Genetic Analysis of the Saccharomyces Cerevisiae Pheromone Response Pathway: a Thesis

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GENETIC ANALYSIS OF THE SACCHAROMYCES CEREVISIAE
PHEROMONE RESPONSE PATHWAY

A Thesis Presented

By

Dmitry B. Blinder

Submitted to the Faculty of the
University of Massachusetts Graduate School of Biomedical Sciences,
Worcester
in partial fulfillment of the requirements for degree of:

DOCTOR OF PHILOSOPHY IN MEDICAL SCIENCES

May 1, 1990

Genetics
GENETIC ANALYSIS OF THE SACCHAROMYCES CEREVISIAE
PHEROMONE RESPONSE PATHWAY

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Dmitry B. Blinder

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May 1 1990
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Dedication

I dedicate this thesis to my family who supported me, even though I became a wrong kind of Doctor.
Acknowledgments

I wish to thank my mentor Duane Jenness for guiding me through this project. Without Duane this work would be impossible. I thank my parents, Boris and Luba and my beloved grandmother Rakhil, for their continued moral and financial support. I am very grateful to my wife Ellina and to my dear daughter Veronika for being very patient and to my parents-in-law, Anna, Alla and Vladimir for physical support. I am forever in debt to my good friend Nancy Hoe for editorial assistance. I am grateful to the members of the laboratory: to Phyllis Spatrick for being my friend and tuning the radio, to Jodi Hirschman for providing many useful discussion and to Mimi Hasson for last minute thesis reading. I am grateful to Gabriella Tirda, Ron, and Jeff ("Tolstoy") Barbon for providing many happy moments with the media. Thanks to all members of the M.G.M. Department for wonderful six years in UMass.
Respective Contributions

Suzanne Bouvier initiated this project by constructing the strains (DJ603-136, DJ603-5-3, and DJ603-4-2) and isolating the first non-sectoring mutant (scg1-5).

Phyllis Spatrick had constructed the pDJ117 plasmid described in Chapter I (Materials and Methods).

Dr. Richard E. Baker and Tim O'Brien constructed the plasmid pDJ119 (Chapter I) for a bet of six-pack of imported beer.
ABSTRACT

The cell division of *Saccharomyces cerevisiae* is controlled by the action of pheromones at the G1 phase of the cell cycle. A general method was developed for the isolation of constitutive mutants in the pheromone response pathway. Recessive alleles of the *SCG1* gene (encoding the α subunit of a G protein) were isolated as well as a dominant mutation in the *STE4* gene (encoding the β subunit of a G protein). Analysis of double mutants suggested that the *STE4* gene product functions after the *SCG1* product but before the *STE5* gene product. Double mutants carrying either *scg1* or *STE4*<sup>Hpl</sup> constitutive alleles together with the temperature-sensitive unresponsive mutation, *ste5-3<sup>ts</sup>* showed arrest and recovery when shifted from 34<sup>°</sup> C to 22<sup>°</sup> C. Recovery from the constitutive signal was independent of the receptor. The *STE4*<sup>Hpl</sup> *sst2 ste5<sup>ts</sup>* triple mutant was not able to recover from arrest, suggesting that an SST2-dependent mechanism is involved in recovery of the *STE4*<sup>Hpl</sup> mutant from constitutive arrest. In contrast, the *scg1-7 sst2 ste5<sup>ts</sup>* triple mutant recovered only partially suggesting that even though SST2 gene product is probably involved in recovery of the *scg1-7* mutant, this mutant can recover by an SST2-independent mechanism. This implies existence of another, SST2-independent postreceptor recovery mechanism. The *scg1*-null mutant do not recover from constitutive arrest (J. Hirschman, personal communication). Both recovery mechanisms probably operate at the G protein step.
Isolation of a constitutive allele of *STE5* allowed the definition of its site of action as being after the *STE4*-controlled step. In addition, constitutive activation of the pheromone pathway by *STE5*<sup>Hp1</sup> mutation was found to be partially dependent on the *STE4* and *STE18* gene products, the β and γ subunits of a G protein. A comprehensive genetic model is presented to explain the mechanisms of signal transduction and recovery.
INTRODUCTION

