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Mutational Activation of the *STE5* Gene Product Bypasses the Requirement for G Protein β and γ Subunits in the Yeast Pheromone Response Pathway

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The *STE5* gene encodes an essential element of the pheromone response pathway which is known to act either after the G subunit encoded by the *STE4* gene or at the same step. Mutations in *STE5*, designated *STE5^{Hyp}*, that partially activate the pathway in the absence of pheromone were isolated. One allele (*STE5^{Hyp}-2*) was shown to cause a single amino acid substitution near the N terminus of the predicted *STE5* protein. Immunoblotting with anti-Ste5 antibodies indicated that the phenotype was not due to an increased level of the mutant *STE5* protein. A multicopy episomal plasmid containing a *STE5^{Hyp}* allele partially suppressed both the block in pheromone-inducible transcription and the sterility phenotype caused by null alleles of the *STE2*, *STE4*, or *STE18* gene, indicating that the *STE5* product acts after the receptor (*STE2* product) and after the G protein β and γ subunits (*STE4* and *STE18* products, respectively). However, the phenotypes of the *STE5^{Hyp}* mutations were less pronounced in *ste4* and *ste18* mutants, suggesting that the *STE5^{Hyp}*-generated signal partially depends on the proposed $G_{\beta\gamma}$ complex. The *STE5^{Hyp}* alleles did not suppress *ste7*, *ste11*, *ste12*, or *fus3 kss1* null mutants, consistent with previous findings that the *STE5* product acts before the protein kinases encoded by *STE7*, *STE11*, *FUS3*, and *KSS1* and the transcription factor encoded by *STE12*. The mating defects of the *ste2* deletion mutant and the temperature-sensitive *ste4-3* mutant were also suppressed by overexpression of wild-type *STE5*. The slow-growth phenotype manifested by cells carrying *STE5^{Hyp}* alleles was enhanced by the *sst2-1* mutation; this effect was eliminated in *ste4* mutants. These results provide the first evidence that the *STE5* gene product performs its function after the G protein subunits.

The pheromone response in the yeast *Saccharomyces cerevisiae* provides a model system for studying general features of peptide-hormone action (see references 59 and 75 for reviews). In this yeast, conjugation of haploid **a** and α cells yields **a**/α diploid cells. Conjugation requires the action of peptide pheromones: **a** cells secrete **a**-factor pheromone and respond to α-factor, whereas α cells secrete α-factor pheromone and respond to **a**-factor. The binding of the pheromones to specific receptors on the target cell initiates the mating program. Specific cellular responses to pheromones include the arrest of cell division in G_1 (34) and the production of cellular factors required for cell aggregation, cell fusion, karyogamy, and pheromone desensitization (17, 18, 54, 60, 72, 79). These responses are mediated, at least in part, by changes in the transcription of cellular genes. Pheromones also provide spatial information which allows cells to locate and choose specific mating partners (42, 43, 51).

Previous genetic analyses of the pheromone response pathway identified a number of components required for signal transduction. The *STE2* and *STE3* genes encode the α-factor and **a**-factor receptors, respectively (4, 35, 46, 57, 58). The receptors are thought to span the plasma membrane seven times, thus sharing the predicted topologies of rhodopsin and the β-adrenergic receptor (7, 9, 13, 31, 65). The GTP-binding regulatory protein (G protein), which has been proposed to mediate the postreceptor signal, appears to be unusual. Genetic experiments indicate that the $G_{\beta\gamma}$ complex, encoded by the *STE4* and *STE18* genes, activates the subsequent events in the pathway, whereas the G_α subunit, encoded by the *GPA1* gene (also designated *SCG1*), regulates the activity of the $G_{\beta\gamma}$ complex (2, 19, 35, 57, 61, 64, 82). The direct target of G protein action is unknown; however, several gene products have been implicated in subsequent steps in the signal transduction pathway. Five serine/threonine protein kinases are encoded by the *STE7*, *STE11*, *STE20*, *FUS3*, and *KSS1* genes (16, 25, 35, 53, 68, 69, 78); the *FUS3* and *KSS1* products are redundant in function (24, 55). A DNA-binding protein encoded by the *STE12* gene (22, 26) binds to a *cis*-acting DNA sequence (the pheromone response element) that mediates pheromone-induced transcription (30, 50, 81). The *STE5* gene product performs a role in signal transduction which is as yet unknown (35, 37, 57, 63, 67).

Mutations that cause constitutive activation of the pheromone response pathway have been invaluable for epistasis tests to determine the temporal order in which the various gene products function. Cell cycle arrest and pheromone-independent transcription of pheromone-inducible genes have been observed for recessive alleles of the *GPA1* gene (2, 19, 44, 61, 65). Constitutive activation also results from special dominant alleles of the *STE4* and *STE11* genes (3, 8,

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TABLE 1. Strains used in this study

Strain[a]	Genotype[b]
381G	*MATa cry1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3^{ts}*
DJ211-12-1	381G *MATa bar1-1 leu2 TYR1 ura3*
DJ213-6-3	381G *MATa bar1-1 leu2 ste2-10::LEU2 TYR1 ura3*
DJ602-136	381G *MATa ade3 leu2 TYR1 ura3*
DJ656	381G *MATa/MATα ade3/ade3 cry1/CRY1 leu2/leu2 TYR1/tyr1 ura3/ura3*
DJ656-2	381G *MATa/MATα ade3/ade3 cry1/CRY1 leu2/leu2 ste4::LEU2/STE4 TYR1/tyr1 ura3/ura3*
DJ656-2-6-2	381G *MATa leu2 ste4::LEU2 TYR1 ura3* containing plasmid pDJ174
DJ656-2-8-3	381G *MATα leu2 ste4::LEU2 ura3* containing plasmid pDJ174
DJ656-2-10-1	381G *MATa leu2 ste4::LEU2 TYR1 ura3* containing plasmid pDJ174
DJ676-2-2	381G *MATa ade3 leu2 sir3^{ts} TYR1 ura3*
DJ656-3	381G *MATa/MATα ade3/ade3 cry1/CRY1 leu2/leu2 ste12Δ::LEU2/STE12 TYR1/tyr1 ura3/ura3*
DJ656-4	381G *MATa/MATα ade3/ade3 cry1/CRY1 leu2/leu2 ste18Δ::LEU2/STE18 TYR1/tyr1 ura3/ura3*
DJ783	381G *MATa/MATα cry1/CRY1 leu2/LEU2 ste4-3/ste4-3 TYR1/tyr1 ura3/ura3*
DJ786-11-1	381G *MATa sst2-1 ade3 leu2 ura3 TYR1^{+}*
DJ787	381G *MATa/MATα ade3/ade3 cry1/CRY1 leu2/leu2 ste2-10::LEU2/STE2 TYR1/tyr1 ura3/ura3*
DJ788-7-1	381G *MATa leu2 sir3^{ts} sst2-1 TYR1 ura3*
DJ789-2-2	381G *MATa leu2 sst2 ste4::LEU2 ura3* containing plasmid pDJ174
DJ789-3-1	381G *MATa leu2 sst2 ste4::LEU2 ura3* containing plasmid pDJ174
DJ789-5-2	381G *MATa leu2 sst2 ste4::LEU2 ura3* containing plasmid pDJ174
DJ789-5-3	381G *MATa leu2 sst2 ste4::LEU2 ura3* containing plasmid pDJ174
DJ1000A	381G *MATa ade3 leu2 ste5Δ1::LYS2 TYR1 ura3*
DJ1003A	381G *MATa ade3 leu2 ste11Δ::hisG TYR1 ura3*
DJ1004-11-1	381G *MATa ade3 leu2 ste4::LEU2 ura3*
DJ1005-9-1	381G *MATa ade3 leu2 ste12Δ::LEU2 TYR1 ura3*
DJ1006-17-2	381G *MATa ade3 leu2 ste18Δ::LEU2 ura3*
DJ1014A	381G *MATa bar1-1 leu2 ste12Δ::LEU2 TYR1 ura3*
DJ1023A	381G *MATa ade3 leu2 ste7::LEU2 TYR1 ura3*
381GULΔSTE4[c]	381G *MATa leu2 ura3 ste4Δ::LEU2*
EMS63	*MATα his2*
YPH499	*MATa ade2-101 his3Δ200 leu2Δ1 trp1Δ63 ura3-52*
YPH499fus3kss1[d]	YPH499 *MATa fus3-6::LEU2 kss1Δ::HIS3*

[a] EMS63 was derived from strain S288c. Other strains are congenic to strain 381G (35) or to strain YPH499 (74), as indicated.

