Molecular Biology of the Cell
Vol. 5, 297–312, March 1994

A Role for Autophosphorylation Revealed by Activated Alleles of *FUS3*, the Yeast MAP Kinase Homolog

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Submitted October 26, 1993; Accepted January 25, 1994
Monitoring Editor: Michael Wigler

We have isolated dominant gain-of-function (gf) mutations in *FUS3*, a *Saccharomyces cerevisiae* mitogen-activated protein (MAP) kinase homolog, that constitutively activate the yeast mating signal transduction pathway and confer hypersensitivity to mating pheromone. Surprisingly, the phenotypes of dominant $FUS3^{gf}$ mutations require the two protein kinases, STE7 and STE11. $FUS3^{gf}$ kinases are hyperphosphorylated in yeast independently of STE7. Consistent with this, $FUS3^{gf}$ kinases expressed in *Escherichia coli* exhibit an increased ability to autophosphorylate on tyrosine in vivo. $FUS3^{gf}$ mutations suppress the signal transduction defect of a severely catalytically impaired allele of *STE7*. This finding suggests that the tyrosine-phosphorylated form of FUS3 is a better substrate for activation by STE7. Furthermore, these results imply that the degree of autophosphorylation of a MAP kinase determines its threshold of sensitivity to upstream signals.

INTRODUCTION

Yeast mating results from the mutual stimulation of **a** and α haploid cells via peptide pheromones and specific cell-surface receptors for these pheromones. Pheromone stimulation results in a switch from vegetative growth to a differentiated state permissive for mating. This switch requires transmission of the signal of pheromone binding at the cell surface to the nucleus through an evolutionarily conserved kinase cascade.

Although the pheromones and pheromone receptors are cell-type specific, the intracellular components involved in transducing the signal of pheromone binding are identical between the two cell types (Bender and Sprague, 1989). These downstream components include a heterotrimeric G protein encoded by *GPA1/SCG1* (Dietzel and Kurjan, 1987; Miyajima *et al.*, 1987), *STE4* and *STE18* (Whiteway *et al.*, 1989), a gene product of unknown function encoded by *STE5* (Mukai *et al.*, 1993; Perlman *et al.*, 1993), several protein kinases, encoded by the genes *STE20* (Leberer *et al.*, 1992), *STE11* (Rhodes *et al.*, 1990), *STE7* (Teague *et al.*, 1986), *FUS3* (Elion *et al.*, 1990) and *KSS1* (Courchesne *et al.*, 1989), and a transcription factor encoded by *STE12* (Dolan *et al.*, 1989; Errede and Ammerer, 1989). Genetic and biochemical evidence suggests that the order of action of the conserved kinase components is: STE11 → STE7 → FUS3/KSS1 (Cairns *et al.*, 1992; Gartner *et al.*, 1992; Stevenson *et al.*, 1992; Errede *et al.*, 1993; Zhou *et al.*, 1993).

The protein kinase FUS3 functions in this cascade to mediate both transcriptional induction of mating-specific genes and cell-cycle arrest in response to pheromone (Elion *et al.*, 1990; 1991a,b). FUS3 is redundant with KSS1 for stimulating mating-specific transcription; however, FUS3 is uniquely required for G1 arrest (Elion *et al.*, 1991a,b). The target of FUS3 for transcriptional induction is STE12, the pheromone-responsive transcription factor (Elion *et al.*, 1993). STE12 binds to pheromone responsive elements (PREs) found in the 5′ promoter sequences of many mating-specific genes (Dolan *et al.*, 1989; Errede and Ammerer, 1989) and is required for both basal and induced expression of mating-specific genes. Mutations in *STE12* that affect induction but not basal activity cause sterility (Kirkman-Correia *et al.*, 1993). STE12 has been shown to be phosphorylated upon addition of pheromone, and this

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phosphorylation correlates with its ability to activate transcription (Song *et al.*, 1991). Epitope-tagged FUS3 co-immunoprecipitates with and phosphorylates the STE12 protein in vitro (Elion *et al.*, 1993). The target of FUS3 for cell-cycle arrest is FAR1, which binds to and presumably inactivates the CDC28/cyclin complex in response to pheromone (Peter *et al.*, 1993). FAR1 is phosphorylated by FUS3 in an in vitro kinase assay and interacts with FUS3 in the two-hybrid assay in vivo (Elion *et al.*, 1993; Errede *et al.*, 1993; Peter *et al.*, 1993).

FUS3 is a member of the family of MAP kinases (mitogen-activated protein kinases; MAPKs) or ERKs (extracellular signal-responsive kinases; Boulton *et al.*, 1990; Gotoh *et al.*, 1991; Her *et al.*, 1991; reviewed in Cobb *et al.*, 1991; Pelech and Sanghera, 1992; Errede and Levin, 1993; Levin and Errede, 1993; Nishida and Gotoh, 1993; Ruderman, 1993). MAP kinases were originally identified as phosphoproteins with molecular weights of ~42 and 44 kD that became phosphorylated in retrovirally transformed cells or upon addition of mitogenic stimuli (Cooper and Hunter, 1981a,b; Cooper *et al.*, 1982; Martinez *et al.*, 1982; Cooper *et al.*, 1984). Induction of MAP kinase activity in animal cells occurs in response to a variety of stimuli, both mitogenic and nonmitogenic (reviewed in Cobb *et al.*, 1991; Chao, 1992; Ruderman, 1993).

Activation of MAP kinases in vivo is associated with phosphorylation of tyrosine and threonine residues that are highly conserved among members of this protein kinase family (Anderson *et al.*, 1990; Gomez *et al.*, 1990; Ahn *et al.*, 1991; Boulton and Cobb, 1991; Payne *et al.*, 1991; reviewed in Nishida and Gotoh, 1993). Recombinant MAP kinases or ERKs produced in *Escherichia coli* autophosphorylate on a tyrosine residue in vitro (Crews *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991). This tyrosine autophosphorylation is intramolecular (Wu *et al.*, 1991) and occurs on the conserved tyrosine residue that is also phosphorylated in stimulated cells (Wu *et al.*, 1991; Rossomando *et al.*, 1992a). In some cases autophosphorylation occurs on serine and/or threonine residues as well (Crews *et al.*, 1991; Seger *et al.*, 1991; Wu *et al.*, 1991; Robbins and Cobb, 1992). Tyrosine autophosphorylation is associated with an increase in kinase activity, although the kinase activity of the recombinant protein is only 1% that of MAP kinase isolated from mitogen-stimulated cells (Seger *et al.*, 1991; Wu *et al.*, 1991). Surprisingly, a catalytically inactive mutant MAP kinase containing a lysine to arginine substitution in the ATP binding site of the protein is fully phosphorylated in vivo on both threonine and tyrosine residues, arguing that in vivo activation can occur by another kinase rather than by autophosphorylation (Posada and Cooper, 1992).

FUS3 is phosphorylated on the conserved threonine and tyrosine residues in vivo in response to α factor (Gartner *et al.*, 1992). As was found for MAP kinases, a catalytically inactive FUS3 allele (K42R) is hyperphosphorylated on these residues even in the absence of pheromone binding (Gartner *et al.*, 1992). This result has been taken to mean that: 1) the normal pathway for phosphorylation of FUS3 is via phosphorylation by another protein kinase and 2) active FUS3 has a role in down-regulating its own phosphorylation.

Recently, MAP kinase kinases (MAPKKs or MEKs, for MAP kinase/ERK kinase) that share homology with *STE7* have been cloned from a number of species (Crews *et al.*, 1992; Seger *et al.*, 1992; Kosako *et al.*, 1993; Zheng and Guan, 1993). Interestingly, MAPKKs have dual specificity, phosphorylating MAP kinases on the appropriate threonine and tyrosine residues (L'Allemain *et al.*, 1992; Rossomando *et al.*, 1992b). This phosphorylation is independent of MAP kinase activity. Consistent with this picture in vertebrates, the yeast MAPKK, STE7, phosphorylates FUS3 in vitro (Errede, *et al.*, 1993) and probably in vivo (Gartner *et al.*, 1992; Errede *et al.*, 1993). From these data, it is clear that MAP kinases can be phosphorylated independent of their ability to autophosphorylate. However, the use of constitutively inactive MAP kinases in these experiments could obscure a role for autophosphorylation: because the MAP kinase is inactive, the consequences of autophosphorylation for activity could not be detected. For example, autophosphorylation could enhance the ability of the MAP kinase to act as a substrate for activation by MAPKK.

In this paper, we describe gain-of-function mutations in the yeast MAP kinase FUS3 that constitutively activate mating-specific transcription. We present evidence that mutations in FUS3 that increase autophosphorylation have dramatic effects on the sensitivity of the cell to extracellular signals.

MATERIALS AND METHODS

Media and Strains

Standard yeast media were prepared as described (Sherman *et al.*, 1986). To maintain plasmids, we grew strains in synthetic complete (SC) medium lacking uracil, leucine and/or tryptophan. For metabolic labeling experiments, cells were grown in modified Wickerham's minimal medium supplemented with nutritional requirements (Brill, 1993). Bacterial strains were grown on LB medium containing 100 μg/ml ampicillin for plasmid selection (Sambrook *et al.*, 1989). Metabolic labeling of *E. coli* cultures was performed in phosphate-depleted LB (Rubin, 1974).

All yeast strains (Table 1) are derived from the S288C strain background and thus contain the naturally occurring *kss1*$^-$ allele and are sterile in the absence of *FUS3* (Elion *et al.*, 1991a). Transformations were by the lithium acetate method (Ito *et al.*, 1983). Plasmids used for gene replacements are listed in Table 2. JBY311 is the isogenic *ura3-52* parent of JBY350, which has been described previously (Elion *et al.*, 1991a). The *lys2::FUS1::lacZ* allele was converted into the *LYS2* locus as follows: A *LYS2* strain was first transformed with a *URA3* plasmid harboring *lys2::FUS1::lacZ* (pJB230), selecting for uracil prototrophy. Lys$^-$ strains in which the *LYS2* locus had been replaced by *lys2::FUS1::lacZ* were subsequently identified as α-amino adipate resistant (Chattoo *et al.*, 1979). Lys$^-$ Ura$^-$ derivatives that had lost pJB230

Table 1. Yeast strains

Strain	Genotype	Source
Strains isogenic to JBY532		
JBY532	*MAT***a** *ura3-52 leu2-3,112 trp1Δ1 his3Δ200 ade2 lys2::FUS1::lacZ kss1*	This work
JBY634	JBY532 *fus3-9::TRP1*	This work
Strains isogenic to JBY534		
JBY534	*MAT***a** *fus3-7::HIS3 ura3-52 leu2-3,112 trp1Δ1 his3Δ200 ade2 lys2::FUS1::lacZ kss1*	This work
JBY602	JBY534 *ste4::URA3*	This work
JBY606	JBY534 *ste7Δ3::URA3*	This work
JBY607	JBY534 *ste11Δ4::URA3*	This work
JBY610	JBY534 *ste5-102::URA3*	This work
JBY627	JBY534 *ste12Δ1::URA3*	This work
JBY789	JBY534 *ste7-101::URA3*	This work
Strains isogenic to JBY597		
JBY597	*MAT***a** *ura3-52 leu2-3,112 trp1Δ63 his3-11,15* or *his3Δ200 ade2 kss1 lys2::FUS1::lacZ* Gal$^+$	This work
JBY600	JBY597 *fus3-7::HIS3*	This work
JBY825	JBY597 *fus3-7::HIS3 ste7-101::URA3*	This work
Tester strains		
JBY367	*MATα sst2-4 ura1* and/or *ura3-52 trp1Δ1 his6 lys2-801 cry1 cyh2 can1 [met? rme1?]*	This work
JBY311	*MATα ura3-52 lys9*	This work
JBY350	*MATα lys9*	Elion *et al.*, 1991a

Strain constructions are described in MATERIALS AND METHODS.

were tested for the ability to be induced for β-galactosidase activity by α factor (Trueheart *et al.*, 1987).

