragment capable of complementing the Ts$^-$ phenotype of *rlm1 rlt1* cells was able to restore the growth inhibition of *MKK1$^{P386}$* overexpression. The nucleotide sequence of the 4.4-kb *Pst*I-*Hin*dIII region was determined. An open reading frame that predicts a protein of 676 amino acids was found (Fig. 4).

The predicted protein sequence of Rlm1 was compared with those in GenBank and EMBL data banks, using the FASTA program (45). The N-terminal 80-amino-acid region of Rlm1 is homologous to the DNA-binding and dimerization domain of SRF-related proteins (46, 59) (Fig. 5). This region of similarity places the Rlm1 protein in the MADS box family of transcription factors, named for Mcm1 (which regulates transcription of many genes in *S. cerevisiae* (2, 44), Agamous and Deficiens (which function as homeotic gene products in plants) (54, 56), and SRF (which confers serum responsiveness and muscle specificity to several genes) (42). In all MADS box proteins, 16 of the first 50 amino acids in the MADS box domain are invariant (Fig. 5). This region of Rlm1 is more similar to that of the myocyte enhancer factor family implicated in the regulation of muscle transcription in vertebrates (6) (70% identical) than to the corresponding region of other MADS box proteins (38 to 56% identical). The strongly conserved N terminus of the domain is rich in polar and basic residues, reminiscent of DNA-binding domains. The basic region has two segments of predicted α helix. The more divergent C-terminal half of the domain is largely hydrophobic (Fig. 5). Outside the first 80 amino acids, Rlm1 shows no significant homology to MADS box family, and the protein is rich in serine and threonine residues (24% in 595 amino acids). Immediately following the MADS box domain of Rlm1 are 14 amino acids, of which 11 are either aspartate or glutamate. In addition, there is a distinctive sequence of consecutive asparagine beginning at amino acid position 567 (Fig. 4).

FIG. 5. Homology between the deduced amino acid sequence of the Rlm1 protein with sequences of the MADS box proteins RSRF (6), SRF (42), Mcm1 (2, 44), Agamous (54), and Deficiens (54). Identical amino acids are indicated by shaded boxes. The asterisks indicate residues conserved among all six proteins.

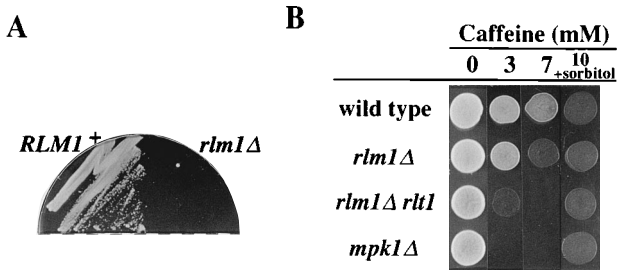


FIG. 6. Caffeine sensitivity. (A) Cells were streaked onto YEPD containing 10 mM caffeine at 30°C. Strains: *RLM1*$^+$, GMY63-5B; *rlm1*Δ, GMY63-5C. (B) Suspensions of approximately $3 \times 10^5$ exponentially growing cells at 25°C were spotted onto indicated plates and photographed after 2 or 3 days of incubation at 25°C. Strains: wild type, GMY63-5B; *rlm1*Δ, GMY63-5C; *rlm1*Δ rlt1, GMY67-5A; *mpk1*Δ, DL456-3B.

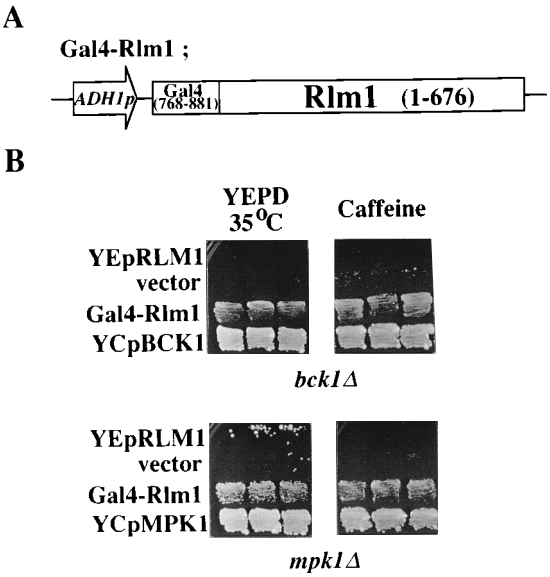


FIG. 7. Effect of the Gal4-Rlm1 fusion protein. (A) Gal4-Rlm1 fusion construct. The GAD (residues 768 to 881) was fused to residues 1 to 676 of Rlm1. This construct was driven by the *ADH1* promoter. (B) Cells were transformed with YEp181-RLM1 or YEp195-RLM1 (YEpRLM1), pACTII (vector), or pACT-RLM1 (Gal4-Rlm1), and then transformants were streaked onto YEPD at 35°C or YEPD containing 2 mM caffeine at 25°C. Strains: *bck1*Δ, DL251; *mpk1*Δ, DL456.