[b] The designation 381G indicates that the genotype is the same as strain 381G except for the markers shown. The *cry1* and *CRY1* alleles determine resistance or sensitivity to the antibiotic cryptopleurine. The temperature-sensitive allele *ste4-3* (35) leads to sterility and α-factor resistance at 34°C. The *sir3^{ts}* mutation was originally designated *ste8-5* (35). The *kss1Δ::HIS3* allele is a partial deletion of the *KSS1* coding sequence (16), and the *fus3-6::LEU2* allele is a deletion of the *FUS3* gene (25).

[c] This strain contains a complete deletion of the *STE4* coding sequence; it was kindly provided by Gary Cole.

[d] This strain was kindly provided by Doreen Ma.

29, 76) and from overproduction of either the wild-type *STE4* (15, 66, 83) or the *STE12* (21) gene product. The order in which the various gene products act in the pheromone response pathway has been inferred from the ability of pheromone-unresponsive mutants to block these constitutive postreceptor signals. If the unresponsive mutant fails to block the constitutive signal, then the corresponding gene product is thought to execute its function before the constitutively active step, whereas if the unresponsive mutant blocks the signal, then it is not possible to distinguish whether it affects the same step or a subsequent step in the pathway.

From this type of epistasis analysis, it has not been possible to determine the gene product that functions immediately after the G protein. The *STE5* product functions before the *STE7*, *STE11*, *FUS3*, and *KSS1* protein kinases (8, 29, 76); however, it is not known whether it functions after the G protein or affects the same step (2). The *STE5* product could conceivably affect the G protein step by controlling the synthesis or structural modifications of the G protein subunits or by influencing the rate of GTP exchange or hydrolysis. In this report, we describe *STE5^{Hyp}* mutations (for hyperactive), which cause partial constitutive activation of the pheromone response pathway. The ability of these mutants to bypass blocks in the pathway imposed by the *ste4* or *ste18* null allele indicates that the *STE5* protein acts after the G_β and G_γ subunits. The inability of the *STE5^{Hyp}* mutants to bypass the defects caused by the *ste7*, *ste11*, or *ste12* null allele or by combined deletions in the *KSS1* and *FUS3* genes is consistent with the established relationship with these protein kinases and the transcription factor (8, 21, 29, 76). The ability of the *sst2* mutation to enhance the phenotype of the hyperactive *STE5* mutant has implications for the role of the *SST2* gene product in adaptation.

MATERIALS AND METHODS

Strains, media, and genetic methods. The strains used in this study are listed in Table 1. YM-1 is a rich liquid medium (33). Minimal medium was yeast nitrogen base (without amino acids; Difco Laboratories) supplemented with ammonium sulfate (1 mg/ml) as the nitrogen source and glucose (2%) as the carbon source. Minimal medium was supplemented with amino acids (40 μg/ml), uracil (20 μg/ml), or adenine (20 μg/ml) as needed. C medium was minimal medium supplemented with adenine, uracil, arginine, aspartic acid, histidine, isoleucine, leucine, lysine, methionine, phenylalanine, serine, threonine, tryptophan, tyrosine, and valine. −Ura and −Ura-Trp media were C medium lacking uracil or lacking both uracil and tryptophan, respectively. −Ura+CAA and −Ura-Trp+CAA media were supple-

mented with 0.1% Casamino Acids (Difco). Standard genetic methods were used for all strain constructions (62).

Plasmid and strain constructions. All yeast transformations utilized the lithium acetate procedure (41). Plasmids pDJ143 and pDJ144 were constructed by ligating a 4.5-kb *Bam*HI-*Pvu*II fragment containing *STE5* from the plasmid pHB3 (37) with the YEp352 (for pDJ143) or YIp352 (for pDJ144) vector (39) digested with *Bam*HI and *Sma*I. The isolation of plasmid pDJ174 is described below; this plasmid is a mutagenized isolate of pDJ143 containing the *STE5^{Hyp}-1* allele. The plasmid pDJ175 was constructed by ligating the *Xba*I fragment of pDJ174 containing *STE5^{Hyp}-1* into YIp352 digested with *Xba*I. The integration of pDJ144 and pDJ175 was targeted to the chromosomal *URA3* locus by digestion with *Apa*I, which cuts the plasmid within the *URA3* gene. Recombinant plasmids of pDJ143 and pDJ174 were constructed as follows (see Fig. 1). For pDJ176, the small *Sac*I-*Afl*II fragment of pDJ174 was ligated to the large *Sac*I-*Afl*II fragment of pDJ143. For pDJ177, the large *Sac*I-*Afl*II fragment of pDJ174 was ligated to the small *Sac*I-*Afl*II fragment of pDJ143. For pDJ178, the small *Asp* 718 (an isoschizomer of *Kpn*I) fragment of pDJ174 was ligated to the large *Asp* 718 fragment of pDJ143. The plasmid pDJ179 was constructed by ligating the small *Kpn*I fragment of pDJ143 with the large *Kpn*I fragment of pDJ176. Large fragments contained vector sequences, while small fragments were pieces of *STE5* DNA.

The *ste2-10*::*LEU2* allele is a nearly complete substitution of the *STE2* gene with *LEU2* (48). Chromosomal deletion of the *STE5* gene was achieved by transforming strain DJ602-136 with an *Xba*I fragment from plasmid pSLYSEA (37), in which *LYS2* replaces 98% of the coding region of *STE5*, to produce the strain DJ1000A. Genetic disruption of the *STE4* gene (DJ656-2) was achieved by transforming the diploid strain DJ656 with a *Pst*I-*Xba*I fragment from the plasmid pDJ154, containing *STE4* with the *LEU2* gene inserted at an internal *Bgl*II site. Deletion of the *STE18* gene (DJ656-4) was achieved by transforming DJ656 with the *Sph*I-*Hin*dIII fragment containing a *LEU2* substitution of the *STE18* gene between the *Nsi*I sites. The M81p12 (*STE4*) and M65p1 (*ste18*::*LEU2*) plasmids were kindly provided by M. Whiteway. Deletions of the *STE12* gene (DJ656-3 and DJ1014A) were constructed by transforming DJ656 and DJ211-12-1 with a *Sac*I-*Sph*I fragment from the plasmid pSUL16, containing *STE12* sequences with *LEU2* replacing an internal *Xba*I fragment (27). The *STE7* gene was disrupted in DJ602-136 to produce strain DJ1023A by transformation with a *Hin*dIII fragment from pDH90 (a gift from D. Higgins and K. Tatchell), containing the *STE7* gene with *LEU2* inserted into a *Bgl*II site. Deletion of the *STE11* gene was accomplished by transformation of strain DJ602-136 with a *Bam*HI-*Xho*I fragment of the plasmid pSURE11, containing *STE11* sequences, with the *URA3* gene, flanked by *Salmonella hisG* repeats, replacing an internal *Hin*dIII fragment. An isolate in which the *hisG* repeats had recombined to remove the *URA3* gene was obtained by selection on 5-fluoro-orotic acid (5) and was named DJ1003A. The plasmid pSURE11 was constructed by inserting a *Bam*HI-*Bgl*II fragment from pNKY51 (1), containing the *URA3* gene flanked by *hisG* repeats, into the *Hin*dIII-cleaved plasmid pBste11Δ1, which was constructed by ligating into *Bam*HI-*Xho*I-cut Bluescript (Stratagene) a *Xho*I-*Bam*HI fragment containing *ste11Δ1*, the *STE11* gene lacking an internal *Hin*dIII fragment (10). In the construction of pBste11Δ1, the 5′ overhangs of both fragments were filled by using the Klenow fragment of DNA polymerase I. Genetic disruptions and deletions were confirmed by Southern blot analysis of sterile isolates (73).