All strain constructions by gene replacement (Rothstein, 1983) were confirmed by Southern analysis (Southern, 1975). *fus3-9::TRP1* was introduced as an *Fsp* I-*Sph* I fragment from pJB228. *fus3-7::HIS3* (pJB225) and *ste5-102::URA3* (pJB221) were introduced as described (Elion *et al.*, 1991a). *ste4::URA3* was introduced from ste4::Tnluk#1 cut with *Bam*HI and *Sph*I. *ste7Δ3::URA3* was derived from pNC149 digested with *Sac*I. *ste7-101::URA3* was obtained from pJB311 cut with *Hin*dIII and *Cla*I. *ste11Δ1::URA3* was obtained from pNC202 cut with *Xba*I. *ste12Δ4::URA3* was derived from pNC163 cut with *Cla*I.

Plasmids and DNA Manipulations

Plasmid constructions (Table 2) were performed by standard methods (Sambrook *et al.*, 1989). pJB230 (*lys2::FUS1::lacZ*) is YCp401 (Ma *et al.*, 1987) with a 4.5 kb *Nco*I fragment of *FUS1::lacZ* from pSB231 (Trueheart *et al.*, 1987) cloned into the *Nco*I site in the middle of *LYS2*. The coding sequence of *FUS1::lacZ* is oriented opposite to that of *LYS2*. pJB228 (*fus3-9::TRP1*) a 0.8 kb *Bam*HI-*Bgl* II fragment of *TRP1* from pJH-W1 (courtesy of John Hill) in the unique *Bgl*II site of pYEE97 (Elion *et al.*, 1991a). pJB311 (*ste7-101::URA3*) is pUC118 carrying the *ste7Δ1* allele on a *Hin*dIII fragment from plasmid p8 (K. Tatchell; Chaleff and Tatchell, 1985) with the *URA3* gene on a 1.1 kb *Xba*I fragment cloned into the unique *Avr*II site. The resulting construct lacks the 0.53 kb *Eco*RV fragment encoding much of the catalytic portion of *STE7*.

pJB236 carries *FUS3* on a *Bam*HI, *Hin*dIII fragment from pYEE81 (Elion *et al.*, 1990), cloned into the *Bam*HI and *Hin*dIII sites of the polylinker of pRS315, a *LEU2, CEN* vector (Sikorski and Hieter, 1989). *FUS3-101* and *fus3-102* were cloned into pRS315 by the same method, generating plasmids pJB237 and pJB241. pJB318 (*STE7, URA3, CEN*; courtesy of H.-P. Liu) was made by subcloning *STE7* on a 2.1 kb *Hin*dIII fragment from pSTE7.4 (Chaleff and Tatchell, 1985) into the

Table 2. Plasmids

Plasmid name	Relevant markers	Source
Plasmids for making isogenic disruption strains		
pJB221	*ste5-102::URA3*	Elion *et al.*, 1991a
pJB225	*fus3-7::HIS3*	Elion *et al.*, 1991a
pJB228	*fus3-9::TRP1*	This work
pJB230	*URA3, CEN, lys2::FUS1::lacZ*	This work
pJB311	*ste7-101::URA3*	This work
pNC149	*ste7Δ3::URA3*	B. Errede
pNC163	*ste12Δ1::URA3*	B. Errede
pNC202	*ste11Δ4::URA3*	B. Errede
ste4::Tnluk#1	*ste4::URA3*	M. Whiteway
Plasmids carrying alleles of *STE7*		
pJB318	*URA3, CEN, STE7*	This work
pJB319	*URA3, CEN, ste7-A220*	This work
pJB320	*URA3, CEN, ste7-A349*	This work
pNC318	*TRP1, CEN, pCYC1::STE7-myc*	Zhou *et al.*, 1993
pNC318-R220	*TRP1, CEN, pCYC1::ste7-R220-myc*	Zhou *et al.*, 1993
pJB321	*TRP1, CEN, pCYC1::ste7-A220-myc*	This work
pJB322	*TRP1, CEN, pCYC1::ste7-A349-myc*	This work
Plasmids carrying alleles of *FUS3*		
pYEE81	*URA3, CEN, FUS3*	Elion *et al.*, 1990
pJB236	*LEU2, CEN, FUS3*	This work
pJB237	*LEU2, CEN, FUS3-101*	This work
pJB27	*LEU2, CEN, FUS3-107*	This work
pJB241	*LEU2, CEN, fus3-102*	This work
pJB278	*LEU2, CEN, fus3-52*	This work
pGAL-FUS3 expression plasmids		
pJB267	*LEU2, CEN, pGAL::FUS3*	This work
pJB268	*LEU2, CEN, pGAL::FUS3-101*	This work
pJB269	*LEU2, CEN, pGAL::fus3-102*	This work
pJB304	*LEU2, CEN, pGAL::fus3-52*	This work
E. coli expression plasmids		
pJB273	*T7•Tag::FUS3*	This work
pJB274	*T7•Tag::FUS3-101*	This work
pJB275	*T7•Tag::fus3-102*	This work
pJB300	*T7•Tag::fus3-52*	This work
pJON210	*T7•Tag::nup2* (antisense; partial clone)	J. Loeb
pJB301	*TrpE::FUS3*	This work

Plasmid constructions are described in MATERIALS AND METHODS.

*Hind*III site of pRS316 (Sikorski and Hieter, 1989). pJB319 (*ste7-A220*) and pJB320 (*ste7-A349*) were made by site-directed mutagenesis of pJB318. pNC318 (*TRP1, CEN, pCYC1::STE7-myc*) and pNC318-R220 (*TRP1, CEN, pCYC1::ste7-R220-myc*) were from B. Errede (Zhou *et al.*, 1993). pJB321 (*TRP1, CEN, pCYC1::ste7-A220-myc*) and pJB322 (*TRP1, CEN, pCYC1::ste7-A349-myc*) were made by subcloning 0.7 kb *Avr*II, *Bgl*II fragments from pJB319 and pJB320 into pNC318.

pJB267 (*LEU2, CEN, pGAL::FUS3*) and related plasmids carrying alleles of *FUS3* under control of the *GAL1, 10* promoter (Johnston and Davis, 1984) were constructed in a vector derived from pRS315, pRS315-gal. A *Bam*HI site 16 nucleotides upstream of the *FUS3* initiation codon was engineered by oligonucleotide mutagenesis, and an *Xba*I site was engineered downstream of the first *Bss*HII site 3′ of the *FUS3* coding sequence in plasmids pJB236 (*FUS3*), pJB237 (*FUS3-101*), pJB241 (*fus3-102*), and pJB278 (*fus3-52*). The resulting 1.3 kb *Bam*HI, *Xba*I fragments containing *FUS3* coding sequences were subcloned into pRS305-gal at the *Bgl*II and *Xba*I sites in the polylinker.

pJB273 (pET::*FUS3 E. coli* expression plasmid) and related plasmids carrying alleles of *FUS3* fused to the 15 N-terminal amino acids of bacteriophage T7 gene 10 (the "T7-Tag") were constructed in the vector pET-3a (Studier and Moffatt, 1986; Rosenberg *et al.*, 1987; Studier *et al.*, 1990). Oligo-directed mutagenesis was used to generate an in-frame *Bam*HI site immediately following the initiator methionine of *FUS3* in plasmids pJB236, pJB237, pJB241, and pJB278. The resulting 2.3 kb *Bam*HI, *Eco*RI fragments containing *FUS3* coding sequences were cloned into the *Bam*HI and *Eco*RI sites of pET-3a, generating plasmids pJB273 (*FUS3*), pJB274 (*FUS3-101*), pJB275 (*fus3-102*), and pJB300 (*fus3-52*). pJB301 (TrpE::*FUS3 E. coli* expression plasmid) was made by cloning the 2.3 kb *Bam*HI, *Eco*RI fragment containing *FUS3* into pATH3 (Koerner *et al.*, 1991).

Mutagenesis of FUS3

Hydroxylamine mutagenesis was performed on 10 μg of pYEE81 or pJB236 DNA as described (Busby *et al.*, 1982). Mutagenized pYEE81 DNA was transformed into *E. coli* strain DH5α to make 12 independent pools of between 20 000 and 100 000 mutagenized plasmids per pool. Mutagenized pJB236 was transformed into *E. coli* strain MC1066 to make four independent pools each containing approximately 100 000 mutagenized plasmids. The frequency of *LEU2*, *URA3*, and *FUS3* knockout mutations among the mutagenized transformants was in each case 1-2%.

Polymerase chain reaction (PCR) mutagenesis of FUS3 was carried out using primers that generate a 1.5 kb PCR product. PCR products from six independent reactions were purified, digested with *Spe*I and *Nsi*I and cloned into the *Xba*I and *Pst*I sites of pRS315 (Sikorski and Hieter, 1989). The resulting plasmids contain single inserts of *FUS3* in the same orientation with respect to the polylinker as that of pJB236. The average knockout frequency for the *FUS3* pools was estimated to be 4% by determining the percentage of plasmids incapable of conferring mating ability on strain JBY534. Sequence analysis of individual mutants revealed an average of one mutation every 800 bp.

fus3-52 (K42R) was generated by oligo site-directed mutagenesis of pJB236. For oligo mutagenesis of *FUS3* and *STE7*, single-stranded plasmid DNA was prepared from an *Hfr dut*$^-$ ung$^-$ bacterial strain, RZ1032, and mutagenized essentially as described (Kunkel, 1985; Vieira and Messing, 1987). Each *FUS3* mutant gene obtained from hydroxylamine or PCR mutagenesis was sequenced in its entirety on both strands. For oligo-directed mutations, one strand of the region of interest was sequenced. *FUS3* sequencing primers were obtained from Oligos, Etc. (Guilford, CT). All other oligonucleotides were synthesized by Sandy Schultz of the Biopolymers Laboratory (Center for Cancer Research, M.I.T., Cambridge, MA).