**Disruption of *RLM1*.** Strains with mutations in the Mpk1 pathway exhibit a variety of defects in growth control. Deletions of *BCK1*, *MKK1* and *MKK2*, or *MPK1* confer the following phenotypes: (i) failure to grow at elevated temperatures, which can be suppressed by the addition of 1 M sorbitol to the medium; (ii) sensitivity to low levels of caffeine in the medium; (iii) failure to grow on glycerol medium; and (iv) sensitivity to nitrogen starvation (9, 11, 26, 30, 31, 37, 38). To examine the phenotypic defect associated with loss of *RLM1* function and to determine whether *RLM1* participates in the same cellular processes as *BCK1*, *MKK1*/*MKK2*, and *MPK1*, a deletion mutant of *RLM1* was constructed in vitro. A 2.1-kb *Acc*I-*Sna*BI fragment encompassing the *RLM1* open reading frame was replaced with the *S. cerevisiae LEU2* gene (Fig. 3A) (see Materials and Methods).

The deletion allele (*rlm1*Δ::*LEU2*) was introduced into wild-type diploid yeast strains 1788 and 15Du. Transformants were tested for possession of the deletion allele by Southern blot analysis (data not shown). Heterozygous diploid transformants were sporulated, and progeny from individual tetrads were dissected onto plates and analyzed. Four spores from each tetrad gave rise to colonies. Unlike Mpk1 pathway mutants, the *rlm1*Δ::*LEU2* segregants grew normally at all temperatures ranging from 14 to 38°C. When the *rlm1*Δ::*LEU2* segregants derived from 1788 and 15Du were crossed with the *rlm1-1 rlt1-1* strain (GMY61-1A), tetrad dissection revealed a 2+:2− segregation for growth on YEPD medium at 38°C in all 23 tetrads. This result confirmed that the complementing plasmid pYW14 carries the *RLM1* gene. The Ts$^-$ phenotype was suppressed by the addition of 1 M sorbitol to the medium, as observed for *bck1*Δ, *mkk1*Δ *mkk2*Δ, and *mpk1*Δ mutants. Microscopic examination of *rlm1 rlt1* mutants revealed a high frequency (66 to 80%) of nonrefractile ghosts at 38°C. Furthermore, an alkaline phosphatase assay on YEPD agar plates containing 5-bromo-4-chloro-3-indolylphosphate (5, 43) showed that *rlm1 rlt1* mutants turned blue at 38°C. These results indicated that cell lysis was occurring at the high temperature.

The *rlm1*Δ::*LEU2* mutants grew on glycerol medium and were not sensitive to nitrogen starvation. However, the *rlm1*Δ::*LEU2* mutation enhanced caffeine sensitivity (Fig. 6). The *rlm1*Δ::*LEU2* mutant was more sensitive than the wild-type strain, but its sensitive phenotype was not as severe as that of *mpk1*Δ::*TRP1* cells. The *rlm1*Δ::*LEU2 rlt1-1* double mutant was more sensitive than *rlm1*Δ::*LEU2*, suggesting that the *rlt1* mutation influences the severity of the *rlm1* phenotype. The caffeine-sensitive phenotype of *rlm1*Δ::*LEU2* and *rlm1*Δ::*LEU2 rlt1-1* cells was suppressed by the addition of 1 M sorbitol medium, as observed for *bck1*Δ and *mpk1*Δ mutants. The caffeine sensitivity of *rlm1*Δ and *mpk1*Δ cells was not additive in the double mutant relative to single mutants (data not shown), suggesting that Rlm1 operates in the Mpk1 pathway.

One explanation for the ability of *rlm1* to suppress the growth-inhibitory effect of Mkk1$^{P386}$ overproduction is that the *rlm1*Δ::*LEU2* mutation would have a negative effect on the expression *MPK1*, thereby reducing the toxicity of Mkk1$^{P386}$ overproduction. To address this possibility, *MPK1* RNA levels in wild-type and *rlm1*Δ::*LEU2* strains were examined by RNA blot analysis (data not shown). Quantitative comparison of *MPK1* RNA levels revealed that the *rlm1*Δ::*LEU2* mutation did not affect *MPK1* transcript levels.

**Genetic evidence suggests that *RLM1* functions in the Mpk1 MAPK pathway.** So far, our experiments demonstrated that Rlm1 is needed for the toxicity caused by *MKK1*$^{P386}$ overexpression. They did not clarify whether the requirement for Rlm1 reflected its direct involvement as a downstream component of the Mpk1 pathway. This possibility was examined by genetic tests. We tested the effect of *RLM1* overexpression on the phenotypes caused by *bck1*Δ and *mpk1*Δ mutations. As shown in Fig. 7B, multiple copies of the *RLM1* gene failed to suppress the Ts$^-$ growth defect of *bck1*Δ and *mpk1*Δ cells and the inhibition of growth by caffeine. Overexpression of *RLM1* driven from the *GAL1* promoter was also unable to suppress *bck1*Δ and *mpk1*Δ (data not shown).

In most cases, MADS box proteins such as SRF and the SRF-related yeast protein Mcm1 seem to be able to bind promoter DNA but unable to activate transcription without an appropriate protein partner (12). If Rlm1 were also involved in the activation of transcription in the Mpk1 pathway by recruiting a regulated TCF, one might expect that overexpression of Rlm1 would be unable to suppress *bck1*Δ and *mpk1*Δ. To test this possibility, we constructed a fusion, in which Rlm1 was directly connected to a strong Gal4 transcriptional activator domain (GAD), whose expression was expressed by the *ADH1* promoter (Fig. 7A). Such a partner-independent Rlm1 fusion

| DNA-binding protein | Activating protein | -His AT | β-galactosidase activity |
|---|---|---|---|
| LexA | GAD | | <0.1 |
| LexA-RLM1ΔC | GAD | | 2.9 |
| LexA | GAD-MPK1 | | <0.1 |
| LexA-RLM1ΔC | GAD-MPK1 | | 31.3 |