Isolation of mutants. Hydroxylamine mutagenesis was performed essentially as described by Rose and Fink (71). Mutagenized plasmids were used directly for transformation of yeast cells. The level of mutagenesis was inferred by monitoring the inactivation of the *URA3* gene when the plasmid was introduced into the Ura$^-$ *Escherichia coli* strain MH6 (*pyrF*). A total of 2.7% of the ampicillin-resistant transformants were Ura$^-$. Yeast strain DJ676-2-2 (*MAT***a** *leu2 ura3 sir3^{ts}*) was transformed with the mutagenized pDJ143 plasmid. Transformants were selected on −Ura plates at 34°C and replica plated onto two −Ura plates, which were incubated at 34 and 22°C, respectively. Plasmid DNA was extracted from colonies that exhibited cold-sensitive growth (40) and were retested by transforming the parent strain, DJ676-2-2. The plasmid isolate designated pDJ174 resulted in both cold-sensitive growth and the appearance of pear-shaped cells in the culture at 22°C. The pDJ174 and pDJ143 plasmids were used to transform strain DJ602-136 (*MAT***a** *ade2 ade3*) carrying the plasmid pDJ117 (*MATα ADE3*). This strain produces red colonies with white sectors resulting from occasional loss of the plasmid; haploid-specific growth defects block the sectoring phenotype of this strain (2). Transformants were selected on −Ura+CAA plates and scored for the ability to grow after spontaneous loss of the pDJ117 plasmid, as indicated by the appearance of sectors. Sectors were apparent in transformants containing pDJ143 but not in those containing pDJ174.

Preparation of whole-cell extracts and immunoblot analysis. Cells growing exponentially in −Ura liquid medium were collected by centrifugation, washed, and resuspended to a density of 0.5 g of cells (wet weight) per ml of lysis buffer (50 mM Tris [pH 6.8], 0.1 mM EDTA, 10% glycerol) containing protease inhibitors [1 mM phenylmethanesulfonyl fluoride, 1 mM benzamidine, 0.5 mM L-1-chloro-3-(4-tosylamido)-7-amino-2-heptanone-HCl, and 5 μM pepstatin] and phosphatase inhibitors (0.1 M sodium pyrophosphate, 0.1 M NaN_3, 0.1 M NaF, 4 mM sodium EDTA, 4 mM sodium metavanadate, and 4 mM sodium orthovanadate). Cells were lysed by agitation with glass beads on a Vortex mixer for 10 30-s intervals separated by 30-s incubations on ice. The extract was removed, and the glass beads were rinsed twice with lysis buffer. Sodium dodecyl sulfate was added to 2% to the combined extract and rinses, and the mixture was boiled for 3 min and then centrifuged for 20 min at 12,000 × *g*. The protein concentration was assayed by using the bicinchoninic acid reagent (Pierce). β-Mercaptoethanol (to 5%) and bromphenol blue (to 0.01%) were added to each extract. A volume containing 150 μg of protein was loaded on a 6.5% polyacrylamide gel (52). Proteins were transferred electrophoretically to a nitrocellulose membrane. The *STE5* gene product was detected with affinity-purified rabbit antiserum (37) and goat anti-rabbit immunoglobulin antibodies coupled to alkaline phosphatase (Bio-Rad) as recommended by the supplier.

Quantitative mating assays. The quantitative mating experiments were performed essentially as described by Hartwell (35). Cells were cultured overnight in −Ura or −Ura+CAA medium at 34°C to a density of between 1×10^6 and 2×10^7 cells per ml, diluted to 1×10^6 cells per ml, and shifted to 22°C for 3 h (to enhance expression of the hyperactive phenotype). To determine mating efficiency, 2×10^6 *MAT***a** cells were mixed with 2×10^6 *MATα* tester cells (strain EMS63). The mixture was collected on a type HA nitrocellulose filter (0.45-μm pore size). The filter was rinsed and

then transferred to a −Ura or −Ura+CAA plate at 22°C. After 6 h at 22°C, the cells were suspended in 2 ml of minimal medium, dispersed by vortexing at high speed for 1 min, and diluted in and plated on unsupplemented minimal medium. Diploid colonies were scored after 2 days at 30°C. The same procedure was used to assay mating at 34°C, except that the cultures were first grown overnight at 34°C, and all solutions and culture media were prewarmed to 34°C.

Plasmid loss assays. The ability of the pDJ174 plasmid to inhibit cell division was inferred from the frequency at which plasmid-deficient cells appeared under nonselective culture conditions. Diploid strains heterozygous for the allele *ste2*::*LEU2*, *ste4*::*LEU2*, *ste12*::*LEU2*, or *ste18*::*LEU2* (strains DJ787, DJ656-2, DJ656-3, and DJ656-4, respectively) were transformed with either the pDJ174 (*STE5^{Hyp}-1*) or the pDJ143 (*STE5*$^+$) plasmid, induced to sporulate, and subjected to tetrad analysis. Diploid strain DJ789 (*ste4*::*LEU2*/+*sst2-1*/+) containing plasmid pDJ174 was processed identically. Tetrads in which the disruption segregated 2 (Leu$^+$):2(Leu$^-$) and the plasmid segregated 4 (Ura$^+$):0(Ura$^-$) were used in plasmid loss experiments. Both *MAT***a** and MATα segregants were analyzed, except in the cases of the *ste2-10*::*LEU2* deletion and *sst2-1* strains, for which only *MAT***a** strains were used. All four segregants in a tetrad were tested for plasmid loss at least twice. Cells were cultured overnight in −Ura medium at 34°C, diluted to 10^5 cells per ml with YM-1, and grown to saturation (4 to 5 days) at 22°C. Serial 10-fold dilutions were spread onto C and −Ura plates. The percentage of plasmid remaining was determined by the ratio of the number of Ura$^+$ colonies to the total number of colonies on the C plates. If more than 15% of the cells retained the plasmid, the plasmid was considered stable. If fewer than 3% of the cells retained the plasmid, it was considered unstable.

Assays of β-galactosidase activity. Assays of β-galactosidase in cell extracts were performed as described by Hagen and Sprague (32). Cultures were grown to exponential phase in −Ura-Trp+CAA at 34°C, diluted to about 5×10^6 cells per ml, and shifted to 22°C 3 h prior to assay. Cells were collected by centrifugation and resuspended in 0.1 volume of Z buffer (10 mM KCl, 1 mM $MgSO_4$, 50 mM β-mercaptoethanol, 0.1 M sodium phosphate, pH 7). To make the cells permeable, toluene (to 1%) and sodium sarcosyl (to 5%) were added, and the mixture was vortexed and incubated at 37°C for 30 min. Enzyme activity was measured at 28°C in 22 mM *o*-nitrophenyl-β-D-galactoside–0.1 M sodium phosphate, pH 7. A one-third volume of Na_2CO_3 was added to terminate the reaction, and the A_{420} was measured after clarification by centrifugation. Units of activity were calculated by using the formula (1,000 × A_{420} of reaction) ÷ (A_{600} of culture × volume [in milliliters] of culture used × time of reaction [in minutes]).

RESULTS

Isolation of the *STE5^{Hyp}-1* allele. We sought a mutant allele of the *STE5* gene that would cause constitutive activation of the pheromone response pathway. The *STE5* gene product is thought to be a positive regulatory element in the pathway, since null alleles result in loss of pheromone responsiveness (35, 37, 57). Hence, it was anticipated that constitutive *STE5* alleles would be associated with a gain of function and would therefore be dominant. Furthermore, since expression of the *STE5* gene is regulated by the mating-type locus (28, 63, 67), the mutant alleles were expected to cause arrest of cell division in haploid cells, which contain either the *MAT***a** locus or the *MAT*α locus, but to have no effect on the growth of diploid cells, which contain both loci. Similarly, haploid cells bearing the *sir3^{ts}* mutation (35, 70) as well as the constitutive *STE5* allele should grow at 34 but not at 22°C, because at the higher temperature *sir3^{ts}* cells express the normally silent mating-type information, α and **a**, located at the *HML* and *HMR* loci, respectively.