Screens for FUS3 Gain-of-Function (gf) Mutations

We devised mutant screens to identify: 1) increased basal expression of *FUS1::lacZ* and 2) haploid-specific lethality (Blinder *et al.*, 1989). The following is a summary of the results of screens for *FUS3*^{gf} mutations (see Figure 1), which are described in detail elsewhere (Brill, 1993). Two alleles that cause elevated expression of *FUS1::lacZ* were identified from a screen of 27 000 hydroxylamine-mutagenized pYEE81 plasmids: *FUS3-101* carries an A to C transversion at nucleotide 481 (I161L); *fus3-102* is a G to A transition at nucleotide 143 (C28Y). *fus3-111* and *fus3-112* were isolated from a screen of approximately 300 PCR-mutagenized plasmids for *FUS3*^{gf} mutations that increase basal *FUS1::lacZ* expression. *fus3-111* is a T to A transversion at nucleotide 26 (I9K). *fus3-112* carries a T to C transition at nucleotide 19 (Y7H). *FUS3-106* and *FUS3-107* were isolated in a screen of approximately 5000 hydroxylamine-mutagenized pJB236 plasmids for *FUS3*^{gf} alleles that bypass the requirement for *STE5* for increasing basal *FUS1::lacZ* expression in strain JBY610 (*fus3*Δ *ste5*Δ). These alleles each carry a single G to A transition, at nucleotides 142 and 679 (D48N and D227N), respectively.

Haploid-specific lethality was assayed for approximately 10 000 plasmid transformants of a *sir3*^{ts} strain. No haploid-lethal alleles of *FUS3* were identified. Roughly 5000 and 1000 hydroxylamine-mutagenized *FUS3* plasmids (pJB236) were screened for increased basal *FUS1::lacZ* expression in strains JBY607 (*fus3*Δ *ste11*Δ) and JBY606 (*fus3*Δ *ste7*Δ), respectively, and approximately 4500 and 15 000 PCR-mutagenized FUS3 plasmids were screened in JBY607 and JBY789 (*fus3*Δ *ste7*Δ), respectively. No *FUS3*^{gf} mutations that bypass the requirement for either *STE11* or *STE7* were isolated from either of these screens.

Halo assays for **a**-factor secretion and for α-factor sensitivity were performed as described (Elion *et al.*, 1990; Sprague, 1991). Patch matings and quantitative mating assays were performed as described (Trueheart *et al.*, 1987; Elion *et al.*, 1991a) except tester strain JBY311 (*MAT*α *ura3-52 lys9*) was employed to abolish the background of prototrophic revertants, and strains were mated overnight for 16-22 h at 30°C. Liquid β-galactosidase assays were performed using whole-cell extracts (Craven *et al.*, 1965). α-factor inductions were performed for 90 min to 2 h (as indicated) at 30°C in synthetic complete medium on washed cell cultures. Filter X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside) assays were performed according to the method of L. Marsh (Albert Einstein College of Medicine, Bronx, NY).

Preparation and Affinity Purification of Anti-FUS3 Polyclonal Antiserum

Recombinant FUS3 protein was prepared for use in the production of rabbit αFUS3 polyclonal antiserum by isopropyl-β-D-thiogalactopyranoside (IPTG) induction of cultures of fresh pJB273 (pET3a::*FUS3*) transformants of *E. coli* strain BL21[DE3]. Cells were boiled in 2× Laemmli sample buffer (2× LSB), electrophoresed on twelve 3 mm preparative 10% Laemmli gels, and gel slices containing FUS3 fusion protein were excised, macerated, and eluted by overnight incubation at 65°C in electrophoresis buffer. The purity and identity of the eluted and concentrated protein were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and reactivity with anti-T7 · Tag antiserum (Novagen, Madison, WI) in an immunoblot using alkaline phosphatase (Promega Corporation, Madison, WI). Rabbits were injected with 500 μg of protein, then boosted twice with 250 μg of protein by Mindy Meadows Donovan at East Acres Biologicals (Southbridge, MA). For affinity purification of anti-FUS3 polyclonal antiserum, TrpE-FUS3 protein was induced in bacteria transformed with pJB301 (pATH::*FUS3*) and partially purified from inclusion bodies as described (Koerner *et al.*, 1991).

Metabolic Labeling and Immunoprecipitation

Yeast strains to be labeled were cultured as described (Brill, 1993). Briefly, 2.5 OD_{600} units of cells grown in modified Wickerham's minimal medium and starved for either sulfate or phosphate were labeled for 1 h at 30°C. ^{32}P labelings were performed by adding 0.8–1.2 mCi ^{32}P-orthophosphate (ICN Biomedicals, Costa Mesa, CA) to each tube.

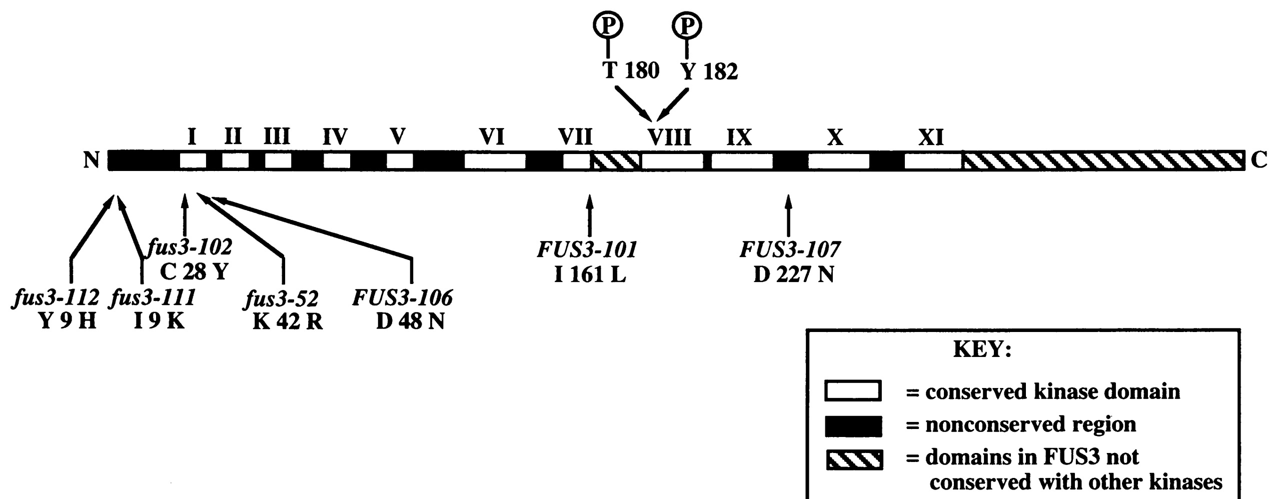

Figure 1. Positions of amino acid substitutions in *FUS3* point mutants. *FUS3-101* (I161L), *FUS3-106* (D48N), and *FUS3-107* (D227N) are dominant *FUS3^{gf}* mutations. *fus3-102* (C28Y), *fus3-111* (I9K), and fus3-112 (Y7H) are recessive *fus3^{gf}* mutations. *fus3-52* is a lysine to arginine substitution (K42R) in the ATP binding site. T180 and Y182 are the conserved phosphorylation sites. The white boxes represent domains of FUS3 that are conserved with other protein kinases. The black boxes represent regions between these domains that are less well conserved. The cross-hatched boxes represent domains that are present in FUS3 but not in other kinases. The entire 353 amino acid protein is drawn approximately to scale.

For ^{35}S-methionine and cysteine labelings, 0.25 mCi Expre^{35}S^{35}S label (DuPont NEN Research Products, Boston, MA) was added to each sample. Pelleted cells were frozen in liquid nitrogen before extract preparation. Cells were vortexed in the presence of glass beads (400–600 μm, Sigma, St. Louis, MO) and ice-cold breaking buffer (50 mM tris(hydroxymethyl)aminomethane [Tris], pH 7.4, 150 mM NaCl, 10 mM $MgCl_2$, 0.5% Triton X-100, 50% glycerol) containing freshly added deoxythiothreitol (DTT; 1 mM final concentration), sodium orthovanadate (0.5 mM final concentration), β-glycerophosphate (15 mM final concentration), *p*-nitrophenyl phosphate (PNPP; 15 mM final concentration), 1× protease inhibitor mix (final concentrations of 5 μg/ml each aprotinin, antipain, leupeptin, and pepstatin A, and 10 μg/ml chymostatin) and 1× phosphatase inhibitor mix (final concentrations of 0.5 mM sodium metavanadate, 1 mM sodium pyrophosphate, 1 mM sodium azide, and 1 mM sodium fluoride). All chemicals including inhibitors were from Sigma. Equal numbers of TCA-precipitable counts were used for immunoprecipitations.

FUS3 was immunoprecipitated from extracts using 10 μl of affinity-purified anti-FUS3 polyclonal antiserum for FUS3 expressed from its own promoter or 20 μl of affinity-purified anti-FUS3 polyclonal antiserum for *GAL*-overproduced FUS3 or FUS3 expressed from the T7 promoter in *E. coli*, using 200 μg of cold *fus3*Δ extract (JBY534) to block nonspecific binding. In some cases, rabbit polyclonal anti-PSTAIR antiserum (Upstate Biotechnology, Lake Placid, NY) was used as an internal control for labeling and immunoprecipitation (IP). IPs were performed in 1 ml RIPA buffer (Harlow and Lane, 1988) containing 0.1% SDS, 1 mM DTT, and protease inhibitor mix. 20 μl of 50% washed protein A-agarose beads (Sigma #P2670) were used for each IP. Immune complexes were collected by brief (<1 s) centrifugation and were then washed three times in RIPA plus 0.1% SDS, two times in RIPA containing 0.5 M NaCl plus 0.1% SDS, and once in distilled water. Samples were resuspended in 2× LSB and boiled for 5 min before separating on 10% Laemmli gels by SDS-PAGE. Gels containing ^{35}S-methionine and cysteine-labeled samples were rinsed in H_2O and treated with either Amplify (Amersham, Arlington Heights, IL) or 1 M sodium salicylate for 30 min prior to drying onto Whatman paper (Whatman, Hillsboro, OR) and exposing to film (Kodak XAR-5).