FIG. 8. Two-hybrid interaction between Mpk1 and Rlm1. The reporter strain L40 (*LYS2*::*lexA-HIS3 URA3*::*lexA-lacZ*) was transformed with various plasmids as indicated. Approximately $3 \times 10^5$ cells of each transformant were spotted onto SC-His medium containing 100 mM 3-aminotriazole (AT). Plates were incubated at 30°C for 4 days. Transformants were assayed for β-galactosidase activity as described previously (13). Plasmids: LexA, pBTM116; LexA-RLM1ΔC, pYW62; GAD, pACTII; GAD-MPK1, pB331. β-Galactosidase activity is expressed in units.

variant should now stimulate transcription without activation by Mpk1, resulting in suppression of the *bck1*Δ and *mpk1*Δ mutations. As shown in Fig. 7B, the Gal4-Rlm1 fusion protein partially suppressed the $Ts^-$ growth defect and caffeine-sensitive phenotypes of *bck1*Δ and *mpk1*Δ cells. Furthermore, Gal4-Rlm1 also suppressed the failure of cells to grow on glycerol medium and the nitrogen starvation sensitivity of these mutants (data not shown). Thus, the Rlm1 fusion protein carrying the GAD suppresses all defects of *bck1*Δ and *mpk1*Δ cells. These genetic tests indicate that Rlm1 functions in the same pathway as Bck1 and Mpk1 and that Rlm1 operates downstream of Mpk1 in the pathway. Therefore, Rlm1 appears to play a role in the transcriptional events controlled by the Mpk1 MAPK.

**Rlm1 and Mpk1 interact in the two-hybrid system.** Genetic analysis suggests that Rlm1 is a downstream component of the Mpk1 pathway. A possible interaction between Mpk1 and Rlm1 was analyzed with the yeast two-hybrid system, which allows the detection of protein-protein interactions in vivo (18). Mpk1 was fused to the GAD (10). A truncated form of Rlm1 (Rlm1ΔC) lacking the COOH-terminal 151 amino acids was fused to the DNA-binding domain of LexA (see Materials and Methods). These proteins were analyzed, singly or in combination, for the ability to activate transcription of *HIS3* and *lacZ* reporter genes containing LexA operators (Fig. 8). Expression of the LexA-Rlm1ΔC fusion, in the absence of a GAD fusion, yielded a relatively high background of expression of reporter genes. Nevertheless, stimulation over this background was observed when the GAD-Mpk1 fusion was coexpressed. Measurement of the level of β-galactosidase induced indicated that this signal was around 10-fold higher than the background level of β-galactosidase expression. This finding suggests that Mpk1 and Rlm1 interact in vivo, supporting the possibility that Rlm1 functions in the Mpk1 pathway.

***RSP5* is an extra-copy suppressor of *rlm1 rlt1*.** By subcloning and complementation analyses, the ability of plasmid pYW52 to complement the $Ts^-$ phenotype was localized to the 4.9-kb *Sal*I-*Sph*I fragment (Fig. 3B). Determination of the DNA sequence of the 1-kb *Bam*HI fragment revealed that this DNA fragment is identical to the sequence of *RSP5* (accession number L1119 [63a]). The *RSP5* gene was identified as a suppressor mutation of the *spt3* mutations (64) and encodes a protein of 809 amino acid residues. At the C-terminal 400 amino acids, Rsp5 has sequence similarity to oncogene product E6-AP, which appears to be involved in ubiquitin-mediated protein degradation. In fact, recent biochemical studies showed that the Rsp5 protein has a ubiquitin-protein ligase activity (25).

To determine the phenotypic consequences of loss of *RSP5* function, a deletion mutant of *RSP5* was constructed as described in Materials and Methods. A 1.0-kb fragment of *RSP5* was replaced with the selectable marker *URA3* (Fig. 3B). This deletion allele (*rsp5*Δ::*URA3*) was transplaced into a diploid strain (15Du) by selecting for $URA^+$. Southern blot analysis of chromosomal DNA from transformants confirmed that the resulting diploid was heterozygous for the wild-type and disrupted *rsp5* (*rsp5*Δ::*URA3*) alleles. Sporulation and tetrad analysis of this diploid showed that only two of the four spores per tetrad were viable at 30°C on rich YEPD medium. All viable spores were $Ura^-$, indicating that the inviable spores contain the *rsp5*Δ::*URA3* replacement. These results show that *RSP5* is essential. Addition of 1 M sorbitol to the medium did not support the growth of *rsp5*Δ::*URA3* cells.

The cloned insert in plasmid pYW52 could represent either the actual *rlt1* locus or a suppressor locus. To distinguish between these possibilities, we crossed the *rlm1*Δ::*LEU2 rlt1-1* strain (GMY67-5A) with the *rlm1*Δ::*LEU2 rsp5*Δ::*URA3* strain (GMY68-3A) carrying plasmid pYW52-4. Plasmid pYW52-4 was cured from the resulting diploid. Because this diploid is heterozygous for the *rlt1-1* mutation, a disruption of the *RSP5* gene should unmask the $Ts^-$ phenotype if the cloned fragment is exclusively *RLT1* coding sequence. However, the diploid grew at 38°C. After sporulation and tetrad dissection of the diploid at 25°C, the viability of the spores was assessed. Only half of the spores were viable; as expected, all of these were $Ura^-$, but some segregants were $Ts^+$. These results demonstrate that *RSP5* at low copy number suppresses the $Ts^-$ growth defect of *rlm1 rlt1* cells.