A high-copy-number episomal plasmid carrying the *STE5* gene, pDJ143, was mutagenized with hydroxylamine and used to transform a *sir3^{ts}* strain (DJ676-2-2). Transformants were selected at 34°C and then transferred to duplicate plates that were incubated at 22 and 34°C, respectively. Further analysis was performed for isolates that grew normally at 34°C but divided more slowly at 22°C and accumulated the pear-shaped cells (shmoos) associated with pheromone-treated cells. From a total of 231 transformants, 12 cold-sensitive colonies were isolated, and their plasmid DNA was used to retransform the parent *sir3^{ts}* strain. Four candidate plasmids conferred a growth defect at 22°C, but only one also resulted in a significant number of pear-shaped cells. This mutant plasmid was designated pDJ174, and the "hyperactive" allele was designated *STE5^{Hyp}-1*.

Haploid-specific inhibition of growth. Additional lines of evidence supported the conclusion that plasmid pDJ174 causes an inhibition of growth that is specific to haploid cells. A colony-sectoring assay was previously used to identify haploid-specific mutants (2). In that method, a *MAT***a** *ade2 ade3* parent strain carrying an *ADE3 MAT*α plasmid (pDJ117) was used; such a strain produces red colonies with white sectors that arise as a result of plasmid instability (49). Mutations that cause a haploid-specific growth defect, such as *gpa1* and *STE4^{Hpl}*, prevent the division of cells lacking the plasmid and thereby block the formation of white sectors (2). For the present study, strain DJ602-136 containing plasmid pDJ117 was transformed with the mutant plasmid pDJ174 and with the control plasmid pDJ143. Isolates carrying the pDJ143 plasmid produced white sectors, but the pDJ174 transformants did not, indicating that the plasmid pDJ174 conveys a haploid-specific growth defect.

Growth inhibition was also evident among the haploid segregants of the diploid strain DJ656 containing plasmid pDJ174. Tetrad analysis of the diploid cells containing the control plasmid pDJ143 gave rise to segregants that showed normal colony size. In contrast, the strain containing pDJ174 yielded spores that exhibited a variety of phenotypes upon germination: single cells with a pear shape, small Ura$^+$ colonies that contained the plasmid, and larger Ura$^-$ colonies that did not contain the plasmid. Presumably, the copy number of the pDJ174 plasmid at the time of germination determined whether the spore exhibited arrest of cell division in the first cycle or produced a small colony. Consistent with this view, diploid cells containing one integrated copy of the *STE5^{Hyp}-1* gene on plasmid pDJ175 never yielded spores which arrested division in the first cycle, and the resulting colonies showed no obvious growth defect. The phenotype of the pDJ174 plasmid appeared to be more extreme at 22 than at 34°C, since a larger fraction of the spores that germinated at the lower temperature arrested in the first cycle. Consequently, in most of our subsequent studies haploid cells that carried a *STE5^{Hyp}* allele were propagated at 34°C and then shifted to 22°C for stronger expression of the hyperactive phenotype.

Sequence analysis of *STE5^{Hyp}-1*. As discussed below, the mutant plasmid pDJ174 suppresses the sterility phenotype of *ste4* mutants; this phenotype was used to map the nucleotide

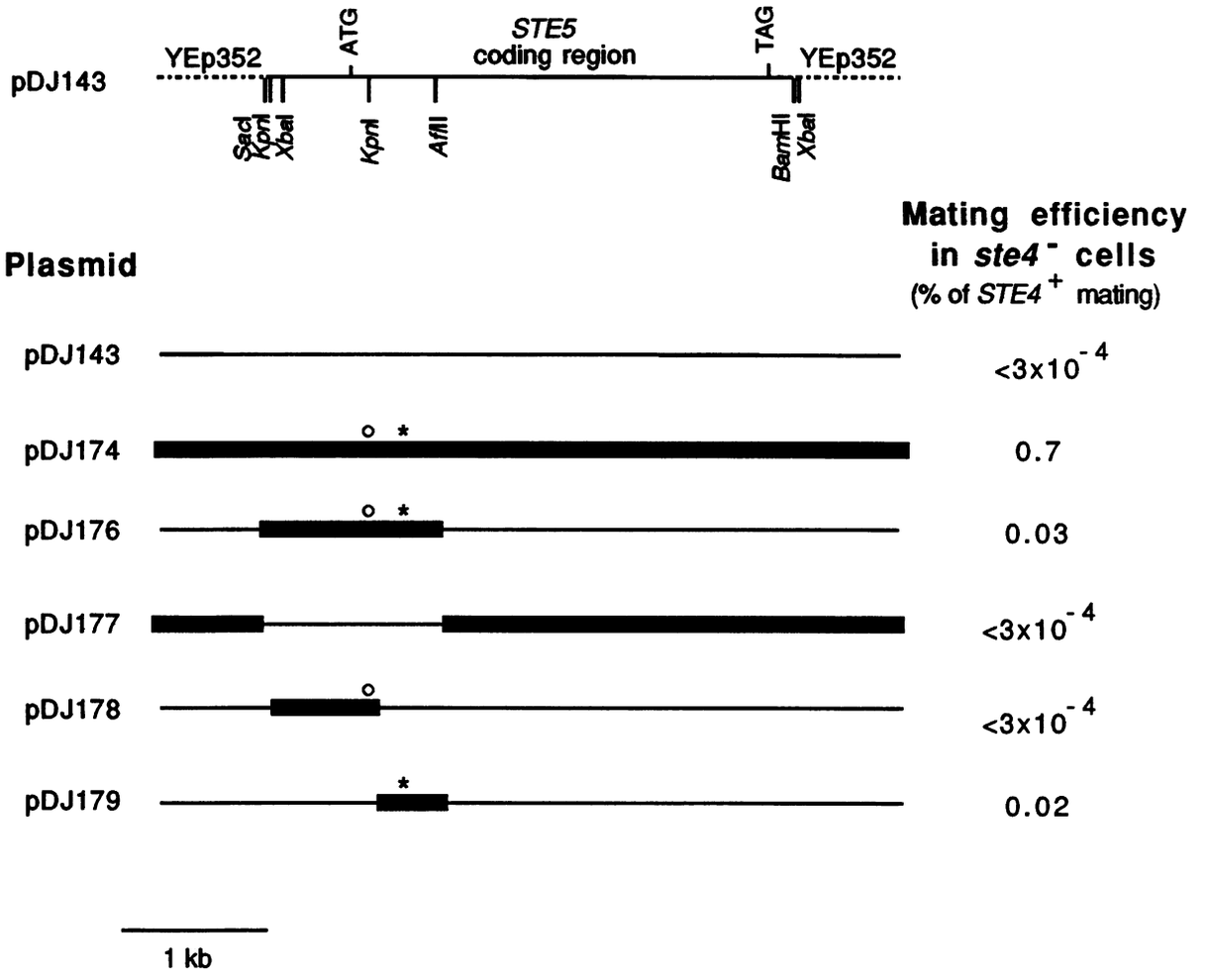

FIG. 1. A single base pair change in the *STE5* coding sequence confers a hyperactive phenotype. A schematic diagram of the restriction map of the *STE5*$^+$ plasmid pDJ143 is shown at the top. The diagram below shows the locations, relative to this map, of wild-type (thin lines) or mutant (thick lines) sequences in the recombinant plasmids. Mutational differences indicated by ○ and * were identified by DNA sequence analysis. The mutation responsible for the hyperactive phenotype (*) is a C-to-T transition in the coding sequence of *STE5* which is predicted to cause a Thr-to-Met change at position 52 of the protein (37, 63, 67). The silent mutation (○) causes a Ser-to-Arg substitution at position 18. The mating efficiency of the strain 381GULΔSTE4 containing each plasmid is indicated, relative to that of a *STE4*$^+$ strain, which produced 3×10^6 diploids in the quantitative mating assay. The assay methods were the same as used for Table 2, except that filters were incubated on −Ura+CAA plates.

change responsible for the phenotype of pDJ174. The presence of pDJ174 improved the mating ability of *ste4* mutants more than 1,000-fold compared with that of cells carrying the control plasmid pDJ143 (Fig. 1). In the recombinant plasmid pDJ176, mutant sequences replaced wild-type sequences in the promoter and approximately the first quarter of the *STE5* coding sequence. This plasmid possessed most of the ability of the original isolate to suppress the sterility of *ste4* strains, while the plasmid containing the opposite combination of mutant and wild-type sequences, pDJ177, had no such ability. When the protein-coding region of the mutant gene contained in plasmid pDJ176 was sequenced, two nucleotide changes were found. These two changes were separated, in plasmids pDJ178 and pDJ179. Only pDJ179 was found to confer suppression (Fig. 1, last two lines); thus, a single mutation in the coding region of the wild-type *STE5* sequence was sufficient to cause a constitutive phenotype. The mutation in plasmid pDJ179, a C-to-T transition, is predicted to cause a change of a threonyl to a methionyl residue at position 52 in the *STE5* gene product (37, 63, 67); this mutant allele was designated *STE5*^{Hyp}*-2*. The stronger phenotype associated with pDJ174 is apparently due to an unidentified mutation(s) elsewhere in the plasmid that enhances the phenotype of *STE5*^{Hyp}*-2*; these mutations must be either in the C-terminal portion of the *STE5* coding region or in vector sequences. Since these mutations in plasmid pDJ177 had no detectable phenotype, they were not analyzed further.