Signal transduction mechanisms are used by cells to sense environmental cues. In a majority of eucaryotic cells the cell cycle is regulated at the G1 step by the action of hormones, nutrients, temperature, etc. The cell's ability to correctly perceive these stimuli, respond to them, and then adapt when the stimuli are withdrawn, is crucial to its survival. In cases of hormonal regulation, cells have evolved specific receptors that recognize agonists and activate post-receptor mechanisms which transmit the signal to its final destination. Stimulation of receptors by hormones often results in the subsequent activation of desensitization pathway(s) which allow cells to fine-tune the response with respect to strength and duration of the stimuli. Many components of the signal transduction pathways remain elusive due to the complexity of higher eucaryotic systems.

The laboratory strains of *Saccharomyces cerevisiae* offer an ideal model system for understanding basic features of signal transduction. Yeast cells secrete and respond to small polypeptides called pheromones. These hormone-like peptides bind to specific receptors on the cell surface; the complexes then trigger activation of the signaling pathway resulting in cell-cycle arrest at the G1 step. Different doses of pheromones elicit different responses with the highest concentration causing the most severe phenotype. Yeast cells
have also developed mechanisms that allow them to adapt and
resume mitotic growth after prolonged exposure to pheromones.
Thus the mechanism of signal transduction and its regulation can be
pursued in a well defined biological system. The ease of handling and
biochemical manipulation in combination with indepth genetic
analysis have caused yeast to be one of the most studied lower
eucaryotic organisms to date. We have employed these advantages to
gain a partial understanding of underlying mechanisms of cell
communication.

Life cycle of *Saccharomyces cerevisiae*

The two haploid cell types in yeast, *a* cells and *α* cells, differ at
a single regulatory locus, designated *MAT*. The *MATα* and *MATα* loci
each contain two open reading frames that encode the *α*1 and *α*2
proteins and the *α*1 and *α*2 proteins, respectively. In *α* cells, it is the
action of the *α*1 and *α*2 regulatory proteins that determines cell type,
whereas *a* cells are determined by the absence of the *α*1 and *α*2
regulatory proteins. *α*1 protein is thought to activate transcription of
*α*-specific genes and *α*2 is thought to repress all *a*-specific
transcripts. There are no known roles for *α*1 and *α*2 regulatory
proteins in the haploid *a* cell (reviewed by Herskowitz, 1988).

The *a* and *α* cells express a specific set of haploid-specific and
cell-type-specific genes which include secreted pheromones,
pheromone receptors and some of the postreceptor machinery for
activation of the pheromone response pathway. It is the expression
of cell-type-specific genes that make \( \text{a} \) cells and \( \alpha \) cells phenotypically different (reviewed by Cross, 1988). Haploid \( \text{a} \) cells secrete \( \text{a} \)-factor pheromone and produce \( \alpha \)-factor receptor which allows them to communicate with \( \alpha \) cells; haploid \( \alpha \) cells secrete \( \alpha \)-factor pheromone and produce \( \text{a} \)-factor receptor permitting them to communicate with \( \text{a} \) cells (Sprague et al., 1983a). Binding of pheromones to specific receptors on the surface of the opposite mating-type cell causes cell-cycle arrest at a point in the G1 stage, termed "START" (Hartwell, 1973). This arrest is thought to synchronize cells for further events in mating such as cell fusion and karyogamy (McCaffrey et al., 1987; Trueheart et al., 1987; Curran and Carter, 1986; Rose et al., 1986). Other responses to pheromones include induction of cell-type-specific agglutinins, the extracellular glycoproteins, that allow cells of opposite mating type to aggregate. Haploid cells that have been exposed to pheromones exhibit increased transcription of several genes: these include genes that are thought to be involved in transducing the signal, promoting cell fusion, and recovery from cell-cycle arrest (reviewed by Nasmyth and Shore, 1987; Dietzel and Kurjan, 1987b).