***RLT1* is identical to *SSD1*/*SRK1*.** Several mutants of the Pkc1-Mpk1 pathway have been shown to be synthetically lethal with *ssd1*/*srk1* (9, 30). Thus, loss of *SSD1* function enhances the phenotypes associated with the Mpk1 pathway mutants. The *SSD1* gene is predicted to encode a protein related to the Dis3 protein of *Schizosaccharomyces pombe*, which has been implicated in protein phosphatase function (58, 63). In view of these facts, we explored the possibility that *rlt1* was allelic to *ssd1*. To determine the phenotypic effect of loss of both *RLM1* and *SSD1*, the *ssd1*Δ::*URA3* strain (KA31-2A-2) (61) was crossed to a strain (GMY63-5C) containing the *rlm1*Δ::*LEU2* mutation. *rlm1*Δ::*LEU2 ssd1*Δ::*URA3* segregants were observed at the expected frequency. Strain KA31-2A-2 grew normally over a range of temperatures and was not sensitive to caffeine (at 10 mM), whereas double mutants exhibited the various defects observed in mutants of the Mpk1 pathway, including the inability to grow at 38°C, inhibition of growth by caffeine (at 5 mM), failure to grow on glycerol medium, and sensitivity to nitrogen starvation (data not shown). The $Ts^-$ growth defect and caffeine-sensitive phenotypes were suppressed by the addition of 1 M sorbitol to the medium. Thus, the *ssd1* mutation enhanced the severity of the *rlm1* phenotype, as observed for *rlm1 rlt1* mutants.

To determine whether *rlt1* is allelic with *ssd1*, the *rlm1*Δ::*TRP1 rlt1-1* strain (GMY70-10B) was crossed to the *rlm1*Δ::*LEU2 ssd1*Δ::*URA3* strain (GMY72-1B), and segregation analysis was conducted on the resulting diploid strain. The diploid was $Ts^-$ for growth at 38°C and exhibited caffeine sensitivity. Tetrad dissection revealed a 0+:4− segregation for growth on YEPD at 38°C and growth on YEPD containing 5 mM caffeine in all 11 tetrads. These results indicate tight linkage of *rlt1* to *ssd1*, suggesting that *RLT1* is identical to *SSD1*.

**Overexpression of *MSG5* suppresses the $Mkk1^{P386}$ overproduction toxicity.** To identify additional components of the Mpk1 pathway, we carried out a screen to isolate genes which, when overexpressed from multicopy plasmids, are capable of

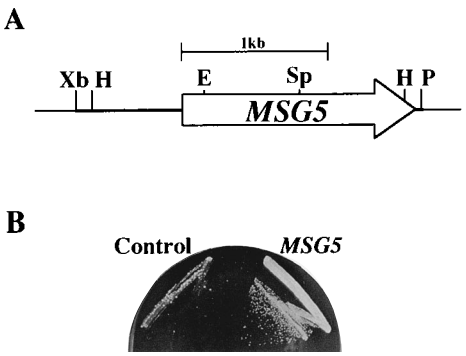


FIG. 9. Overexpression of the *MSG5* gene suppresses the growth-inhibitory effect of overexpression of $MKK1^{P386}$. (A) Restriction map of insert of pSPG14 (*MSG5*). The arrow represents the open reading frames and direction of transcription of the gene. Restriction enzymes: E, *Eco*RI; H, *Hin*dIII; P, *Pst*I; Sp, *Sph*I; Xb, *Xba*I. (B) Cells of GMI1783 ($GAL1p\text{-}MKK1^{P386}$::*TRP1*) were transformed with YEp13 (Control) or pSPG14 (*MSG5*), and then transformants were streaked onto YEPG containing galactose at 30°C.

suppressing the growth-inhibitory effect of $MKK1^{P386}$ overexpression. It seemed probable that genes identified by this screen would encode proteins which negatively regulate the Mpk1 pathway. The selection was performed by using a yeast genomic library cloned in the multicopy vector YEp13 (contains *S. cerevisiae LEU2*; provided by Y. Ohya). The library was transformed into strain GMI1783, which carries a $GAL1p\text{-}MKK1^{P386}$ construct integrated at the *trp1* locus. A total of 4,000 $Leu^+$ transformants were obtained and subsequently screened for the ability to grow in the presence of galactose. Seventeen transformants were capable of forming colonies on galactose-containing medium. The suppressing plasmids were recovered in *E. coli* and shown to rescue the $GAL1p\text{-}MKK1^{P386}$ phenotype when reintroduced into GM1783 cells (Fig. 9B). Of the 17 candidates, 10 clones did not suppress the toxic phenotype of *ACT1* overexpression under the control of the *GAL1* promoter, indicating that their effects were not related to control of transcription from the *GAL1* promoter.

The 10 plasmids contained identical restriction fragments (Fig. 9A). Sequence analysis of subclones containing the suppressing activity revealed that this DNA fragment is identical in sequence to *MSG5*. The *MSG5* gene was identified as a multicopy suppressor of growth arrest caused by a *gpa1* mutation (13). *MSG5* encodes a dual-specificity protein phosphatase that dephosphorylates and inactivates Fus3 MAPK acting in the mating pheromone signal transduction (13). Therefore, our results suggest that overexpression of *MSG5* suppresses the toxic effect caused by hyperactivation of the Mpk1 pathway by dephosphorylating and inactivating Mpk1 MAPK. Thus, Msg5 phosphatase appears to act on Mpk1 under overexpressed conditions.