To test whether pDJ179 confers a growth defect, we measured the growth rates of a wild-type haploid strain, YPH499, when it was carrying either the parent vector YEp352, the *STE5*$^+$ plasmid pDJ143, or the *STE5*^{Hyp}*-2* plasmid pDJ179. For two independent transformants of each plasmid, the culture doubling times at 34°C were determined and averaged. The strain carrying the vector alone grew with a generation time of 1.8 ± 0.2 h. The wild-type *STE5* gene in multiple copies did not affect the growth rate perceptibly (1.9 ± 0.2 h). However, the presence of the single base change in *STE5*^{Hyp}*-2* lengthened the generation time to 2.8 ± 0.6 h.

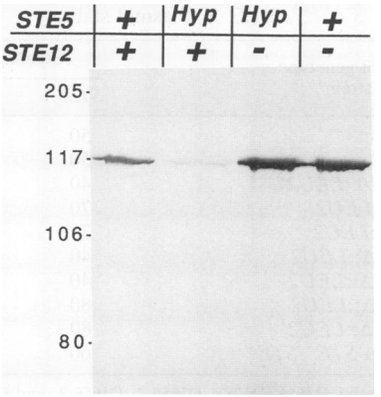

FIG. 2. Levels of *STE5* protein in mutant and wild-type strains. Cell extracts (150 μg of total protein) were resolved by sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and the proteins were transferred electrophoretically to nitrocellulose and probed with an affinity-purified antiserum that recognizes the *STE5* gene product. Strains used were DJ211-12-1 (*STE12*$^+$) and DJ1014A (*ste12*). Cells carry the *STE5* plasmid pDJ143 (+) or pDJ179 (*Hyp*) as indicated; the latter contains the *STE5*^{Hyp}*-2* allele. The positions of the protein molecular weight markers (kilodaltons) are indicated.

The lower growth rate of the strain containing pDJ179 was apparently due to a longer period in the G_1 phase of the division cycle. As judged from flow cytometry of propidium iodide-stained cells, the proportion of cells in the G_1 phase was 45% for the culture containing pDJ179, while it was 32 and 26% for the cultures containing pDJ143 and YEp352, respectively.

Levels of *STE5* gene product. We considered the possibility that phenotypes associated with the *STE5*^{Hyp} mutants were a consequence of an increased accumulation of the gene product. Immunoblot analysis was performed on extracts of cells carrying either the *STE5*$^+$ plasmid pDJ143 or the *STE5*^{Hyp}*-2* plasmid pDJ179 (Fig. 2). The antiserum (37) was raised against a C-terminal fragment of the *STE5* product that does not include the residue altered in the mutant protein. We found that the amount of *STE5* gene product recovered from cells carrying pDJ179 was in fact less than the amount recovered from the control cells carrying pDJ143. The lower level of mutant protein may be due to a growth advantage for the cells that carry fewer copies of the plasmid. Consistent with this view, plasmids pDJ143 and pDJ179 led to equal amounts of *STE5* protein in a *ste12* strain, in which *STE5*^{Hyp} does not cause a growth disadvantage (see below). Hence, the phenotype of the *STE5*^{Hyp}*-2* mutant is not due simply to a higher level of the gene product; rather, the phenotype persists despite a relatively low level of the mutant protein.

Suppression of sterility. The combined phenotype caused by mutations affecting two different steps in a cellular process can provide information about the order in which the two gene products function (38, 45). Constitutive activation of the pheromone response pathway caused by a *STE5*^{Hyp} mutation should bypass the mating defect imposed by a block of an earlier step of the pathway. This approach was used by other investigators (14, 44, 77) to show that the constitutive *gpa1*, *srm1*, and *cdc72* mutants could bypass the block caused by a deletion of the receptor structural gene, *STE2*. Similarly, cells containing both the constitutive *STE4*^{Hpl} mutation and the conditionally unresponsive *ste5-3* mutation do not respond to pheromone and fail to exhibit the constitutive-arrest phenotype of *STE4*^{Hpl} at the restrictive temperature for *ste5-3* (2). This result suggests either that the *STE5* gene product functions after the *STE4* product or that the two proteins affect the same event in the signal transduction pathway. The *STE5*^{Hyp} mutant permitted a resolution of this ambiguity. Analysis of double mutants was also used to examine the relationships of the *STE5* product to the α-factor receptor, encoded by *STE2*; the G_γ subunit, encoded by *STE18*; a DNA-binding protein, encoded by *STE12*; and protein kinases involved in pheromone signal transduction, encoded by *STE7*, *STE11*, *FUS3*, and *KSS1*.

TABLE 2. Mating efficiency

Relevant genotype of strain[a]	Diploids produced[b]				Temp (°C)
	YEp plasmid		YIp plasmid		
	STE5	*STE5*^{Hyp}	*STE5*	*STE5*^{Hyp}	
STE$^+$	3×10^6	1×10^6	2×10^6	2×10^6	22
ste2-10::*LEU2*	4×10^2	1×10^3	<10	<10	22
ste4::*LEU2*	<10	1×10^3	<10	<10	22
*ste4*Δ::*LEU2*	<10	2×10^3	ND[c]	ND	22
ste7::*LEU2*	<10	<10	ND	ND	22
*ste11*Δ::*hisG*	<10	<10	ND	ND	22
*ste12*Δ::*LEU2*	<10	<10	ND	ND	22
*ste18*Δ::*LEU2*	<10	2×10^4	ND	ND	22
ste4-3	2×10^3	3×10^5	30	4×10^4	34
STE$^+$ (YPH499)	4×10^6	ND	ND	ND	22
fus3-6::*LEU2* *kss1*Δ::*HIS3*	<10	<10	ND	ND	22

[a] Strains used in these experiments were derived as haploid *MAT***a** segregants of the diploid strain DJ656, DJ787, DJ656-2, DJ656-3, DJ656-4, or DJ783. Other strains were transformants of the haploid strain 381GULΔSTE4, DJ1023A, DJ1003A, YPH499, or YPH499fus3kss1. For wild-type strains, the strain name is in parentheses. All other strains except YPH499fus3kss1 and YPH499 are congenic to 381G. Strain YPH499fus3kss1 is derived from YPH499.

[b] Data are the number of colonies growing on the unsupplemented minimal plates multiplied by the dilution factor. Strains carried one of the following plasmids, as indicated: pDJ143 (YEp *STE5*); pDJ174 (YEp *STE5*^{Hyp}), containing *STE5*^{Hyp}*-1* for all strains except the *fus3 kss1* strain, which carried pDJ179, containing *STE5*^{Hyp}*-2*; pDJ144 (YIp *STE5*); or pDJ175 (YIp *STE5*^{Hyp}), containing *STE5*^{Hyp}*-1*.

[c] ND, not determined.