E. coli cultures starved for phosphate for 2 h at 36° were induced with IPTG for 2 h and labeled with 100 μCi ^{32}P-orthophosphate for 0.5 h at 37°. Cell extracts were obtained by boiling cells in 2× LSB. Either 40μl or Forty microliters of extract were immunoprecipitated in RIPA containing protease inhibitors. Quantitation of labeled immunoprecipitated proteins was on a Fuji BAS 2000 Bio-Image Analyzer (Tokyo, Japan).

Protein Gels, Immunoblotting, and Phosphoamino Acid Analysis

Protein extracts for Westerns were made as described for metabolically labeled samples. For Western blotting, gels were transferred to either 0.45 μm nitrocellulose (Schleicher and Schuell) for ^{125}I-protein A detection or to Amersham (Arlington Heights, IL) ECL nitrocellulose for chemiluminescent detection. Incubations were performed in Blotto, which is PBS containing 0.05% Tween-20 (Sigma; PBST) and 5% non-fat dried milk. Antibodies were used at the following dilutions: affinity-purified anti-FUS3 polyclonal antiserum, 1:500; anti-myc 9E10 (Evan *et al.*, 1985) ascites culture supernatant, 1:3. For radioactive detection, anti-myc westerns were incubated with rabbit anti-mouse secondary antibody (Jackson Laboratories, Bar Harbor, ME) diluted 1:500. Blots were then incubated with ^{125}I-Protein A (Amersham) at a 1:2000 dilution. Autoradiography was at −80°C with an enhancing screen. ECL westerns (Amersham) were performed using donkey anti-rabbit, or sheep anti-mouse horseradish peroxidase-conjugated secondary antibody (Amersham) diluted 1:1000. To reduce background, blots were occasionally rewet for 30 s in the used ECL reagents before re-exposing to film.

Phosphoamino acid analysis was performed on either T7 · Tag::FUS3 from 20 μl of ^{32}P-labeled *E. coli* extracts or FUS3 immunoprecipitated from ^{32}P-labeled yeast extracts. Samples were electrophoresed on 10% Laemmli gels and electroblotted in alkaline transfer buffer to PVDF+ membranes (Kamps and Sefton, 1989; MicronSeparations, Incorporated, Westboro, MA). Protein-containing bands were identified by staining with India Ink, excised and subjected to acid hydrolysis and TLC electrophoresis essentially as described (Nairn and Greengard,

1987; LeGendre and Matsudaira, 1989). Spots containing cold phosphoamino acids were detected by staining with 0.25% ninhydrin dissolved in 95% ethanol. Quantitation of radioactive phosphoamino acids and of ^{125}I-Protein A Westerns was performed on a Fuji Bio-Image Analyzer (Tokyo, Japan).

RESULTS

FUS3^{gf} Mutations Increased Basal *FUS1* Expression

To better understand the role of *FUS3* in yeast mating signal transduction, we isolated gain-of-function (gf) mutations in *FUS3* from randomly mutagenized pools of plasmid DNA that had been transformed into appropriate yeast strains and screened for their ability to confer a constitutive mating response. Transformants were screened for one of two phenotypes: 1) constitutive elevation of mating-specific transcription in the absence of pheromone or 2) constitutive cell-cycle arrest resulting in haploid-specific lethality. Six *FUS3^{gf}* mutations causing constitutive expression of the mating-specific *FUS1::lacZ* reporter construct (Trueheart *et al.*, 1987) were isolated and no haploid-lethal alleles were obtained (see MATERIALS AND METHODS). We tested the dominance of the *FUS3^{gf}* mutations (see MATERIALS AND METHODS) and found that three are dominant for increased *FUS1::lacZ* expression (*FUS3-101, FUS3-106,* and *FUS3-107*) and three are recessive (*fus3-102, fus3-111,* and *fus3-112*). The amino acid substitutions determined by sequence analysis of the *FUS3^{gf}* alleles are depicted in Figure 1. With the exception of *fus3-102* (C28Y), these mutations fall at residues not highly conserved among the family of MAP kinases. For most experiments, *FUS3-101* and *fus3-102* were examined as representative dominant and recessive alleles, and a catalytically impaired allele with a lysine to arginine substitution in the ATP binding site, *fus3-52* (K42R), was used as a negative control.

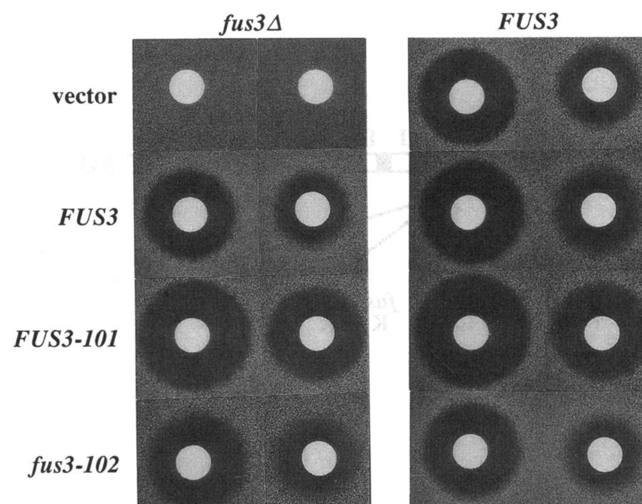

Figure 2. α-factor sensitivity of strains expressing *FUS3^{gf}* alleles. Nonisogenic JBY534 (*fus3*Δ) and JBY532 (*FUS3*) strains were transformed with plasmids bearing different alleles of *FUS3:* pRS315 (vector), pJB236 (*FUS3*), pJB237 (*FUS3-101*), and pJB241 (*fus3-102*). α-factor sensitivity was measured by growth inhibition of these strains in an α-factor halo assay. For each strain, filter disks were spotted with 7 μl of either 1.5 mM (left) or 0.5 mM α factor in DMSO (see MATERIALS AND METHODS).

Table 3. *FUS1::lacZ* expression in *FUS3^{gf}* strains

	Units of β-galactosidase activity[a]			
Strain[b]	*fus3*Δ		*FUS3*	
α factor	−	+	−	+
FUS3 plasmid[c]				
vector	0.1	0.1	0.7	144
FUS3	0.5	135	0.7	126
FUS3-101	2.7	166	2.5	198
fus3-102	3.8	158	1.1	170
fus3-52	0.2	0.1	0.2	84

[a] β-galactosidase units are calculated as mmol ONPG cleaved/min/mg protein. α-factor induction (90 min), extract preparation and enzyme assays were performed as described in MATERIALS AND METHODS. Numbers are averages from a typical experiment performed in duplicate on two independent transformants.

[b] Isogenic *fus3*Δ (JBY634) and *FUS3* (JBY532) strains were used. Complete genotypes are given in Table 1.

[c] Plasmids pRS315 (*CEN, LEU2* vector) and derivatives pJB236 (*FUS3*), pJB237 (*FUS3-101*), pJB241 (*fus3-102*), and pJB278 (*fus3-52*).

Phenotypic characterization of *FUS3-101* and *fus3-102* revealed that neither mutation causes a growth defect in a wild-type strain and that strains bearing these mutations mate normally. *FUS3-101* and *fus3-102* increase basal *FUS1::lacZ* expression roughly fivefold and *FUS3-101* is dominant for this effect (Table 3). *FUS3-101* also exhibits dominant hypersensitivity to α factor, as revealed by a halo assay (Figure 2). With the exception of its effect on *FUS1::lacZ*, the recessive *fus3^{gf}* mutant *fus3-102* shows no differences from *FUS3* strains for all phenotypes tested and has been included in experiments solely for comparison with *FUS3-101*.

FUS3^{gf} Mutations Require Signaling Pathway Components for Their Function

To determine the order of action of FUS3 with respect to other components of the signaling pathway, we constructed a series of isogenic double mutant strains (see MATERIALS AND METHODS), each bearing a *FUS3^{gf}* allele and a deletion of another gene required for mating signal transduction. These double mutant strains were tested for: 1) increased basal expression of *FUS1::lacZ* and 2) mating. Increased basal *FUS1::lacZ* in *FUS3-101* strains is partially independent of *STE4* and *STE5*; however, *FUS3-101* requires STE4 and *STE5* for diploid for-

mation, a more stringent test of mating pathway activation (Figure 3). Increased *FUS1::lacZ* expression and mating of *FUS3-101* strains depend on the kinases encoded by *STE11* and *STE7* as well as the transcription factor encoded by *STE12* (Figure 3). In addition, overproduction of *FUS3-101* from the inducible *GAL1, 10* promoter does not bypass the requirement for *STE7* for diploid formation (Figure 4C).

Dominant FUS3^{gf} Mutations Constitute Hyperactive Alleles of FUS3

Genetic evidence suggests that *FUS3-101* encodes an activated kinase: First, *FUS3-101* is dominant (Figure 2; Table 3), which would be expected for a mutation causing an increase in enzymatic activity. Second, *FUS3-101* strains are hypersensitive to α factor (Figure 2), which might be expected of a mutant that is already partially activated. Third, *FUS3-101* complements the mating defect of *fus3Δ* better than either *FUS3* or *fus3-102* when their expression from the heterologous *GAL* promoter is limited to the same extent because no inducer was present (on raffinose growth medium; Figure 4B). This difference is not seen on galactose, which activates expression to a high level (Figure 4C).

Dominant FUS3^{gf} Kinases Are Hyperphosphorylated in Yeast

Since increased kinase activity is associated with phosphorylation of FUS3 and other MAP kinases, we examined whether FUS3^{gf} kinases are constitutively hyperphosphorylated in the absence of pheromone. The activated kinase, FUS3-101, is hyperphosphorylated in yeast in the absence of pheromone in a *fus3Δ* strain (Figure 5A, lane 8), and this hyperphosphorylation is dominant (Figure 5A, lane 3). The difference in the degree of phosphorylation between FUS3-101 and wild type is about twofold after taking into account differences in the amount of wild-type and mutant proteins observed by either Western blotting or by parallel immunoprecipitations of FUS3 from ^{35}S-methionine and cysteine-labeled extracts. This extent of hyperphosphorylation was observed in five independent experiments, based on quantitation with a phospho-image analyzer (see MATERIALS AND METHODS). As previously described, the phosphorylation of fus3-52 is roughly 10-fold higher than FUS3 protein in a *fus3Δ* strain but not in a *FUS3* strain (Figure 5A; compare lanes 5 and 10; Gartner *et al.*, 1992). By contrast, fus3-102 is not hyperphosphorylated in either *fus3Δ* or *FUS3* (Figure 5A, lanes 4 and 9). Thus hyperphosphorylation is observed for the dominant but not the recessive FUS3^{gf} kinase.