Prolonged exposure to pheromones causes morphological changes (shmoo formation) in which a projection extends toward a cell of the opposite mating type, presumably toward higher pheromone concentration (Tkacz and MacKay, 1979; Moore, 1983). Such adjacent cells can undergo cellular and nuclear fusion to form a diploid cell. The diploid will now contain both \( \text{MATa} \) and \( \text{MATa} \).
regulatory loci. Presence of these loci in the same cell will repress all haploid-specific and cell-type-specific transcription thus making diploids unresponsive to pheromones and unable to mate. It is thought that combination of α1-α2 proteins act as a transcriptional regulator to repress haploid-specific and activate diploid-specific transcription (reviewed by Herskowitz, 1989).

Diploid cells will grow mitotically with only subtle phenotypic differences from haploid cells. The diploid cells are somewhat larger than haploids and their pattern of bud formation is polar rather than equatorial (Freifelder, 1960). Under conditions of nutrient limitation, i.e. nitrogen starvation and poor carbon source, diploid cells undergo meiosis and sporulation. The result is four haploid products, two α-cell spores and two α-cell spores which remain dormant until favorable nutrient conditions arise.

**Recovery of Cells from Pheromone-Induced Arrest**

Haploid cells possess mechanisms for recovery from the action of pheromones (Chan, 1977; Moore, 1984). After prolonged exposure to pheromones, cells can overcome cell-cycle arrest, resume mitotic growth and become insensitive to the same or lower concentration of mating factors. There are at least three independent intracellular mechanisms for adaptation (desensitization in the presence of pheromones) which are induced upon exposure to pheromones (Konopka et al, 1988; Reneke et al, 1989; Blinder and Jenness, 1989; Dietzel and Kurjan, 1987b; Chan and Otte, 1982a). Defects in two
separate components of the pheromone response pathway impaire the cell's ability to recover from the pheromone-induced arrest (Konopka et al., 1988; Reneke et al., 1988; Chan and Otte, 1982). Mutations in the SST2 gene as well as carboxy-terminal truncation of the $\alpha$-factor receptor ($STE2$) exhibit $\alpha$-factor supersensitive phenotype. In addition, the genetic evidence presented in this thesis predicts the existence of yet another recovery mechanism that operates on postreceptor-induced signal (Blinder and Jenness, 1989). Also, a cells constitutively secrete $\alpha$-factor specific protease ($BAR1$) which degrades $\alpha$-factor in the medium (Sprague and Herskowitz, 1981). Production of the protease is induced to higher levels upon exposure to $\alpha$-factor (Kronstad et al., 1987). Mutations in Bar1 protease are known to cause defects in recovery (Chan and Otte, 1982b).

There is a number of possible reasons for complicated recovery mechanisms. Selection of the mating partner in the conjugation mix must depend on the relative proximity of the cells and, therefore, on pheromone concentration (Jackson and Hartwell, 1990). Recovery mechanisms can help to eliminate the pheromone "noise" from distant cells and prevent lethal G1 arrest in the case of an unsuccessful mating event. In addition, recovery mechanisms are required for the newly-formed zygote to resume mitotic cycle, since the two haploid cells that formed the zygote were arrested in G1 prior to fusion.
Genetic Analysis of the Pheromone Response Pathway

Successful mating in yeast depends on expression of both constitutive and pheromone inducible functions. In the absence of pheromones, these functions are expressed at the basal level, however, in the presence of pheromones, expression of the inducible functions is increased several-fold. Both haploid cell types constitutively produce pheromones and gene products necessary for their processing and secretion. Constitutive functions also include expression of the pheromone receptors on the surface of each cell type. Pheromone inducible functions include induction of cell surface agglutinins (Fehrenbacher et al, 1978) and increased transcription of several genes including genes involved in signal transduction and cell fusion among others (Hartig et al, 1986; McCaffrey et al, 1987; Trueheart et al, 1987; Van Arsdell et al, 1987). Pheromones induce cell-cycle arrest in G1 (Bucking-Throm et al, 1973) and after prolonged exposure cause morphological changes (shmoo formation) (Moore, 1983).