Double mutants were generated in two ways: (i) *ste* mutants were transformed directly with pDJ174, pDJ179, or pDJ143, and two independent transformants of each plasmid were tested for mating ability; or (ii) diploid strains heterozygous at a *STE* locus were transformed with the *STE5* plasmids and induced to sporulate, the resulting asci were dissected, and *ste* segregants that carried a plasmid were tested for mating ability. The results are presented in Table 2. The mating defect of the *ste2* strain was partially suppressed by either the plasmid pDJ174, containing *STE5*^{Hyp}*-1*, or the plasmid pDJ143, containing *STE5*$^+$. The *ste4* and *ste18* null mutants were partially suppressed by pDJ174, while pDJ143 did not increase mating to a detectable level. *STE5*^{Hyp}*-1* could not suppress the mating defect caused by a disruption of the *STE7* gene or by deletion of the *STE11* or *STE12* gene, nor could *STE5*^{Hyp}*-2* suppress a combined deletion of the *FUS3* and *KSS1* genes. These results suggest that the *STE5* gene product acts after the G protein on the signal transduction pathway and that it acts either before or at the same step as the protein kinases and the transcription factor. These results are in agreement with recent reports that *ste5* mutants are unable to block the phenotype of a constitutive *STE11* allele (8, 29, 76).

When the integrating plasmid pDJ175 or pDJ144 was introduced into the chromosome at the *URA3* locus, we found no suppression of the *ste2-10*::*LEU2* or the *ste4*::*LEU2* null allele (Table 2). Apparently, a high dosage of the $STE5^{Hyp}$-*1* gene is required in order to provide a signal sufficient for mating in the absence of those gene products. In contrast, the mating defect of the temperature-sensitive *ste4-3* mutant was suppressed by the $STE5^{Hyp}$-*1* allele when it was integrated at the chromosomal *URA3* locus in a single copy (Table 2). We also found that multiple copies of $STE5^{+}$ could suppress the temperature-sensitive allele of *STE4*, in agreement with the previous observation of MacKay (56). Thus, less $STE5^{Hyp}$-*1* product is needed for suppression of *ste4-3* than for suppression of a disruption of the gene; even the wild-type *STE5* gene product, at high enough levels, suffices. The allele-specific suppression of the *ste4* mutants is consistent either with a direct interaction between the *STE4* and *STE5* products or with a weak signal generated by the *ste4-3* product at the restrictive temperature which can be amplified in a separate step either by the $STE5^{Hyp}$ product or by overproduction of the $STE5^{+}$ product. If direct interactions do occur, they are unnecessary for the constitutive signal generated by the $STE5^{Hyp}$-*1* and $STE5^{Hyp}$-*2* products when they are expressed from high-copy-number plasmids, since these plasmids suppressed a *ste4* deletion mutant (Table 2 and Fig. 1).

Suppression of the slow growth phenotype by *ste* mutations. Because cells containing mutant plasmid pDJ174 ($STE5^{Hyp}$-*1*) or pDJ179 ($STE5^{Hyp}$-*2*) grew more slowly than cells containing the control plasmid pDJ143, we wished to determine whether normal growth rates could be restored by blocking specific steps in the pheromone response pathway. The ability of the mutant pDJ174 to inhibit cell division was inferred from the frequency at which cells lacking the plasmid appeared under nonselective culture conditions. The percentage of cells containing the plasmid should be a sensitive measure of the relative growth rate of cells with and without the plasmid, because cells that have lost the plasmid will grow faster and take over the population. The relative abilities of the *ste* mutant and STE^{+} cells to retain plasmids are summarized in Table 3. Plasmid pDJ174 ($STE5^{Hyp}$-*1*) was maintained poorly in STE^{+} and *MAT***a** *ste2* cells (≤3% retention), whereas it was relatively stable (≥20% retention) in cells containing *ste4*, *-5*, *-7*, *-11*, or *-12* or in **a**/α diploid cells. In contrast, the stability of control plasmid pDJ143 ($STE5^{+}$) was unaffected by *ste* mutations or the *MAT* locus. Plasmid pDJ179 ($STE5^{Hyp}$-*2*) was also unstable in a wild-type host (data not shown). Hence, mutations in the *STE4*, *STE12*, and *STE18* genes block the ability of pDJ174 to inhibit cell division. The effects of the *ste4* and *ste18* mutations were unexpected, since pDJ174 permits mating in the complete absence of *STE4* and *STE18* gene function (Table 2). Together, the data indicate that the product of the $STE5^{Hyp}$-*1* gene can activate the pheromone pathway in the absence of the pheromone receptor and the G protein but that for full activity it requires $G_{\beta\gamma}$.

Increase in transcription from a pheromone-inducible promoter. A pheromone-inducible reporter gene was used to examine the activation of the pheromone response pathway in the various *ste* mutants. The reporter plasmid pDJ10 (80) carries an α-factor-inducible promoter (containing eight copies of the pheromone response element) fused to the coding sequence of the *lacZ* gene. When this construct is expressed in wild-type *MAT***a** cells, α-factor pheromone stimulates the production of β-galactosidase by more than 10-fold (80). Both plasmids pDJ143 ($STE5^{+}$) and pDJ179 ($STE5^{Hyp}$-*2*) led to constitutive transcription of the reporter gene in the STE^{+} strain as judged by the increased level of β-galactosidase activity in the absence of pheromone (Table 4). Furthermore, the effect of the $STE5^{Hyp}$-*2* mutation on constitutive expression is independent of the presence or absence of normal *STE5* at the chromosomal location (compare STE^{+} with *ste5Δ1*::*LYS2*). In other experiments (data not shown), either a single copy (pDJ175) or multiple copies (pDJ174) of the $STE5^{Hyp}$-*1* allele also caused an approximately 10-fold increase in transcription. When the pheromone response pathway was blocked in *ste2*, *ste4*, and *ste18* mutants, the presence of the pDJ179 plasmid still resulted in an increased accumulation of β-galactosidase activity, whereas it was unable to effect an increase in this activity in the *ste7*, *ste11*,

TABLE 3. Plasmid stability

Relevant genotype of strain[a]	Stability of plasmid[b]	
	STE5	$STE5^{Hyp}$
*MAT***a** STE^{+}	50	2
*MAT*α STE^{+}	50	2
*MAT***a** *ste2-10*::*LEU2*	40	3
*MAT***a** *ste4*::*LEU2*	70	20
*MAT*α *ste4*::*LEU2*	30	20
*MAT***a** *ste12Δ*::*LEU2*	40	20
*MAT*α *ste12Δ*::*LEU2*	40	20
*MAT***a** *ste18Δ*::*LEU2*	80	40
*MAT*α *ste18Δ*::*LEU2*	80	20
*MAT***a**/*MAT*α STE^{+}/STE^{+}	60	50

[a] Diploid strains DJ656, DJ787, DJ656-2, DJ656-3, and DJ656-4, heterozygous for the *ste* allele indicated, were transformed with plasmid pDJ143 or pDJ174. Tetrads from these strains in which the disruption segregated 2 (Leu^{+}):2 (Leu^{-}) and the plasmid segregated 4 (Ura^{+}):0(Ura^{-}) were used in plasmid loss experiments. In the case of the *ste2-10*::*LEU2* deletion, only *MAT***a** strains were used. All four segregants in a tetrad (two *ste* mutant and two STE^{+}) were tested in parallel for plasmid loss at least twice, and results of the independent determinations were averaged.

[b] Data are the percentage of cells retaining plasmid pDJ143 (*STE5*) or pDJ174 ($STE5^{Hyp}$-*1*), as determined after the cells had been cultured for a defined period in the absence of selection for the plasmid. Strains retaining less than 10% of the plasmid were considered unstable.

TABLE 4. Effect of *STE5* activity on transcription from a pheromone-inducible promoter

Relevant genotype of strain[a]	β-Galactosidase activity[b] (U)		
	Vector	*STE5*	$STE5^{Hyp}$-*2*
STE^{+} (DJ602-136)	3	20	50
ste2-10::*LEU2*	10	60	90
ste4::*LEU2*	0.2	0.2	20
ste18Δ::*LEU2*	0.3	0.3	20
ste5Δ1::*LYS2*	0.3	30	60
ste7::*LEU2*	0.04	0.04	0.04
ste11Δ::*hisG*	0.04	0.05	0.05
ste12Δ::*LEU2*	0.02	0.02	0.02
STE^{+} (YPH499)	2	20	30
fus3-6::*LEU2 kss1Δ*::*HIS3*	0.05	0.05	0.04

[a] The *MAT***a** strains DJ602-136, DJ213-6-3, DJ1004-11-1, DJ1000A, DJ1023A, DJ1003A, DJ1005-9-1, DJ1006-17-2, YPH499, and YPH499fus3kss1 were used. For wild-type strains, the strain name is in parentheses. All strains except YPH499 and YPH499fus3kss1 were congenic to strain 381G.