Hyperphosphorylation of FUS3^{gf} Kinases Is Independent of STE7

To determine whether the hyperphosphorylation of FUS3-101 in yeast requires STE7, FUS3 proteins overproduced from the *GAL* promoter were examined for their phosphorylation state in a *ste7Δ* strain. As seen by immunoprecipitation of FUS3 proteins from yeast cells grown in the presence of ^{32}P-orthophosphate, FUS3-101 is more highly phosphorylated than either FUS3 or fus3-102 (Figure 5B; compare lane 3 with lanes 2 and 4). Approximately equal amounts of protein were expressed, as determined by Western blotting performed on extracts from parallel unlabeled cultures, and immunoprecipitated, as verified by Coomassie staining. As an additional control for metabolic labeling and immunoprecipitations, polyclonal anti-PSTAIR antibody was used to immunoprecipitate CDC28, a protein whose phosphorylation is invariant over the cell cycle and is not affected by mating signal transduction (Moll *et al.*, 1991; Gartner *et al.*, 1992). The extent of hyperphosphorylation of FUS3-101 is approximately threefold after normalization to the amount of phospho-CDC28.

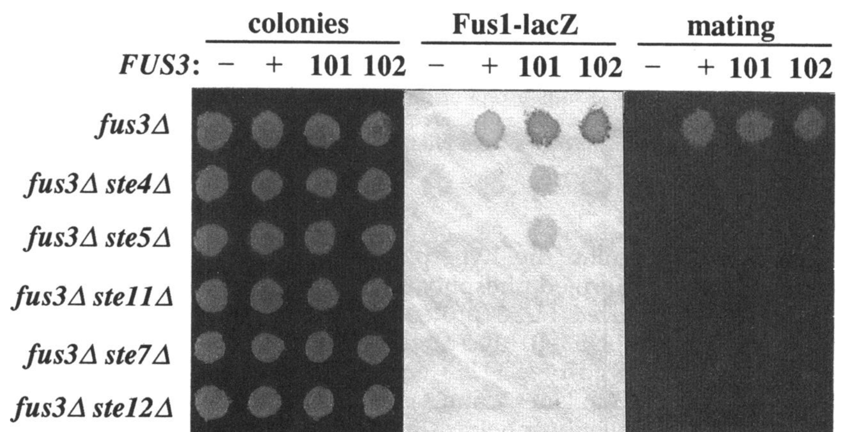

Figure 3. Epistasis tests of *FUS3^{gf}* mutations in strains lacking various *STE* genes. The left-hand panel shows patches of transformants of the following isogenic strains: JBY534 (*fus3Δ*), JBY602 (*fus3Δ ste4Δ*), JBY610 (*fus3Δ ste5Δ*), JBY607 (*fus3Δ ste11Δ*), JBY789 (*fus3Δ ste7Δ*), and JBY627 (*fus3Δ ste12Δ*). Plasmids were pRS315 (vector; "–"), pJB236 (*FUS3*; "+"), pJB237 (*FUS3-101*), and pJB241 (*fus3-102*). The middle panel shows basal *FUS1::lacZ* expression in these strains, as determined by 30 h X-gal assays (see MATERIALS AND METHODS). Complementation of *fus3Δ* for mating was assayed by 20-h patch matings, as shown in the panel on the right (see MATERIALS AND METHODS).

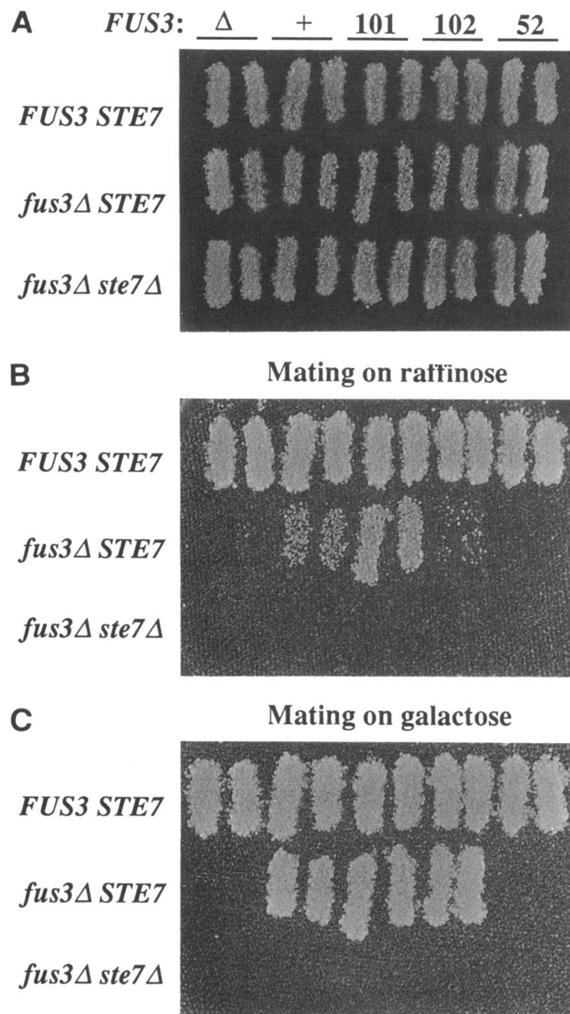

Figure 4. Mating ability of *GAL*-promoted *FUS3*^{sf} mutations. (A) Pairs of patches of plasmid transformants of Gal$^+$ strains. Plasmids were as follows: pRS315-gal (vector; "Δ"); pJB267 (*FUS3;* "+"); pJB268 (*FUS3-101*); pJB269 (*fus3-102*); and pJB304 (*fus3-52*). Strains were JBY597 (*FUS3 STE7*), JBY600 (*fus3*Δ *STE7*), and JBY825 (*fus3*Δ *ste7*Δ). (B) Mating ability of *GAL*-promoted *FUS3* transformants on raffinose (uninduced). Patches pregrown on selective SC raffinose plates were replica-mated for 4 h to a wild-type tester lawn (JBY350) on YPRaff plates, followed by printing to minimal medium to select for diploids. (C) Mating of these transformants on galactose. Patch mating assays were performed exactly as in B, except matings were performed on YPGal. The scattered papilli in B and C are prototrophic revertants of the mating tester strain. Plates were photographed 3 days after printing to select for diploids.

To further examine the hyperphosphorylation of FUS3-101, we performed phosphoamino acid analysis on *GAL*-overproduced FUS3 proteins expressed in a *fus3*Δ *ste7*Δ strain. FUS3-101 has more phosphotyrosine than does either wild-type FUS3 or fus3-102 (Figure 5C; compare lane 3 to lanes 2 and 4). In contrast, fus3-52 has no detectable phosphotyrosine (Figure 5C; lane 5). It is noteworthy that none of the proteins has detectable phosphothreonine, given that FUS3 and other MAP kinases require both threonine and tyrosine phosphorylation for full activation. All four proteins examined have a small amount of phosphoserine. Because fus3-52 is catalytically impaired, it is likely that this

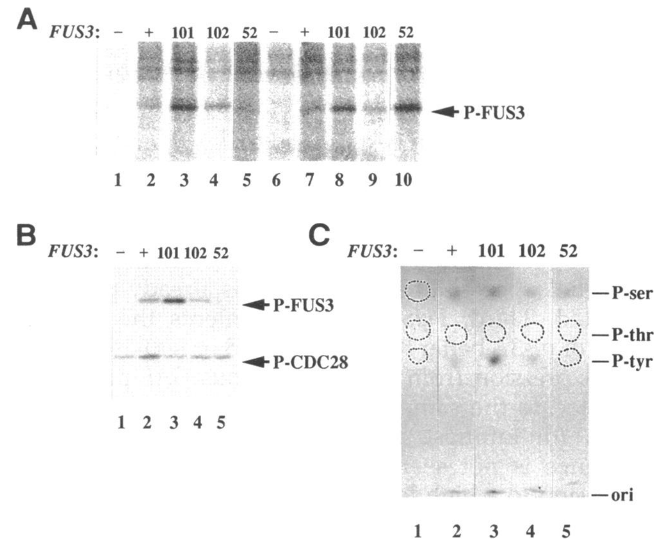

Figure 5. Hyperphosphorylation of FUS3-101 in yeast. (A) Phosphorylation state of FUS3 kinases expressed in isogenic *FUS3* and *fus3*Δ strains. Lanes 1–5, JBY532 (*FUS3*); lanes 6–10, JBY634 (*fus3*Δ). Plasmids were pRS315 (vector; "–"; lanes 1 and 6); pJB236 (*FUS3;* "+"; lanes 2 and 7); pJB237 (*FUS3-101;* lanes 3 and 8); pJB241 (*FUS3-102;* lanes 4 and 9); and pJB278 (*fus3-52;* lanes 5 and 10). Plasmid-containing strains were grown selectively in modified Wickerham's minimal medium labeled with ^{32}P-orthophosphate as described (see MATERIALS AND METHODS). Immunoprecipitations were carried out in the absence of unlabeled *fus3*Δ blocking extract and thus contain non-FUS3-specific bands. The band corresponding to phospho-FUS3 is indicated. Note that expression of *FUS3* from the *CEN* plasmid pJB236 is approximately 10-fold higher than that from the chromosome (compare lanes 1 and 2). (B) *STE7*-independent phosphorylation of *GAL*-overproduced FUS3 kinases. Strain JBY825 (*fus3*Δ *ste7*Δ) was transformed with the following plasmids (lanes 1–5): pRS315-gal (vector; "–"), pJB267 (*FUS3*), pJB268 (*FUS3-101*), pJB269 (*fus3-102*), and pJB304 (*fus3-52*). *GAL* inductions and metabolic labelings were performed as described (see MATERIALS AND METHODS). FUS3 and CDC28 were simultaneously immunoprecipitated using affinity-purified anti-FUS3 polyclonal antiserum and polyclonal anti-PSTAIR antiserum (see MATERIALS AND METHODS). Four hundred μg or micrograms of cold *fus3*Δ extract was used to block non-specific binding. This extract reduces the intensity but not the relative amount of the CDC28 band as determined by parallel immunoprecipitations using anti-PSTAIR alone. Roughly equal amounts of FUS3 protein were immunoprecipitated in lanes 2–5, as determined by Coomassie staining. (C) Phosphoamino acid analysis on samples prepared as in B. Phosphoamino acid analysis was performed as described (see MATERIALS AND METHODS). India Ink staining was used to verify that approximately equal amounts of labeled FUS3 protein were hydrolyzed. The identities of the phosphoamino acids are indicated as follows: P-ser (phosphoserine), P-thr (phosphothreonine), and P-tyr (phosphotyrosine). The ninhydrin staining pattern of phosphoamino acids for which no radioactivity was detected is indicated by hand-drawn circles.