Sterile mutants

Genetic analysis of the pheromone response in yeast began with the isolation of mutants that failed to mate with the opposite mating type cells (MacKay and Manney, 1974a, b). Haploid cells of each mating type were mutagenized and later challenged to mate with cells of the opposite mating type. Those cells that failed to form prototrophic diploids were selected as mutant candidates. Nonmaters
were designated *ste* for sterile. In addition to initial phenotypic characterization, further complementation analysis was impaired since the mutant cells formed diploids at very low frequency. However, from a large number of mutants several classes were identified. In particular, two classes of mutants, of which *ste2* and *ste3* are representative, exhibited a nonmating phenotype only in one cell type (α and α cells, respectively). Other classes such as *ste4* and *ste5* exhibited a sterile phenotype in both α and α cells. All of the above mutants failed to mate, however, they retained a partial level of the constitutive functions such as pheromone production and secretion. *ste1* mutations were linked to the *MATα* locus; they are apparently alleles of the *MATα1* gene. Thus they are involved in mating-type control and affect the mating pathway indirectly (MacKay and Manney, 1974b).

Further genetic analysis was undertaken by selecting for the α-factor unresponsive phenotype (Manney and Woods, 1976; Hartwell, 1980). In these studies, the unresponsive phenotype was required to be conditional (temperature-sensitive) to facilitate genetic analysis (Hartwell, 1980). Eight complementation groups were identified among temperature-sensitive, *MATα* unresponsive mutants. They were also designated sterile (*ste*) in accordance with the nomenclature of MacKay and Manney (1974a). Mutants in the *ste2* complementation group produced sterility only in α cells and *ste4, 5, 7, 8, 9, 11, and 12* were mating defective in both α and α cells. The *ste2* mutants were defective only for the two inducible properties,
division arrest and agglutination, whereas *ste4, 5, 7, 11,* and *12* were defective to varying degrees in the constitutive functions (α-factor destruction, α-factor production) as well as the inducible functions. The *ste8* and *ste9* mutants were strongly defective in all aspects of mating; in addition, they produced a polar budding pattern similar to diploid cells, rather than the equatorial pattern which is characteristic of haploids. These mutants were later shown to be allelic to *sir3* and *sir4* mutations, respectively, and are thought to be involved in mating-type regulation through repression of silent *MAT* cassettes (Rine and Herskowitz, 1987).

Since *ste2* and *ste3* mutants were cell-type-specific mutations, they were likely to contain defects in cell-type-specific functions such as expression of pheromone receptors or pheromone secretion. Direct evidence that *STE2* encodes the receptor came from the α-factor binding experiments where radioactively-labelled α-factor bound to a cells in a specific, saturable manner. The binding depended on the *STE2* gene product (Jenness et al, 1983; 1986; Blumer et al, 1988). Temperature-sensitive alleles of *STE2* directed the synthesis of a thermolabile pheromone-binding activity. Furthermore, response to *Saccharomyces cerevisiae* α-factor in *Saccharomyces kluveri* cells was dependent on the expression of *Saccharomyces cerevisiae STE2* gene (Marsh and Herskowitz, 1988). Binding of α-factor to α-factor receptors was used to establish that receptors are down-regulated in response to the pheromone,
probably due to internalization and degradation (Jenness and Spatrick, 1986; Chvatchko et al, 1986).

The α-factor receptor was suggested to be encoded by the \textit{STE3} gene by indirect evidence since α-factor binding assays have not been developed (Hagen et al, 1986). The \textit{ste3} mutation produced cell-type specific sterility exclusively in \textit{MATa} cells (MacKay and Manney, 1974a). The \textit{STE3-β}-galactosidase fusion protein fractionates into the particulate fraction of yeast cell extracts, suggesting that \textit{STE3} is a membrane protein (Hagen et al, 1986). Significantly, the sequence of the \textit{STE3} gene resembles that of the \textit{STE2} gene in that both gene products are predicted to contain seven hydrophobic stretches of amino acids capable of spanning the membrane bilayer (Burkholder and Hartwell, 1985; Nakayama et al, 1985; Hagen et al, 1986). This common structure of the \textit{STE2} and \textit{STE3} receptors is shared by G-protein coupled receptors such as rhodopsin, the β-adrenergic receptor, and the muscarinic acetylcholine receptors. Receptors of this class are thought to bind their ligand within the hydrophobic segments (Strader et al, 1987).