[b] Results are the averages of those from at least two independent transformants containing both the reporter plasmid JD10 (80) and the plasmid indicated. When treated with 2.3×10^{-6} M synthetic α-factor for 45 min, the wild-type strain DJ602-136 carrying YEp352 gave 30 U of β-galactosidase activity. Strains contained the YEp-based plasmid YEp352 (vector), pDJ143 (*STE5*), or pDJ179 ($STE5^{Hyp}$-*2*).

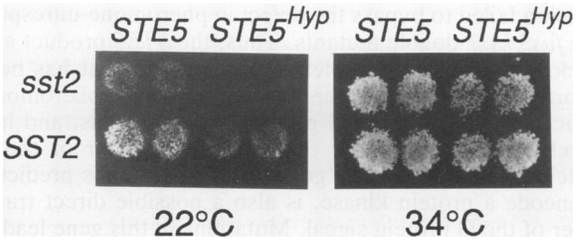

FIG. 3. The *sst2-1* mutation enhances the growth defect of the *STE5^{Hyp}-1* mutant. Strains DJ676-2-2 (*sir3^{ts}*) and DJ788-7-1 (*sir3^{ts} sst2*) were transformed with plasmid pDJ174, which contains *STE5^{Hyp}-1*, or pDJ143, which contains *STE5^{+}*. Two independent transformants (10 μl of a stock grown to saturation at 34°C) were spotted on a plate, incubated overnight at 22°C, and replica plated twice at 22 or 34°C.

ste12, or *fus3 kss1* strain. These results are consistent with the results of the assays for suppression of sterility and for plasmid loss, namely, that the *STE5^{Hyp}* mutants can partially bypass the absence of the $G_{\beta\gamma}$ subunit (*ste4* and *ste18*) but not the absence of the kinases (*ste7*, *ste11*, and *fus3 kss1*) or of the transcription factor (*ste12*). The mutant *STE5^{Hyp}* product appears to be qualitatively different from the wild-type product, since pDJ143 (*STE5^{+}*) has no detectable effect in cells containing null alleles of *STE4* or *STE18*, whereas pDJ179 (*STE5^{Hyp}-2*) increases transcription 100-fold. Thus, the single amino acid change encoded by pDJ179 seems to make the protein at least partially independent of upstream events in the signal transduction pathway.

The basal level of transcription, observed in the strains carrying the vector plasmid, was influenced by the mutations listed in Table 4. The level of β-galactosidase activity was reduced in the *ste4*, *ste5*, and *ste18* mutants, and the level was reduced still further in the *ste11*, *ste12*, and *fus3 kss1* mutants. The basal level of transcription in the *ste2* mutant was consistently higher than that in the *STE^{+}* control. Similar results were obtained for basal levels of agglutinin production in *ste2* mutants (44); thus, the receptor appears to have a weak negative influence on the response pathway in the absence of pheromone.

Effects of the *sst2* mutation. The fact that cells containing *STE5^{Hyp}* are able to divide (albeit slowly) may be in part a consequence of the adaptation mechanisms that permit cells to recover from the pheromone-induced arrest of cell division. The wild-type *SST2* gene product is required for cells to recover from pheromones (11, 12); it is also necessary for recovery from the signal generated in the *STE4^{Hpl}* mutant (3). We therefore examined the effect of an *sst2* mutation on the growth of the cells carrying the *STE5^{Hyp}* alleles. An *sst2 sir3^{ts}* strain was constructed and transformed with plasmids pDJ174 (*STE5^{Hyp}-1*), pDJ179 (*STE5^{Hyp}-2*), and pDJ143 (*STE5^{+}*). Growth of the transformants was examined at temperatures that were permissive (22°C) and nonpermissive (34°C) for the *sir3^{ts}* mutant (Fig. 3). At 22°C the *SST2^{+} sir3^{ts}* strain containing the *STE5^{Hyp}-1* plasmid pDJ174 grew more slowly than the same strain containing the *STE5^{+}* plasmid pDJ143. The *sst2 sir3^{ts}* strain carrying pDJ174 showed the most severe growth defect, an observation that is consistent with the idea that the *SST2* gene product is involved in attenuation of the signal caused by the *STE5^{Hyp}-1* mutation; such attenuation might affect the pathway either upstream or downstream of the *STE5*-controlled step. Moderate slowing of growth by the pDJ143 plasmid in an *sst2 sir3^{ts}* strain probably reflects the partial activation of the signalling pathway caused by increased expression of *STE5* (Table 4). Plasmid pDJ179, containing the *STE5^{Hyp}-2* allele, had generally the same effect as pDJ174, but its effect was slightly less pronounced (36), and the weak effect of *sst2* in the strain containing plasmid pDJ143 (YEp *STE5^{+}*) was not observed with the vector control plasmid YEp352 (36). At 34°C, the restrictive temperature for *sir3^{ts}*, all strains exhibited normal growth.

We attempted to test the effect of the *sst2* mutation in a *SIR3^{+}* strain carrying pDJ174, but we were unable to construct such a strain, presumably because of an extreme growth defect. In subsequent crosses it became apparent that this extreme growth defect was suppressible by the *ste4* mutation. When the *ste4* strain DJ656-2-8-3 carrying pDJ174 was crossed with the *sst2* strain DJ786-11-1, we found that the *sst2* mutation was absent in the 12 *STE4^{+}* segregants carrying pDJ174, whereas it was present in the *ste4* segregants and in *STE4^{+}* segregants lacking the pDJ174 plasmid (data not shown). In an additional experiment, we tested whether the *ste4* mutation was required for the *sst2* mutation to influence the growth defect caused by plasmid pDJ174. When we constructed congenic *sst2 ste4* and *SST2^{+} ste4* strains (DJ656-2-6-2, DJ656-2-10-1, DJ789-2-2, DJ789-3-1, DJ789-5-2, and DJ789-5-3) containing plasmid pDJ174, we found that the plasmid stabilities in these strains were indistinguishable. Together these results indicate that the effect of the *sst2* mutation on the *STE5^{Hyp}* phenotype requires the activity of the *STE4* product, raising the possibility that the action of the *SST2* gene product on the pheromone response pathway is mediated at the level of the G protein.

DISCUSSION

In this report, we describe an alteration of the *S. cerevisiae STE5* gene which results in partial constitutive activation of the pheromone response pathway. We exploited this phenotype for the purpose of determining the relative order of events in the pathway. The *STE5^{Hyp}-1* allele was identified by mutagenizing a plasmid containing the *STE5* gene and screening yeast transformants for a haploid-specific growth defect. The *STE5^{Hyp}-2* allele, containing a single mutation present in *STE5^{Hyp}-1*, differs from the wild-type gene at a single base pair yet confers all of the phenotypes of the original *STE5^{Hyp}-1* allele. In addition to the haploid-specific growth phenotype, both *STE5^{Hyp}* alleles led to increased transcriptional activity of a pheromone-inducible gene in the absence of pheromone. Although the phenotype caused by *STE5^{Hyp}-2* is slightly weaker than that of the original isolate, no other mutation in the plasmid containing *STE5^{Hyp}-1* had an independent effect that was detectable in our assays. The phenotypes of both alleles are highly dependent on plasmid copy number, since single integrated copies of *STE5^{Hyp}* caused no growth defect and were unable to suppress the sterility of *ste4* and *ste18* deletion strains. Furthermore, the majority of the segregants which inherited YEp *STE5^{Hyp}* in tetrad analysis arrested growth as single pear-shaped cells, whereas other segregants were able to divide at a rate that was slower than that of the wild type. These slowly growing segregants as well as simple transformants were used to analyze the effects of the plasmid on mating and growth.