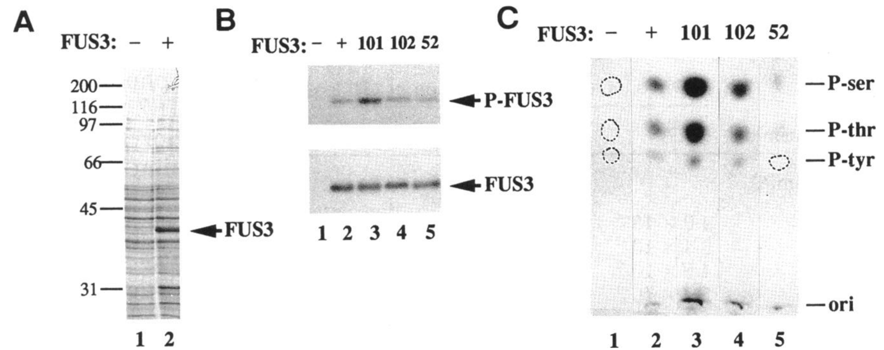

Figure 6. Increased autophosphorylation of FUS3-101 expressed in *E. coli*. (A) Expression of T7·Tag::FUS3 in *E. coli:* lane 1, pET-3a (T7·Tag vector; "–"); lane 2, pJB273 (T7·Tag::FUS3; "+"). Transformants of *E. coli* strain BL21[DE3] were induced with IPTG as described (see MATERIALS AND METHODS). (B) Phosphorylation of recombinant T7·Tag::FUS3 proteins expressed in *E. coli* (see MATERIALS AND METHODS). Induction conditions were the same as in A. Plasmids used were (lanes 1–5): pJON210 (pET-3a vector carrying a cloned fragment that does not make a fusion protein; "–"); pJB273 (T7·Tag::FUS3; "+"); pJB274 (T7·Tag::FUS3-101); pJB275 (T7·Tag::fus3-102); and pJB300 (T7·Tag::fus3-52). The top panel shows the phosphorylation state of T7·Tag::FUS3 proteins immunoprecipitated from extracts of *E. coli* cultures grown on ^{32}P-orthophosphate. Affinity-purified anti-FUS3 polyclonal antiserum was used for immunoprecipitations as described (see MATERIALS AND METHODS). Recombinant proteins were induced to a similar extent, as shown by a Western blot of equal volumes of unlabeled extracts (bottom panel), and approximately equal amounts of protein were immunoprecipitated in lanes 2–5, as determined by Coomassie staining. (C) Phosphoamino acid analysis of recombinant T7·Tag::FUS3 proteins produced in *E. coli*. Samples 1–5 were prepared as described in B. Phosphoamino acid analysis was performed as described (see MATERIALS AND METHODS). The positions of phosphoserine (P-ser), phosphothreonine (P-thr), and phosphotyrosine (P-tyr) are indicated. Hand-drawn circles indicate the positions of ninhydrin-stained cold phosphoamino acids for which no radioactivity was detected.

serine phosphorylation is due to the activity of some kinase other than FUS3, perhaps a consequence of FUS3 overexpression. The presence of phosphoserine serves as a useful internal control; the ratio of phosphotyrosine to phosphoserine in FUS3-101 is threefold higher than for either FUS3 or fus3-102. Thus the phosphoamino acid profile for FUS3-101 is consistent with enhanced tyrosine autophosphorylation.

Dominant FUS3^{gf} Kinases Exhibit Increased Autophosphorylation

To test the hypothesis that dominant FUS3^{gf} kinases have an increased ability to autophosphorylate, we examined the phosphorylation state of recombinant wild-type and mutant FUS3 kinases expressed in *E. coli.* T7·Tag::FUS3 fusion proteins expressed from the T7 promoter were induced (Figure 6A) and immunoprecipitated from extracts of *E. coli* cultures grown in the presence of ^{32}P-orthophosphate (see MATERIALS AND METHODS). FUS3-101 is more highly phosphorylated than FUS3 or fus3-102 (Figure 6B; compare lane 3 with lanes 2 and 4). Quantitation of the extent of this increased phosphorylation in four independent metabolic labeling experiments revealed that FUS3-101 is at least fourfold more highly phosphorylated than either FUS3 or fus3-102.

Phosphoamino acid analysis on phospho-FUS3 expressed in *E. coli* showed that recombinant FUS3-101 has twofold more phosphotyrosine than either FUS3 or fus3-102 (Figure 6C; compare lane 3 with lanes 2 and 4). This result was reproducible in three independent metabolic labeling experiments. As *E. coli* has no known tyrosine kinase activity, and fus3-52 shows no detectable tyrosine phosphorylation (Figure 6C, lane 5), autophosphorylation must account for the tyrosine phosphorylation observed for FUS3, FUS3-101, and fus3-102.

Unlike FUS3 expressed in yeast (Figure 5C), FUS3 from *E. coli* shows a substantial amount of serine and threonine phosphorylation (Figure 6C). FUS3-101 exhibits a greater degree of serine and threonine phosphorylation than do FUS3 and fus3-102 (Figure 6C; compare lane 3 with lanes 2 and 4). fus3-52 shows minimal phosphorylation on serine and threonine (Figure 6C, lane 5), suggesting that the serine and threonine phosphorylation observed for FUS3, FUS3-101 and fus3-102 is due to a FUS3-specific activity. Thus the relative increase in serine and threonine phosphorylation observed for FUS3-101 is likely to correspond to increased kinase activity.

Dominant FUS3^{gf} Mutations Reveal Activity in ste7-R220 Strains

FUS3^{gf} mutations require *STE7* and *STE11* for their function in vivo (Figure 3), despite their enhanced ability to autophosphorylate (Figure 6). To examine their requirement for *STE7,* we coexpressed *FUS3^{gf}* mutations with three catalytically impaired alleles of *STE7:* 1) *ste7-R220,* which carries a lysine to arginine substitution in the ATP binding site; 2) *ste7-A220,* which carries a lysine to alanine substitution at this same residue; and 3) *ste7-A349,* which carries an aspartate to alanine substitution at a residue that is also required for ATP binding and catalysis. *FUS3 ste7-R220, FUS3 ste7-A220,* and *FUS3 ste7-A349* strains are sterile. Surprisingly, *FUS3-101 ste7-R220* and *FUS3-107 ste7-R220* strains mate (Table 4).

Table 4. Quantitative mating assays on *FUS3*^{gf} *ste7-R220* strains

	Diploids formed as a fraction of viable cells[a]		
STE7 plasmid[b]	*STE7*	*ste7-R220*	Vector
FUS3 plasmid[c]			
Vector	4.0×10^{-9}	$<1 \times 10^{-9}$	$<1 \times 10^{-9}$
FUS3	3.4×10^{-1}	5.5×10^{-8}	$<1 \times 10^{-9}$
FUS3-101	4.5×10^{-1}	7.9×10^{-3}	2.0×10^{-9}
FUS3-107	5.7×10^{-1}	1.3×10^{-2}	2.0×10^{-9}
fus3-102	6.4×10^{-1}	2.3×10^{-8}	$<1 \times 10^{-9}$
fus3-52	1.7×10^{-6}	3.7×10^{-8}	$<1 \times 10^{-9}$

Double-plasmid transformants of JBY789 (*fus3Δ ste7Δ*) were used for this experiment. *LEU2*-marked plasmids[b] carried alleles of *FUS3*. *TRP1*-marked plasmids[b] carried alleles of *STE7*.

[a] Quantitative mating assays were performed as described in MATERIALS AND METHODS. $<1 \times 10^{-9}$ indicates that no prototrophic colonies were observed out of 10^9 viable colonies plated. Numbers represent the average of two experiments plated in duplicate.

[b] *STE7, TRP1* plasmids: pNC318 (*STE7*); pNC318-R220 (*ste7-R220*); and pRS200 (vector).

[c] *FUS3, LEU2* plasmids: pRS315 (vector) and derivatives pJB236 (*FUS3*), pJB237 (*FUS3-101*), pJB27 (*FUS3-107*), pJB241 (*fus3-102*), and pJB278 (*fus3-52*).

Quantitative mating assays show that *FUS3*^{gf} alleles increase the mating efficiency of *ste7-R220* strains by roughly five orders of magnitude (Table 4). In contrast, neither *FUS3-101* nor *FUS3-107* suppresses the mating defect of either *ste7-A220* or *ste7-A349*. Two lines of evidence indicate that the failure of *FUS3*^{gf} mutations to suppress these alleles is not due to a problem in synthesis of ste7-A220 or ste7-A349: 1) the amount of STE7, ste7-R220, ste7-A220, and ste7-A349 protein is roughly equivalent in *FUS3-101* strains (Figure 7A); and 2) in a *FUS3 STE7* strain, ste7-A220 and ste7-A349 are phosphorylated in response to α factor to the same extent as ste7-R220 (Figure 7B; compare lanes 6, 8, and 10). Hence, the allele-specific suppression of *ste7-R220* by *FUS3*^{gf} mutations is most likely due to residual kinase activity of ste7-R220.

Consistent with this, genetic evidence shows that mating-specific transcription is activated upon the addition of pheromone to *FUS3*^{gf} *ste7-R220* strains. First, although *FUS3-101 ste7-R220* and *FUS3-107 ste7-R220* strains have no *FUS1::lacZ* expression under basal conditions (Table 5), *FUS1::lacZ* expression is induced in *FUS3*^{gf} *ste7-R220* strains in the presence of α factor (Table 5). This induced expression is 16- and 36-fold higher, respectively, than that found in *fus3Δ ste7-R220* or *FUS3 ste7-R220* strains (Table 5). Second, *FUS3*^{gf} *ste7-R220* strains induce sufficient expression of **a** factor to cause cell-cycle arrest of a *MATα sst2* tester lawn (Figure 8B). Thus the transcription of at least two mating-specific genes is induced in *FUS3*^{gf} *ste7-R220* strains.