Besides the α-factor and α-factor receptors, what other components of the signal transduction machinery are cell-type specific? This question was answered by expressing the pheromone receptors in cell types that normally do not express them, i.e., α-factor receptor in α cells and α-factor receptor in α cells. The idea was that cells lacking any other cell-type specific component of the signal transduction apparatus will not respond to respective
pheromones. Although, \textit{STE2} and \textit{STE3} gene products did not completely compensate for the lack of appropriate receptors, the mating was sufficient to exclude any other essential component (Bender and Sprague, 1986). This result suggests that pheromone receptors are the only necessary cell-type-specific components of the signal transduction mechanism.

Analysis of the cell-type-nonspecific sterile mutants revealed important components of the mating pathway that directly influence the signal. Initial characterization of the \textit{ste4} and \textit{ste5} mutants revealed moderate defects in constitutive and strong defects in inducible functions (MacKay and Manney, 1974a, b; Hartwell, 1980). The \(\alpha\)-factor destruction and \(\alpha\)-factor production in these mutants were clearly decreased compared to the \textit{ste2} mutants, however, \textit{ste4} and \textit{ste5} mutants expressed constitutive functions better then \textit{ste7}, \textit{11}, and \textit{12} mutants (Hartwell, 1980). Constitutive secretion of the Bar1 protease was decreased only 5-fold in \textit{ste4} and \textit{ste5} mutants, as compared to 25-fold decrease in \textit{ste7}, \textit{11}, and \textit{12}. The moderate effect of \textit{ste4} and \textit{ste5} on constitutive functions may result from a block in the basal level of signal ("noise") in the absence of pheromones. Strong defects in \(\alpha\)-factor inducible functions and moderate defects in constitutive functions suggest that \textit{STE4} and \textit{STES5} are directly involved in signal transduction. Moreover, \textit{ste4} and \textit{ste5} mutants show significant \(\alpha\)-factor binding, but do not respond to the pheromones, suggesting their involvement in postreceptor functions. Affinity of the \(\alpha\)-factor receptor for its ligand was
somewhat decreased in \textit{ste4} mutants while in \textit{ste5} mutants the receptor binding was unaffected (Jenness et al, 1987). This observation suggests a possible direct or indirect interaction between the \textit{STE2} and \textit{STE4} gene products.

Suppressor analysis of the \textit{ste2}, \textit{ste4} and \textit{ste5} mating defects revealed different patterns of suppression. The \textit{ste2-3} mutation was suppressed by the \textit{sst2-1} mutation, which causes supersensitivity to pheromones. Several temperature-sensitive alleles of \textit{ste4} were suppressed by a combination of \textit{sst2-1} with any of the three \textit{ros1}, 2 or 3 (relaxation of sterility) mutations. On the other hand, the temperature-sensitive alleles of \textit{ste5} were suppressed only by \textit{ros1}, with \textit{sst2} mutation having no effect (Jenness et al, 1987). The different suppression patterns suggest that \textit{STE2}, \textit{STE4}, and \textit{STE5} gene products might act at separate steps in the mating pathway.

Mating defects caused by some alleles of \textit{ste4} and \textit{ste5} can also be suppressed by \textit{cdc36} or \textit{cdc39} mutations (Shuster, 1982), which cause cell cycle arrest at the pheromone arrest point, "START" (Reed, 1980). \textit{cdc39} was shown to be allelic to \textit{ros1} (Jenness et al, 1987).

Cloning of the \textit{STE} genes was achieved by complementation of the nonmating phenotype. Sequencing of the \textit{STE4} and newly identified \textit{STE18} genes revealed amino-acid homology to the \(\beta\) and \(\gamma\) subunits of mammalian G protein, respectively (Whiteway et al, 1989). The yeast homolog of the \(\alpha\) subunit of the G protein has also been identified (Deitzel and Kurjan, 1987a; Miyajima et al, 1987; Jahng et al, 1988). Work presented in this thesis as well as work of
others (Chapter II; Whiteway et al, 1989) suggests that in yeast it is the β and γ subunits of the G protein that activate the response to pheromones as opposed to mammalian systems where it is the α subunit that causes the activation of the pathway. Cloning and sequencing of the STE5 gene has so far been uninformative about its mode of action (M. Hasson and J. Thorner, personal communication).