The single base change in *STE5^{Hyp}-2* is predicted to cause the substitution of a methionyl for a threonyl residue at position 52 in the coding sequence of the wild-type *STE5* gene product (37, 63, 67). In the wild-type *STE5* product, Thr-52 is found in the sequence Lys-Lys-Trp-Thr-Glu-Lys,

which is a consensus site for phosphorylation by both cyclic AMP-dependent protein kinase and protein kinase C. However, when cells containing plasmid pDJ143 (*STE5*$^+$) or pDJ179 (*STE5*^{Hyp}*-2*) were labelled in vivo with $^{32}P_i$ and the *STE5* products were purified, we detected no difference between the two-dimensional tryptic phosphopeptide maps for the mutant and wild-type proteins (36); furthermore, a phosphorylated synthetic version of the wild-type peptide was resolved from the labelled tryptic fragments (data not shown). We conclude that Thr-52 is not a major phosphorylation site and that the mutant phenotype is probably not due to alteration of the phosphorylation state of the *STE5* product. However, we cannot rule out the possibility that minor or very labile phosphorylation events are responsible for the phenotype.

The phenotype of the *STE5*^{Hyp} alleles is in some respects similar to that of the overproduced *STE5*$^+$ gene. Both the *STE5*^{Hyp} alleles and multiple copies of the *STE5*$^+$ gene increase the rate of transcription from a pheromone-inducible promoter, and both suppress the sterility phenotype of the *ste2* deletion mutant and the temperature-sensitive *ste4-3* mutant. However, the *STE5*^{Hyp} alleles differ from the *STE5*$^+$ allele in their ability to bypass deletions of the *STE4* and *STE18* genes. In both the suppressor analysis and the transcriptional assay, multiple copies of the *STE5*$^+$ gene had no effect in *ste4* or *ste18* deletion cells, while *STE5*^{Hyp}*-2* increased mating and transcriptional activity 100-fold. Thus, the *STE5*^{Hyp} product, unlike the *STE5*$^+$ product, can act in the absence of $G_{\beta\gamma}$ complex. Furthermore, the hyperactivity is not due to increased steady-state levels of *STE5* gene product, as shown by comparing immunoblots that were prepared from extracts of cells expressing the *STE5*$^+$ or *STE5*^{Hyp} alleles. In fact, for cells that contain an intact signal transduction pathway, the level of mutant protein was much lower than that of the wild type. This low level of mutant protein was apparently not due to mutations which inhibited replication or segregation of the plasmid, because plasmid pDJ179 contains only a single base pair change in the *STE5* coding region. Furthermore, the level of *STE5* protein produced from pDJ179 was the same as that of the wild type when both were expressed in a *ste12* strain, in which the growth rate was unaffected by the *STE5*^{Hyp} mutation. The observed low level of *STE5*^{Hyp}*-2* product in the *STE12*$^+$ strain may result either from a growth advantage of cells that contain fewer copies of the plasmid or from a *STE12*-dependent feedback mechanism that reduces the levels of *STE5* product.

The steps of the pheromone response pathway are defined genetically by null alleles of the *STE* genes that block signal transduction. The ability of the *STE5*^{Hyp} alleles to bypass these blocks was monitored by the suppression of sterility and the acquisition of pheromone-independent transcription from a pheromone-inducible promoter. Our results indicated that the *STE5* product acts after the α-factor receptor (encoded by the *STE2* gene) and the $G_{\beta\gamma}$ complex (encoded by *STE4* and *STE18*). The work of others (8, 21, 29, 76) indicates that the *STE5* product functions before four protein kinases (encoded by *STE7*, *STE11*, *KSS1*, and *FUS3*) and a transcription factor (encoded by *STE12*). Our results are consistent with these findings; however, we are unable to distinguish between the possibilities that the *STE5* product functions before or functions at the same step as the *STE7*, *-11*, *-12*, or *FUS3* product. Other published work (6, 23) indicates that simple overexpression of *STE5* can partially suppress certain *fus3* single mutants; this result is not inconsistent with the proposed order, since *STE5* overexpression failed to bypass the defect in pheromone-unresponsive *fus3 kss1* double mutants. Thus, the *STE5* product acts as close to the $G_{\beta\gamma}$ complex as any protein that has been reported to participate in the transduction of the pheromone-induced signal from the G protein to the nucleus, and it is therefore a candidate for the direct transducer of the G protein signal. The *STE20* gene product, which is predicted to encode a protein kinase, is also a possible direct transducer of the G protein signal. Mutations in this gene lead to a block in the pheromone response pathway that can be suppressed by *STE5*^{Hyp}*-1* and *STE5*^{Hyp}*-2* but not by overproduction of the *STE4*$^+$ product (53); these results suggest that the *STE20* gene product acts at or after the $G_{\beta\gamma}$ step and before the *STE5* gene product. Although a constitutive *STE20* mutant has been described (68), the relationship of this mutant to the G protein remains unresolved, since the authors report that it is unclear whether the phenotype is specific to the pheromone response pathway.

Several lines of evidence indicate that the pheromone response pathway is partially activated at a low basal level in the absence of pheromones. As depicted in Table 4, the basal activity of the pheromone-inducible promoter that remains in the various *ste* null mutants correlates with the position of the defective step in the response pathway. The earlier blocks in the pathway result in higher levels of basal signal, suggesting that spontaneous signals ("noise") arising at the earlier steps in the pathway are propagated through the later steps. The fact that the *ste5* null mutant showed a higher level of basal signal than the *ste7*, *ste11*, or *fus3 kss1* mutant provides suggestive evidence that the *STE5* product acts before the protein kinase products of these genes in the pathway. An early source of noise in the pathway may result from a small degree of spontaneous disassociation of the G protein subunits, causing partial activation of the response pathway in the absence of pheromone (82). Such a model is consistent with the observation that plasmids containing *STE5*^{Hyp} are more stable and cause lower levels of pheromone-inducible transcription in the *ste4* and *ste18* mutants, even though the G_β and G_γ products appear to act upstream. The *STE5*^{Hyp} product, while partially independent of this upstream signal in that it leads to partial restoration of fertility and basal transcription in the *ste4* and *ste18* mutants, is activated further by the small amount of free $G_{\beta\gamma}$ in wild-type cells. In a sense, the *STE5*^{Hyp} product amplifies the noise that is generated at the earlier step in the pathway.

The *SST2* gene product regulates the pheromone response pathway and allows cells to resume the division cycle after persistent exposure to pheromone (3, 12, 18). Our results indicate that this adaptive response promoted by the *SST2* product is required for the growth of strains containing an activated *STE5* gene product. Although the precise action of the *SST2* product is presently unknown, a number of observations are consistent with the notion that it regulates G protein function. (i) The *sst2* mutation can suppress conditionally unresponsive alleles of *STE4* (*ste4-3*, *ste4-5*, and *ste4-6*) in combination with either *ros1*, *-2*, or *-3* mutations, but it has no effect on the *ste5-3* allele, suggesting that the *SST2* product may affect the G protein but not the *STE5* gene product (47). (ii) The inability of cells to recover from the growth-inhibitory effects of *gpa1* null mutations (even though the *SST2* gene is fully induced) is consistent with a role for the *GPA1* product in adaptation (3, 20). (iii) The requirement of the *SST2* gene product for recovery from *STE4*^{Hpl}-induced cell cycle arrest suggests that the *SST2* product negatively regulates the G protein or a subsequent

step in the pathway (3). (iv) Here we show that the *sst2* mutation enhances the growth defect of the $STE5^{Hyp}$-*1* mutant and that this effect of *sst2* on $STE5^{Hyp}$ is blocked in strains harboring a deletion of the *STE4* gene. This result suggests that the *SST2* product acts before the G protein or at the same step. However, other interpretations are possible. Since we also show that *ste4* mutations reduce the severity of the defects in the $STE5^{Hyp}$ mutants, the possibility remains that the *STE4* product acts before the *SST2* product and that the activity of the *STE4* product is necessary to enhance the phenotype of the $STE5^{Hyp}$ product enough to allow us to detect the effect of the *sst2* mutation.

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