## DISCUSSION

In this study, a screen for suppressor mutations of the growth inhibition caused by an activated mutation of *MKK1* was used to identify downstream components of the Mpk1 pathway. While overexpression of an activated form of protein kinase can sometime be nonspecifically toxic, the growth-inhibitory effect of $MKK1^{P386}$ overexpression appeared to be specific, in as much as it was suppressed by the disruption of *MPK1*, which functions downstream of *MKK1*, but not by the disruption of *BCK1*, which functions upstream of *MKK1*. These results suggest that overexpression of $MKK1^{P386}$ causes toxicity through the hyperactivation of the Mpk1 kinase and consequent excessive phosphorylation of target(s) of Mpk1. According to this hypothesis, mutations in a component that functions downstream of Mpk1 will relieve the inhibition caused by $MKK1^{P386}$ overexpression. On the basis of this assumption, we selected mutations that relieved inhibition. Our selection yielded mutations in a new gene, termed *RLM1*. Genetic and phenotypic analysis suggests that Rlm1 is a downstream component of the Mpk1 pathway.

The *RLM1* gene potentially encodes a protein belonging to the MADS box family of transcription factors. The MADS box motif is found in several regulatory proteins, including *S. cerevisiae* Mcm1 (2, 44) and Arg80 (15, 16), human SRF (42), human proteins that bind muscle control elements and growth factor-regulated genes (6), and the plant homeotic gene products Deficiens and Agamous (54, 56). As a member of the MADS box family, the Rlm1 protein is predicted to regulate the expression of a target gene(s) in response to Pkc1-Mpk1 signaling.

The DNA-binding properties of MADS box proteins as homo- or heterodimers have been demonstrated for SRF and Mcm1 (8, 39, 42, 46). The N-terminal basic part (first 30 amino acids) of the MADS box domains within these proteins mediates the sequence specificity of DNA binding, while the divergent C-terminal portion directs dimerization and interaction with coregulators. Mcm1 controls the expression of a number of different genes by recruiting specific transcription regulatory proteins: $\alpha$1 to activate $\alpha$-cell-type-specific gene expression, $\alpha$2 to repress **a**-cell-type-specific gene expression, and Ste12 to activate pheromone-dependent expression of **a**-cell-type-specific genes (22). SRF is essential for the serum-inducible transcriptional activation of the c-*fos* nuclear proto-oncogene of mammals. At the serum response element of c-*fos*, SRF can form a ternary complex with another protein, $p62^{TCF}$, which by itself exhibits no detectable DNA-binding activity (60). Thus, Mcm1 and SRF recruit additional factors to regulate gene expression. It is tempting to speculate that, in analogy to Mcm1 and SRF, Rlm1 may be able to recruit a ternary complex factor(s).

In mammalian cells, activation of the MAPK pathway by growth factor signals leads to phosphorylation of the SRF accessory factor TCF/Elk-1 and activation of transcription (23, 60). This pathway has several parallels to gene induction in the pheromone signaling pathway of *S. cerevisiae*. Pheromone also activates the MAPK pathway that activates transcription factor Ste12 by phosphorylating it (17). Ste12, often in association with Mcm1, then activates transcription (22). By analogy with the MAPK pathways regulating mammalian SRF–Elk-1 and yeast Mcm1-Ste12, it seems reasonable to speculate that the Mpk1 MAPK pathway may phosphorylate a transcription factor analogous to Elk-1 and Ste12, which activates transcription by association with Rlm1. It would likewise be interesting to determine whether proteins that interact with Rlm1 exist. Since a mammalian gene encoding TCF/SAP-1 was isolated by using the yeast system (12), yeast genes encoding the proteins interacting with Rlm1 may be represented in a cDNA collection obtained by two-hybrid screening.

Although overexpression of *RLM1* was unable to suppress the *mpk1*$\Delta$ mutation, the fusion of Rlm1 to the Gal4 activator domain caused suppression of the phenotypes of *mpk1*$\Delta$. We suggest two possible explanations for this observation. The activity of Rlm is activated by the Mpk1 pathway, and the Rlm1 fusion protein carrying the GAD is active independently of the Mpk1 pathway. Alternatively, Rlm1 associates with an accessory protein, which is activated by the Mpk1 pathway and functions as an activator of Rlm1. Such a partner-independent

Rlm1 fusion variant is active without activation of the Mpk1 pathway. In either case, the Gal4-tagged derivative of Rlm1 is expected to be constitutively active. Hyperactivation of the Mpk1 pathway by *MKK1*$^{P386}$ overexpression caused growth arrest associated with morphological alterations, whereas wild-type cells expressing the Gal4-Rlm1 fusion protein grew normally but morphological alterations were slightly induced (data not shown). Therefore, the activity of Gal4-Rlm1 may be lower than that of Rlm1 activated by the Mpk1 pathway.

Yeast strains with mutations in the Mpk1 pathway display the following phenotypes (9, 11, 26, 30, 31, 37, 38): caffeine sensitivity, a temperature-dependent cell lysis defect, glycerol growth defect, and starvation sensitivity. *rlm1*Δ mutants display a caffeine-sensitive phenotype, but this sensitivity is not as severe as that of *bck1*Δ or *mpk1*Δ cells. Unlike Mpk1 pathway mutants, the *rlm1*Δ cells grow normally at any temperature, grow on glycerol medium, and are not sensitive to nitrogen starvation. One possible explanation for this finding is that Rlm1 is an important downstream component of the Mpk1 pathway, but additional Rlm1-like proteins that prevent *rlm1*Δ cells from acquiring the growth defects may be present. Precedent exists for such a situation in the Mpk1 pathway. For example, there are two sets of functionally redundant genes, *MKK1*/*MKK2* and *NHP6A*/*NHP6B* (10, 26). This model posits that partial loss of activity of downstream components by the *rlm1*Δ mutation is enough to cause resistance to the growth-inhibitory effect caused by hyperactivation of the Mpk1 pathway. Consistent with this possibility, the Gal4-Rlm1 fusion protein suppressed not only caffeine sensitivity but also other growth defects of *bck1*Δ and *mpk1*Δ cells.