Analysis of the ste7, 11, and 12 mutants implicates their gene products in transcriptional regulation of haploid-specific genes (Fields and Herskowitz, 1987; Fields et al, 1988). Mutations in this group of genes are strongly defective in both constitutive and inducible functions. Transcription of STE2 and STE3 genes as well as pheromone structural genes MFα1, MFα1, and MFα2 is reduced in ste7, 11, or 12 backgrounds. Any double mutant combination fails to decrease transcription further, suggesting that the STE7, 11, or 12 gene products may act in the same pathway (Fields et al, 1988). The sterility of ste7, 11, and 12 mutants can therefore be attributed at least in part to the defect in synthesis of pheromones and pheromone receptors which make cells unable to elicit a response or respond in the mating reaction. Interestingly, the STE5 transcript level is unaffected in these mutants (Fields et al, 1988). Cloning and sequencing of STE7 and STE11 revealed amino-acid homology to the family of protein kinases (Chaleff and Tatchell, 1985; Teague et al, 1986; B. Errede, unpublished observations). The STE12 protein has been shown to bind to the DNA sequence element responsible for pheromone induction (Errede and Ammerer, 1989; Dolan et al, 1989).
The current model for \textit{STE7}, \textit{11}, and \textit{12} action predicts transcriptional regulation of pheromone-inducible genes by \textit{STE7} and \textit{11} kinases through the \textit{STE12} gene product (reviewed by Herskowitz, 1989).

In summary, analysis of \textit{ste} mutants revealed components of the pheromone response pathway which directly influence the signal such as \textit{STE2} and \textit{STE3}, the \textit{a-} and \textit{\alpha-}factor receptors, \textit{STE4} and \textit{STE18}, the \textit{\beta} and \textit{\gamma} subunits of a G protein, and \textit{STE5}, the function of which is as yet unknown. The \textit{STE7, 11, and 12} gene products may influence the pathway indirectly by transcriptionally regulating other components in the mating pathway; however, their direct involvement in signal transduction is not ruled out.

\textbf{Supersensitive Mutants}

Isolation of mutants which are more sensitive to mating factors permitted further genetic dissection of the pheromone response pathway. Eight independently isolated mutants which are supersensitive (\textit{sst}) to the G1 arrest induced by the \textit{\alpha-}factor were identified by screening mutagenized \textit{MATa} cells for increased growth inhibition by the pheromone. These mutants carried lesions in two complementation groups, \textit{sst1} and \textit{sst2} (Chan and Otte, 1982a, b).

The \textit{sst1} mutation caused supersensitivity only in \textit{a} cells and had no effect in \textit{\alpha} cells. The \textit{SST1} gene product appeared to act extracellularly since \textit{MATa SST1} cells alleviated the supersensitivity effect of \textit{MATa sst1} cells in mixed cultures. The \textit{sst1} mutations are
alleles of the *BAR1* locus, which encodes an α-cell-specific extracellular protease that degrades α-factor pheromone (Chan and Otte, 1982b; Sprague and Herskowitz, 1981; Manney, 1983).

In contrast, the *sst2* mutations affected both a and α cells and the supersensitivity phenotype was not corrected by exogeneously added wild-type cells (Chan and Otte, 1982b). About 10% of the cells in growing cultures of *sst2* mutants accumulate as aberrant schmoo cells, even in the absence of pheromones. Cell-cycle arrest in *sst2* mutants can be triggered by α-factor concentrations two orders of magnitude lower than that necessary to cause the same effect in wild-type cells (Chan et al., 1983). Thus, the *SST2* gene product probably acts either by increasing receptor sensitivity or by affecting some post-receptor step in the signalling pathway. Cloning and sequencing of the *SST2* gene did not reveal any apparent homologies or its mode of action. *SST2* transcription is haploid-specific and significantly induced after treatment with pheromones (Deitzel and Kurjan, 1987b). The latter observation suggests that the *SST2* gene product is part of a desensitization pathway which is induced by pheromones.