Biochemical evidence supports the conclusion that FUS3^{gf} kinases are activated by ste7-R220 in response to pheromone. In addition to its role in FUS3 activation, STE7 is also a substrate for FUS3-dependent phosphorylation. Pheromone-dependent hyperphosphorylation of catalytically impaired ste7-R220 normally requires both FUS3 and STE7; ste7-R220 is not hyperphosphorylated in a *FUS3 ste7Δ* strain (Figure 9, lanes 5 and 6; Zhou *et al.*, 1993). Surprisingly, ste7-R220 expressed in a *FUS3-101 ste7Δ* strain is phosphorylated upon the addition of α factor (Figure 9; compare lanes 7 and 8). The degree of this phosphorylation is less than that observed for STE7 (Figure 9; compare the ratio of phosphorylated to unphosphorylated bands in lanes 2 and 4 to that observed in lane 8); however, since the observed phosphorylation of ste7-

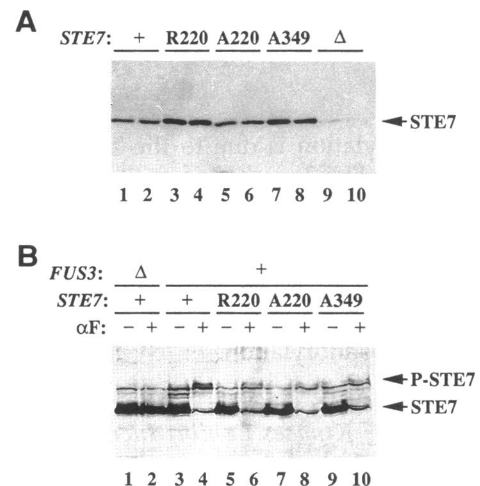

Figure 7. Expression and phosphorylation of ste7-A220 and ste7-A349 proteins. (A) Expression of *STE7* alleles in yeast. The samples are pairs of identical double transformants of strain JBY789 (*fus3Δ ste7Δ*) carrying pJB237 (*LEU2, FUS3-101*). The *TRP1, pCYC1::STE7* plasmids were as follows: lanes 1 and 2, pNC318 (*STE7*; "+"); lanes 3 and 4, pNC318-R220 (*ste7-R220*); lanes 5 and 6, pJB321 (*ste7-A220*); lanes 7 and 8, pJB322 (*ste7-A349*); lanes 9 and 10, pRS200 (vector control; "Δ"). Strains were grown in selective SC raffinose medium (to induce *STE7* expression from the *CYC1* promoter) and extracts were prepared as described (see MATERIALS AND METHODS). STE7 proteins are fused to the myc epitope tag (Evan *et al.*, 1985). ^{125}I-protein A Westerns were performed using anti-myc mouse monoclonal antibody (see MATERIALS AND METHODS). The faint band in lanes 9 and 10 is a cross-reacting protein that migrates slightly faster than STE7-myc and was seen in all experiments. (B) α factor-dependent phosphorylation of STE7 proteins. Double transformants of strain JBY534 (*fus3Δ*) were used as described in A, except the *LEU2* plasmids were as follows: pRS315 (*fus3Δ*; lanes 1 and 2) and pJB236 (*FUS3*; lanes 3–10). Strains grown in liquid culture were incubated with or without 5 μM α factor for 2 h and extracts were prepared as described (see MATERIALS AND METHODS). The presence or absence of α-factor ("αF") induction is indicated by "+" or "−". The unphosphorylated form of STE7 is indicated as "STE7". The phosphorylated form of STE7 that appears upon α-factor addition is indicated by "P-STE7". In A and B, 100 μg of protein was electrophoresed on 7.5% Laemmli gels as described (see MATERIALS AND METHODS).

Table 5. *FUS1::lacZ* expression in *FUS3ˢᶠ ste7-R220* strains

	Units of β-galactosidase activity[a]					
STE7 plasmid[b]	*STE7*		*ste7-R220*		Vector	
α factor	−	+	−	+	−	+
FUS3 allele[b]						
Vector	0.1	0.1	0.1	0.1	0.2	0.1
FUS3	0.6	89	0.1	0.1	0.1	0.2
FUS3-101	2.2	91	0.1	1.6	0.2	0.1
FUS3-107	1.2	131	0.2	3.6	0.1	0.1
fus3-102	1.7	94	0.1	0.1	0.1	0.1
fus3-52	0.1	0.1	0.2	0.1	0.2	0.1

[a] β-galactosidase units are calculated as described in Table 3. Two hour α-factor induction, extract preparation and enzyme assays were performed as described in MATERIALS AND METHODS. Numbers are the average of two experiments performed in duplicate.

[b] Double-plasmid transformants of JBY789 (*fus3Δ ste7Δ*) were used for this experiment. Plasmids are the same as those used in Table 4.

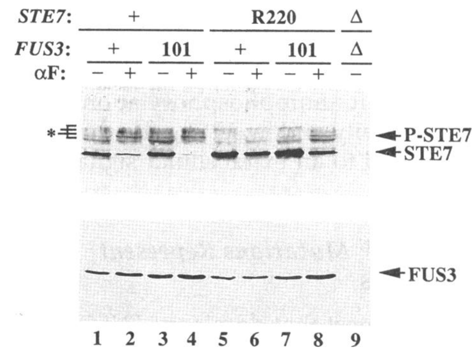

Figure 9. α factor-induced phosphorylation of ste7-R220 by FUS3-101. Double transformants of strain JBY789 (*fus3Δste7Δ*) carrying the following plasmids were used: pNC318 (*STE7;* "+"; lanes 1–4); pNC318-R220 (*ste7-R220;* lanes 5–8); pJB236 (*FUS3;* "+"; lanes 1, 2, 5, and 6); pJB237 (*FUS3-101;* lanes 3, 4, 7 and 8). The extract in lane 9 is untransformed JBY789, and was prepared from cells grown in YPD instead of SC. α-factor inductions were performed and indicated as described for Figure 7. Parallel ECL Westerns were carried out using anti-myc mouse monoclonal antibody (top panel) or affinity-purified anti-FUS3 polyclonal antiserum (bottom panel) as described (see MATERIALS AND METHODS). Laemmli gels (10%) were loaded with 100 μg of extract per lane. Four STE7 phosphoprotein bands are observed under these conditions (indicated by short lines to the left of the figure). Upon the addition of α factor, the middle two bands (especially the lower of these bands; indicated by the asterisk) become more intense and the unphosphorylated form becomes less prominent.

R220 in the presence of FUS3-101 occurs only in response to α factor, FUS3-101, unlike FUS3, must become activated in a *ste7-R220* strain.

DISCUSSION

Activated FUS3, like other MAP kinases from stimulated eukaryotic cells, is highly phosphorylated on tyrosine and threonine residues that are conserved among all members of this protein kinase family (Payne *et al.*, 1991; Gartner *et al.*, 1992; Errede *et al.*, 1993; reviewed in Nishida and Gotoh, 1993). Catalytically defective mutant MAP kinases with a lysine to arginine substitution in the ATP binding site are also phosphorylated in vivo on both the threonine and tyrosine residues (Posada and Cooper, 1992; Gartner *et al.*, 1992). Dual-specificity MAP kinase kinases (MEKs) can activate MAP kinases by phosphorylating these two residues in vitro (L'Allemain *et al.*, 1992; Rossomando *et al.*, 1992b; Errede *et al.*, 1993). By contrast, MAP kinases expressed in bacteria autophosphorylate on the conserved tyrosine residue, but this autophosphorylation results in <1% of full kinase activity (Seger *et al.*, 1991; Wu *et al.*, 1991).

Three models for the activation of MAP kinases in vivo have been proposed: 1) enhancement of an intrinsic autophosphorylation activity (Wu *et al.*, 1991; Seger *et al.*, 1991; L'Allemain *et al.*, 1992; Robbins and Cobb, 1992); 2) a combination of autophosphorylation and phosphorylation by one or more other kinases (Anderson *et al.*, 1990; Payne *et al.*, 1991; Posada and Cooper, 1992); or 3) phosphorylation by other kinase(s) with no role for autophosphorylation (Gartner *et al.*, 1992). In

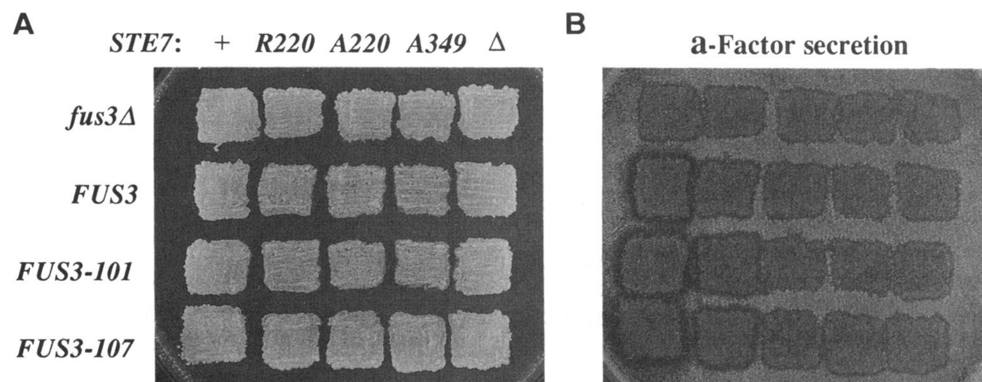

Figure 8. **a**-factor secretion by *FUS3ˢᶠ ste7-R220* strains. (A) Patches of double transformants of yeast strain JBY789 (*fus3Δ ste7Δ*). The *TRP1, CEN, pCYC1-STE7* plasmids were as follows: pNC318 (*STE7;* "+"); pNC318-R220 (*ste7-R220*); pJB321 (*ste7-A220*); pJB322 (*ste7-A349*); and pRS200 (vector; "Δ"). *LEU2, CEN, FUS3* plasmids were pRS315 (vector), pJB236 (*FUS3*), pJB237 (*FUS3-101*), and pJB27 (*FUS3-107*). (B) a-factor secretion by these strains, as assayed by a halo of growth inhibition of JBY367 (*MATα sst2*).

this paper, we describe gain-of-function mutations in *FUS3* that result in an increase in tyrosine autophosphorylation. These *FUS3^gf* alleles have proved useful in suggesting a role for autophosphorylation in regulating MAP kinase activity and in setting the threshold of the response of a cell to an extracellular signal.

Dominant FUS3^gf Mutations Represent Activated Alleles

Genetic data suggest that dominant *FUS3^gf* alleles have increased activity in vivo: 1) *FUS3^gf* alleles increase basal *FUS1::lacZ* expression in a manner that is both dominant and partially independent of *STE4*- and *STE5* (Table 3); 2) *FUS3^gf* alleles cause dominant hypersensitivity to α factor (Figure 2); and 3) under conditions where FUS3 expression is limited, FUS3-101 is better able to complement the mating defect of a *fus3Δ* strain than is FUS3 (Figure 4B). These mutations are considered gain-of-function alleles because they are dominant and because the range of phenotypes affected overlaps with those affected by wild-type *FUS3* as a part of the normal response to pheromone.