*S. cerevisiae* has at least four separate MAPK activation pathways that mediate distinct responses to different extracellular or cell autonomous signals. The Hog1 MAPK pathway is involved in osmoregulation and regulated by an Sln1-Ssk1 two-component system (34). Disruption of *SLN1* results in constitutive activation of the Hog1 pathway and lethality (34). Thus, hyperactivation of two different MAPKs, Mpk1 and Hog1, induces the growth-inhibitory effect. However, disruption of *RLM1* did not suppress *sln1*Δ mutations (data not shown), suggesting that the causes for these toxic effects induced by hyperactivation of the Mpk1 and Hog1 pathways may be different. Overexpression of *PTP2* was able to suppress growth defects caused by the *sln1*Δ mutation (34). This result raises the possibility that Ptp2 dephosphorylates phosphotyrosine of the Hog1 MAPK, resulting in its inactivation. Previously, we identified Msg5 as a dual-specificity phosphatase that acts on Fus3 (13). In addition, Msg5 is likely to act on Mpk1, because overexpression of *MSG5* suppressed the growth-inhibitory effect of *MKK1*$^{P386}$ overexpression. In contrast, *MSG5* overexpression failed to suppress the lethality of *sln1*Δ mutations (14). Therefore, Msg5 appears to act on Fus3 and Mpk1 but not on Hog1. Taken together, the data suggest that there are two separate types of MAPK phosphatases that have some specificity for MAPKs.

## ACKNOWLEDGMENTS

We thank Y. Suzuki for help in some of the experiments. We also thank J. Cooper, Y. Kikuchi, D. Levin, T. Maeda, Y. Ohya, and M. Snyder for plasmids and strains, and we thank C. Brenner, J. L. Horecka, and R. Ruggieri for reading the manuscript and helpful discussions.

This work was supported by the Ministry of Education, Science and Culture of Japan (K.I. and K.M.).