Search for suppressors of the *sst2* supersensitive phenotype identified another component of the signal transduction pathway. The *KSS1* (kinase supressor of *sst2*) gene present on a high-copy-number plasmid suppresses an *sst2* mutation. The sequence of *KSS1* predicts strong homology to the Cdc28 family of protein kinases. However, deletion of the *KSS1* locus has a very general phenotype of
slow growth (Courchesne et al, 1989). At present it is still unclear how KSS1 affects the pheromone response pathway.

Another class of supersensitive mutants was recovered by analysis of the STE2 receptor gene. Carboxy-terminal truncations of 105 amino acids caused a supersensitive phenotype when expressed in a cells (Konopka et al, 1988; Reneke et al, 1989). This supersensitivity was additive in combination with sst2 mutation, therefore, suggesting that the two mutations affect different pathways of desensitization.

**Cell-Division-Cycle Mutants In Pheromone Response Pathway**

Cell division cycle mutants (CDC) were isolated as temperature-sensitive lethal mutants that exhibit growth arrest at a unique point in the cell cycle at the restrictive temperature (Hartwell et al, 1974). These cdc mutants were ordered throughout the cell cycle with respect to each other (reviewed by Hartwell, 1978). It was therefore possible to determine the position in the cell cycle in which mating occurs. cdc mutant cells that were blocked for division at the nonpermissive temperature were challenged to mate with wild-type cells. Only cells arrested at the mating-competent step in the cell cycle will form diploids. Such experiments revealed that cells are able to conjugate only at the cdc28-sensitive step, which was designated "START" (Reid and Hartwell, 1977). One of the most salient features of cdc28 and pheromone-induced arrest at "START"
is that the spindle-pole body remains unduplicated (Byers and Goetsch, 1975). Duplication of the spindle-pole body signifies initiation of another cell cycle and mating beyond this point is somehow prevented.

Search for more cdc mutations causing arrest at "START" revealed three other complementation groups, cdc36, 37, and 39, in addition to cdc28 (Reed, 1980). These cdc mutant cells arrested at the nonpermissive temperature, formed shmoos similar to α-factor arrested cells, and remained mating competent (Reed, 1980). In addition, mating defects caused by ste4 and ste5 mutations were relieved by cdc39 (ros1) in combination with sst2 for ste4 or by itself in case of ste5 (Jenness et al, 1987; Shuster, 1982).

Interestingly, a/α cdc36/cdc36 and a/α cdc39/cdc39 homozygous diploids do not show a Cdc phenotype, that is, there is no stage specific cell cycle arrest, instead cells are arrested at all stages of the cycle. In contrast, cdc28 and cdc37 cause pheromone-like arrest at "START" even in a/α homozygous diploids. Also the cdc36, 37, and 39 phenotype depend on genetic background and carbon source (Shuster, 1982; Jenness et al, 1987).

To summarize, it is likely that CDC28, 36, 37, and 39 gene products are involved in cell cycle regulation at "START". However, CDC28 and 37 are probably directly involved in cell cycle control in haploids as well as in diploids. On the other hand, CDC36 and 39 probably play a role in the pheromone signal transduction pathway,
even though they have other essential functions that are required in a \textit{MATa/MAT\alpha} diploid cell as well.

Biochemical analysis of the \textit{CDC28} gene product also implicated it in cell cycle regulation (Mendenhall et al, 1987; Wittenberg and Reed, 1988). The \textit{CDC28} protein has strong homology to the \textit{cdc2} protein kinase of \textit{Schizosaccharomyces pombe} and shows pheromone-dependent activity (Mendenhall et al, 1987; Reed et al, 1985). \textit{In vitro} phosphorylation of a 40-kDa protein that coimmunoprecipitates with the \textit{CDC28} gene product is eliminated by pretreatment of cells with \alpha-factor pheromone (Mendenhall et al, 1987). The active form of the \textit{CDC28} kinase is a complex of approximately 160-kDa containing the 40-kDa substrate. Cell cycle arrest during G1 results in inactivation of the protein kinase and disassembly of the complex (Wittenberg and Reed, 1988).