Our biochemical evidence suggests that FUS3^gf kinases have an increased ability to autophosphorylate on tyrosine. FUS3-101 is more highly phosphorylated than FUS3 when expressed in *E. coli* (Figure 6B). Importantly, some of this increased phosphorylation occurs on tyrosine, a residue not phosphorylated by *E. coli* kinases. Tyrosine autophosphorylation of the mutant kinase does not involve a novel activity but rather an enhancement of the activity of the wild-type protein (Figure 6C). In yeast, FUS3-101 is constitutively hyperphosphorylated in a manner that is independent of STE7, the kinase that phosphorylates and activates FUS3 (Figure 5B). This hyperphosphorylation corresponds to an increase in tyrosine phosphorylation and is dependent on FUS3 kinase activity, as the inactive fus3-52 mutant contains no detectable phosphotyrosine (Figure 5C).

The increased tyrosine autophosphorylation of FUS3-101 correlates with increased kinase activity: In the *E. coli* labeling experiment, a large proportion of the phosphorylation of FUS3, FUS3-101 and fus3-102 occurs on serine and threonine residues, and this heightened serine and threonine phosphorylation is not observed for the inactive fus3-52 (Figure 5C). The high levels of serine and threonine phosphorylation relative to tyrosine phosphorylation is not observed in FUS3 obtained from yeast (Gartner *et al.*, 1992; Errede *et al.*, 1993). As FUS3-101 exhibits a greater ratio of phosphoserine and phosphothreonine to phosphotyrosine than does FUS3 or fus3-102, the increased serine and threonine phosphorylation found in FUS3-101 may be due to its increased kinase activity.

FUS3^gf Kinases Require STE7 for Their Activation in Vivo

Dominant *FUS3^gf* mutations require the kinases encoded by *STE11* and *STE7* both for mating and for *FUS1::lacZ* expression. The requirement of *FUS3^gf* alleles for these kinases may be explained as follows: either STE11 or STE7 has additional substrates that cannot be activated by FUS3, or one or both of these kinases plays a structural or biochemical role in FUS3 function that cannot be bypassed by *FUS3^gf* mutations.

Since *STE7* encodes the kinase that phosphorylates and activates FUS3 in response to pheromone (Errede *et al.*, 1993; Zhou *et al.*, 1993), we examined in detail the requirement of FUS3 for STE7. Interestingly, *FUS3^gf* mutations can suppress the sterility of a *ste7-R220* mutation (Table 4). This result is consistent with models invoking either a structural requirement for STE7 or residual activity of the ste7-R220 mutant kinase. The failure of *FUS3^gf* mutations to suppress the mating defect of either *ste7-A220* or *ste7-A349* rules out a model in which formation of a complex between FUS3 and STE7 is sufficient for activation of FUS3. *FUS3^gf* alleles are able to dispense with *STE7* in rare matings (2×10^{-9} diploids/viable cell; Table 4), lending further credence to the idea that STE7 does not serve a structural role. Thus the ability of *FUS3^gf* alleles to suppress *ste7-R220* shows that ste7-R220 is likely to have sufficient kinase activity to phosphorylate and activate FUS3^gf kinases in response to pheromone.

ste7-R220 replaces the lysine residue in the ATP binding site with arginine, a substitution that has generally been considered to generate a "null" allele (see, for example, Kamps and Sefton, 1986; Jove *et al.*, 1987). Biochemical studies by Errede *et al.* (1993) failed to reveal any kinase activity by ste7-R220 immunoprecipitated from yeast; however, a very small amount of residual kinase activity is unlikely to have been detected in this assay. Several lines of evidence suggest that substitutions at the conserved lysine residue of protein kinases do not necessarily result in complete loss of function. For example, a mutant cAMP-dependent protein kinase carrying a lysine to alanine change at this residue has residual catalytic activity (Gibbs and Zoller, 1991; reviewed in Taylor *et al.*, 1993). In the case of *FUS3*, the lysine to arginine mutation in *fus3-52* is not "null" for mating ($\approx 10^{-6}$ diploids formed/viable cell), as compared to *fus3Δ* ($<10^{-9}$ diploids/viable cell; Table 4). In agreement with our findings, *GAL*-promoted overproduction of *ste7-R220* was previously reported to complement *ste7Δ* at a very low level, suggesting the existence of a small amount of residual activity in the protein (Cairns *et al.*, 1992).

Autophosphorylation of FUS3 Affects Its Ability to Amplify the Mating Signal

Our results show a correlation between the hypersensitive phenotypes of dominant *FUS3^gf* alleles and in-

creased tyrosine autophosphorylation. Previous experiments by Gartner *et al.* (1992) showed that FUS3 does not have to be active to be fully phosphorylated on threonine and tyrosine, leading to their speculation that autophosphorylation is not physiologically relevant. However, these experiments do not address the role for autophosphorylation by an active FUS3 kinase. A role for autophosphorylation can be inferred from experiments in which MAP kinase activity is retained. In experiments by Posada and Cooper (1992), it was noted that the efficiency of threonine phosphorylation of MAP kinase appeared to be enhanced by presence of the phosphotyrosine. In more quantitative experiments, tyrosine-phosphorylated MAP kinase was shown to be a better substrate for threonine phosphorylation by MAP kinase activator in vitro than is the unphosphorylated protein (Haystead *et al.*, 1992).

In this paper, we have shown that activating mutations in *FUS3* that cause increased tyrosine autophosphorylation (Figure 6C) significantly increase the magnitude of both basal and pheromone-induced signal transduction: 1) *FUS3^{gf}* mutations increase basal expression of mating-specific genes (Table 3); 2) in an otherwise wild-type cell, the presence of *FUS3^{gf}* alleles causes hypersensitivity to pheromone, as seen by the size of the halo of growth inhibition caused by α factor (Figure 2); 3) in a situation in which FUS3 protein is expressed in limited quantities, *FUS3-101* is better able to complement *fus3Δ* than is wild-type *FUS3* (Figure 4B); and, 4) the ability of dominant *FUS3^{gf}* mutations to suppress a catalytically impaired *ste7-R220* allele suggests that the small amount of signal transduced by ste7-R220 is sufficient to activate tyrosine-phosphorylated FUS3 (FUS3-101), but not the unphosphorylated (wild-type) FUS3 protein.

These results suggest that the activation of FUS3 can be viewed as a two-step process (Figure 10). First, autophosphorylation results in the presence of a small quantity of tyrosine-phosphorylated FUS3 (B1). Second, signal transduction leading to activation of FUS3 proceeds through STE11 to STE7 (A), which in turn phosphorylates both unphosphorylated and tyrosine-phosphorylated forms of FUS3 (B1 and B2). STE7 can phosphorylate FUS3 on both threonine and tyrosine. As autophosphorylation of FUS3 occurs only on tyrosine, STE7-dependent phosphorylation of the threonine residue is absolutely required for FUS3 activation and efficient phosphorylation of target substrates, including STE12, FAR1, and STE7 (C). By this model, tyrosine autophosphorylation of FUS3 is not required for its activation by STE7. However, the concentration of tyrosine-phosphorylated FUS3 molecules in the cell will affect the rapidity of FUS3 activation. By analogy to MAP kinases (Haystead *et al.*, 1992), tyrosine phosphorylation of FUS3 would make it a better substrate for phosphorylation by STE7, thereby explaining why *FUS3^{gf}* alleles can suppress a *STE7* mutation with reduced function. One prediction of these results is that tyrosine-phosphorylated FUS3 will be a better substrate for phosphorylation by both ste7-R220 and STE7 in vitro.

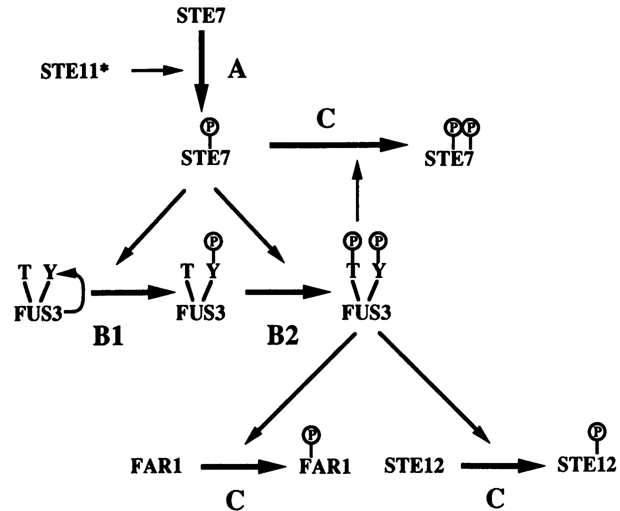

Figure 10. A model for FUS3 activation in response to pheromone. (A) Activated STE11 activates STE7 by phosphorylating it. Although this reaction has not been demonstrated biochemically and the phosphorylation sites on STE7 have not been determined, there is much evidence suggesting that this phosphorylation step is likely to occur. (B) Phosphorylation of FUS3 occurs in two steps. (B1) Tyrosine phosphorylation at Tyr-182 occurs through either autophosphorylation (indicated by curved arrow) or phosphorylation by STE7. (B2) Threonine phosphorylation at Thr-180 requires the activity of STE7. (C) Following activation, FUS3 phosphorylates at least 3 substrates: FAR1, which is required for cell-cycle arrest; STE12, which is required for transcriptional induction of mating-responsive genes; and STE7, whose phosphorylation by FUS3 (on as yet unidentified sites) is thought to result in down-regulation of the signal-transduction pathway.

The role for autophosphorylation in the regulation of MAP kinases has proved puzzling, especially since tyrosine autophosphorylation does not result in their full activation (Seger *et al.*, 1991; Wu *et al.*, 1991). Moreover, phosphorylation of MAP kinases by MAP kinase kinases is independent of MAP kinase activity (Gartner *et al.*, 1992; L'Allemain *et al.*, 1992; Rossomando *et al.*, 1992b). The dominant FUS3^{gf} kinases described in this paper exhibit increased autophosphorylation and cause hypersensitivity to yeast mating pheromone. Although it is clear that autophosphorylation cannot in itself supply the mating function and full activation of FUS3, the properties of FUS3^{gf} kinases imply that the degree of autophosphorylation by a MAP kinase determines its threshold of sensitivity to phosphorylation by MAP kinase kinase and hence the threshold of sensitivity of the cell to extracellular signals involved in growth control.

ACKNOWLEDGMENTS

The authors thank S. Reed, M. Whiteway, M. Hasson, and J. Loeb for gifts of plasmids, with special thanks to B. Errede, who kindly

provided both plasmids and unpublished results prior to publication. We are grateful to members of the Fink laboratory for insightful discussion and to J. Ashkenas, L. Guarente, H. Lodish, and T. Orr-Weaver for comments on the manuscript. This work was supported by National Institute of General Medical Sciences GM-40266-07. G.R.F. is an American Cancer Society Professor of Genetics.

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