## REFERENCES

1. **Alvarez, E., I. C. Northwood, F. A. Gonzales, D. A. Latour, A. Seth, C. Abata, T. Curran, and R. J. Davis.** 1991. Pro-Leu-Ser/Thr-Pro is a consensus primary sequence for substrate protein phosphorylation. J. Biol. Chem. **266:** 15277–15285.
2. **Ammerer, G.** 1990. Identification, purification, and cloning of a polypeptide (PRTF/GRM) that binds to mating-specific promoter elements in yeast. Genes Dev. **4:**299–312.
3. **Ammerer, G.** 1994. Sex, stress, and integrity: the importance of MAP kinases in yeast. Curr. Opin. Genet. Dev. **4:**90–95.
4. **Blumer, K. J., and G. L. Johnson.** 1994. Diversity in function and regulation of MAP kinase pathways. Trends Biochem. Sci. **19:**236–240.
5. **Cabib, E., and A. Duran.** 1975. Simple and sensitive procedure for screening yeast mutants that lyse at nonpermissive temperatures. J. Bacteriol. **124:** 1604–1606.
6. **Chambers, A. E., S. Kotecha, N. Towers, and T. J. Mohun.** 1992. Muscle-specific expression of SRF-related genes in the early embryo of *Xenopus laevis*. EMBO J. **11:**4981–4991.
7. **Chen, R. H., C. Abata, and J. Blenis.** 1993. Phosphorylation of the c-Fos transrepression domain by mitogen-activated protein kinase and 90-kDa ribosomal S6 kinase. Proc. Natl. Acad. Sci. USA **90:**10952–10956.
8. **Christ, C., and B. K. Tye.** 1991. Functional domains of the yeast transcription/replication factor MCM1. Genes Dev. **5:**751–763.
9. **Costigan, C., S. Gehrung, and M. Snyder.** 1992. A synthetic lethal screen identifies SLK1, a novel protein kinase homolog implicated in yeast cell morphogenesis and cell growth. Mol. Cell. Biol. **12:**1162–1178.
10. **Costigan, C., Kolodrubetz, D., and M. Snyder.** 1994. *NHP6A* and *NHP6B*, which encode HMG1-like proteins, are candidates for *SLT2* mitogen-activated protein kinase pathway. Mol. Cell. Biol. **14:**2391–2403.
11. **Costigan, C., and M. Snyder.** 1994. SLK1, a yeast homolog of MAP kinase activators, has a RAS/cAMP-independent role in nutrient sensing. Mol. Gen. Genet. **243:**286–296.
12. **Dalton, S., and R. Treisman.** 1992. Characterization of SAP-1, a protein recruited by serum response factor to the c-fos serum response element. Cell **68:**597–612.
13. **Doi, K., A. Gartner, G. Ammerer, B. Errede, H. Shinkawa, K. Sugimoto, and K. Matsumoto.** 1994. MSG5, a novel protein phosphatase promotes adaptation to pheromone response in *S. cerevisiae*. EMBO J. **13:**61–70.
14. **Doi, K., and K. Matsumoto.** Unpublished data.
15. **Dubois, E., J. Bercy, F. Descamps, and F. Messenguy.** 1987. Characterization of two new genes essential for vegetative growth in *Saccharomyces cerevisiae*: nucleotide sequence determination and chromosome mapping. Gene **55:** 265–275.
16. **Dubois, E., J. Bercy, and F. Messenguy.** 1987. Characterization of two genes, *ARGRI* and *ARGRIII* required for specific regulation of arginine metabolism in yeast. Mol. Gen. Genet. **207:**142–148.
17. **Elion, E. A., B. Satterberg, and J. E. Kranz.** 1993. FUS3 phosphorylates multiple components of the mating signal transduction cascade: evidence for STE12 and FAR1. Mol. Biol. Cell **4:**495–510.
18. **Fields, S., and O.-K. Song.** 1989. A novel gene system to detect protein-protein interactions. Nature (London) **340:**245–246.
19. **Gietz, D., A. St. Jean, R. A. Woods, and R. H. Schiestl.** 1992. Improved method for high efficiency transformation of intact yeast cells. Nucleic Acids Res. **20:**1425.
20. **Hanahan, D.** 1983. Studies on transformation of *Escherichia coli* with plasmids. J. Mol. Biol. **166:**557–580.
21. **Herskowitz, I.** 1995. MAP kinase pathways in yeast: for mating and more. Cell **80:**187–197.
22. **Herskowitz, I., J. Rine, and J. N. Strathern.** 1992. Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*, p. 583–656. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular biology and cellular biology of the yeast *Saccharomyces*: gene expression. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
23. **Hill, C. S., and R. Treisman.** 1995. Transcriptional regulation by extracellular signals: mechanisms and specificity. Cell **80:**199–211.
24. **Hill, C. S., J. Wynne, and R. Treisman.** 1994. Serum-regulated transcription by serum response factor (SRF): a novel role for the DNA binding domain. EMBO J. **13:**5421–5432.
25. **Huibregtse, J. M., M. Scheffner, S. Beaudenon, and P. M. Howley.** 1995. A family of proteins structurally and functionally related to the E6-AP ubiquitin protein ligase. Proc. Natl. Acad. Sci. USA **92:**2563–2567.
26. **Irie, K., M. Takase, K. S. Lee, D. E. Levin, H. Araki, K. Matsumoto, and Y. Oshima.** 1993. *MKK1* and *MKK2*, encoding *Saccharomyces cerevisiae* mitogen-activated protein kinase-kinase homologs, function in the pathway mediated by protein kinase C. Mol. Cell. Biol. **13:**3076–3083.
27. **Janknecht, R., W. H. Ernst, V. Pingoud, and A. Nordheim.** 1993. Activation of TCF Elk-1 by MAP kinases. EMBO J. **12:**5097–5104.
28. **Kortenjann, M., O. Thomae, and P. E. Shaw.** 1994. Inhibition of v-*raf*-dependent c-*fos* expression and transformation by a kinase-defective mutant of the mitogen-activated protein kinase Erk2. Mol. Cell. Biol. **14:** 4815–4824.
29. **Lee, K. S., L. K. Hines, and D. E. Levin.** 1993. A pair of functionally redundant yeast genes (*PPZ1* and *PPZ2*) encoding type 1-related protein phosphatases function within the PKC1-mediated pathway. Mol. Cell. Biol. **13:**5843–5853.

30. **Lee, K. S., K. Irie, Y. Gotoh, Y. Watanabe, H. Araki, E. Nishida, K. Matsumoto, and D. E. Levin.** 1993. A yeast MAP kinase homolog (MPK1) mediates signalling by protein kinase C. Mol. Cell. Biol. **13:**3067–3075.
31. **Lee, K. S., and D. E. Levin.** 1992. Dominant mutations in a gene encoding a putative protein kinase (*BCK1*) bypass the requirement for a *Saccharomyces cerevisiae* protein kinase C homolog. Mol. Cell. Biol. **12:**172–182.
32. **Li, L., S. J. Elledge, C. A. Peterson, E. S. Bales, and R. J. Legerski.** 1994. Specific association between the human DNA repair proteins XPA and ERCC1. Proc. Natl. Acad. Sci. USA **91:**5012–5016.
33. **Liu, H., J. Krizek, and A. Bretscher.** 1992. Construction of a *GAL1*-regulated yeast cDNA expression library and its application to the identification of genes whose expression causes lethality in yeast. Genetics **132:**665–673.
34. **Maeda, T., S. M. Wurgler-Murphy, and H. Saito.** 1994. A two-component system that regulates an osmosensing MAP kinase cascade in yeast. Nature (London) **369:**242–245.
35. **Marshall, C. J.** 1994. MAP kinase kinase kinase, MAP kinase kinase, and MAP kinase. Curr. Opin. Genet. Dev. **4:**82–89.
36. **Marshall, C. J.** 1995. Specificity of receptor tyrosine kinase signaling: transient versus sustained extracellular signal-regulated kinase activation. Cell **80:**179–185.
37. **Martin, H., J. Arroyo, M. Sanchez, M. Molina, and C. Nombela.** 1993. Activity of the yeast MAP kinase homologue Slt2 is critically required for cell integrity at 37°C. Mol. Gen. Genet. **241:**177–184.
38. **Mazzoni, C., P. Zarzov, A. Rambourg, and C. Mann.** 1993. The *SLT2* (*MPK1*) MAP kinase homolog is involved in polarized cell growth in *Saccharomyces cerevisiae*. J. Cell Biol. **123:**1821–1833.
39. **Mueller, C. G., and A. Nordheim.** 1991. A protein domain conserved between yeast MCM1 and human SRF directs ternary complex formation. EMBO J. **10:**4219–4229.
40. **Nakajima, T., S. Kinoshita, T. Sasagawa, K. Sasaki, M. Naruto, T. Kishimoto, and S. Akira.** 1993. Phosphorylation at threonine-235 by a ras-dependent mitogen-activated protein cascade is essential for transcription factor NF-IL6. Proc. Natl. Acad. Sci. USA **90:**2207–2211.
41. **Ninomiya-Tsuji, J., S. Nomoto, H. Yasuda, S. I. Reed, and K. Matsumoto.** 1991. Cloning of a human cDNA encoding a CDC2-related kinase by complementation of a budding yeast *cdc28* mutation. Proc. Natl. Acad. Sci. USA **88:**9006–9010.
42. **Norman, C., M. Runswick, R. Pollock, and R. Treisman.** 1988. Isolation and properties of cDNA clones encoding SRF, a transcription factor that binds to the *c-fos* serum response element. Cell **55:**989–1003.
43. **Paravicini, G., M. Cooper, L. Friedli, D. J. Smith, J.-L. Carpentier, L. S. Klig, and M. A. Payton.** 1992. The osmotic integrity of the yeast cell requires a functional *PKC1* gene product. Mol. Cell. Biol. **12:**4896–4905.
44. **Passmore, S., R. Elble, and B. K. Tye.** 1989. A protein involved in minichromosome maintenance in yeast binds a transcriptional enhancer conserved in eukaryotes. Genes Dev. **3:**921–935.
45. **Pearson, W. R., and D. J. Lipman.** 1988. Improved tools for biological sequence comparison. Proc. Natl. Acad. Sci. USA **85:**2444–2448.
46. **Pollock, R., and R. Treisman.** 1991. Human SRF-related proteins: DNA-binding properties and potential regulatory targets. Genes Dev. **5:**2327–2341.
47. **Posas, F., A. Casamayor, and J. Arino.** 1993. The PPZ protein phosphatases are involved in the maintenance of osmotic stability of yeast cells. FEBS Lett. **318:**282–286.
48. **Pulverer, B. J., J. M. Kyriakis, J. Avruch, E. Nikolakaki, and J. R. Woodgett.** 1991. Phosphorylation of *c-jun* mediated by MAP kinase. Nature (London) **353:**670–674.
49. **Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink.** 1987. A *Saccharomyces cerevisiae* genomic bank based on a centromere-containing shuttle vector. Gene **60:**237–243.
50. **Rose, M. D., F. Winston, and P. Hieter.** 1990. Methods in yeast genetics. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
51. **Rothstein, R.** 1983. One-step gene disruption in yeast. Methods Enzymol. **101:**202–211.
52. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
53. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **83:**5463–5467.
54. **Schwarz-Sommer, Z., P. Huijser, W. Nacken, H. Saedler, and H. Sommer.** 1990. Genetic control of flower development by homeotic genes in *Antirrhinum majus*. Science **250:**931–936.
55. **Seth, A., F. A. Gonzalez, S. Gupta, D. L. Raden, and R. J. Davis.** 1992. Signal transduction within the nucleus by mitogen-activated protein kinase. J. Biol. Chem. **267:**24796–24804.
56. **Sommer, H., J.-P. Beltran, P. Huijser, H. Pape, W.-E. Lonning, H. Saedler, and Z. Schwarz-Sommer.** 1990. Deficiens, a homeotic gene involved in the control of flower morphogenesis in Antirrhinum majus: the protein shows homology to transcription factors. EMBO J. **9:**605–613.
57. **Sugimoto, K., and K. Matsumoto.** Unpublished data.
58. **Sutton, A., D. Immanuel, and K. T. Arndt.** 1991. The *SIT4* protein phosphatase functions in late $G_1$ for progression into S phase. Mol. Cell. Biol. **11:**2133–2148.
59. **Treisman, R.** 1990. The SRE: a growth factor responsive transcriptional regulator. Sem. Cancer Biol. **1:**47–58.
60. **Treisman, R.** 1994. Ternary complex factors: growth factor regulated transcription activators. Curr. Opin. Genet. Dev. **4:**96–101.
61. **Uesono, Y., A. Fujita, A. Toh-e, and Y. Kikuchi.** 1994. The *MCS1*/*SSD1*/*SRK1*/*SSL1* gene is involved in stable maintenance of the chromosome in yeast. Gene **143:**135–138.
62. **Vojtec, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
63. **Wilson, R. B., A. A. Brenner, T. B. White, M. J. Engler, J. P. Gaughran, and K. Tatchell.** 1991. The *Saccharomyces cerevisiae SRK1* gene, a suppressor of *bcy1* and *ins1*, may be involved in protein phosphatase function. Mol. Cell. Biol. **11:**3369–3373.
63a. **Winston, F.** Unpublished data.
64. **Winston, F., and P. L. Minehart.** 1986. Analysis of the yeast SPT3 gene and identification of its product, a positive regulator of Ty transcription. Nucleic Acids Res. **14:**6885–6900.
65. **Yashar, B., K. Irie, J. A. Printen, B. J. Stevenson, R. Ruggieri, S. G. Macdonald, F. McCormick, G. F. Sprague, Jr., K. Matsumoto, and B. Errede.** Yeast MEK-dependent signal transduction: response thresholds and parameters affecting fidelity. Submitted for publication.