Cyclins have also been implicated in the execution of "START". The dominant, \alpha-factor resistant mutants \textit{DAF1 (WHI1-1)}, are defective in pheromone arrest and size control (Cross, 1989; Nash et al, 1988). The phenotype of this mutant suggests a close connection between these two pathways in G1 regulation.. Sequence analysis of the wild-type \textit{daf1 (WHI1)} gene revealed homology to the marine invertebrate cyclin protein, suggesting its involvement in cell-cycle regulation by pheromones (Richardson et al, 1989). Two other cyclin homologs were identified in yeast, \textit{CLN1} and \textit{CLN2}. Disruption of all three cyclin homologs causes G1 cell cycle arrest at "START", whereas any other combination of disruptions has little effect. The hypothesis
for *CLN1, CLN2*, and *CLN3 (daf1/WHI1)* function predicts that they regulate *CDC28* activity in a cell cycle dependent manner (Richardson et al, 1989). This is based on the precedent that cyclins interact directly with homologs of Cdc28 in other organisms (Booher and Beach, 1988; Booher et al, 1989).

**Mutants Defective For Cell Fusion and Karyogamy**

Cell fusion results from a series of morphological events that occur after initial cell contact between opposite mating type cells. One or both cells produce projections toward each other, and the adjacent cell walls become attached at the fusion junction and can no longer be separated by sonication (Hartwell, 1973). Mutations in three genes, *fus1, fus2* and *fus3 (dac2)* have been isolated that arrest the mating process at this stage. The mating defect is stronger when both fusion partners are mutant, this is termed a bilateral effect (Trueheart et al, 1987; McCaffrey et al, 1987; Elion et al, 1990; Fujimura, 1990).

Detailed study of the *fus1* mutant revealed a defect within the conjugation bridge (McCaffrey et al, 1987). These structures, designated "prezygotes", contained a partition that prevented nuclear fusion and mixing of organelles. Membrane fractionation and protease protection experiments indicate that *FUS1* spans the plasma membrane, with its glycosylated amino-terminus projecting into the periplasmic space (McCaffrey et al, 1987; Trueheart and Fink, 1989). Moreover the *fus1*-LacZ fusion, strongly induced by pheromones, was
localized to the tip of the projections, shmoos, as judged by immunofluorescence (Trueheart et al., 1987; Trueheart and Fink, 1989). \textit{FUS1} and \textit{FUS2} functions are somewhat redundant since \textit{fus2} mutants can be suppressed by overexpression of \textit{FUS1} from a plasmid and vice versa (Trueheart et al., 1987). Both \textit{FUS1} and \textit{FUS2} might encode distinct degradative activities against different components of the cell wall; overexpression of either \textit{FUS1} or \textit{FUS2} is sufficient for complete hydrolysis of both respective components.

The \textit{fus3} (\textit{dac2}) mutation is also defective for zygote formation at the septum degradation step (Elion et al., 1990; Fujimura, 1990). The sequence of \textit{FUS3}, however, predicts homology to the \textit{CDC28} family of protein kinases. Overexpression of \textit{FUS3} leads to increased pheromone sensitivity, suggesting its role in the pheromone response pathway. The fusion defect in \textit{fus3} could arise from an inability to synchronize cell cycles with the mating partner (Fujimura, 1990).

The yeast nuclear envelope does not break down during mitosis or conjugation, therefore, two intact nuclei must fuse during conjugation (Byers and Goetsch, 1975). Several mutations have been isolated that prevented nuclear fusion during conjugation (Conde and Fink, 1976). \textit{kar1} is the best characterized mutation that prevents nuclear fusion with unilateral effect, i. e., only one partner must carry the mutation (Rose and Fink, 1987). The nuclear fusion defect in \textit{kar1} mutants can be explained as a secondary consequence of other pleiotropic effects of the mutation, such as abnormal distribution of microtubules, defective spindle plaque, chromosome
non-disjunction and a \textit{cdc}-like arrest at the unduplicated spindle pole body step (Rose and Fink, 1987).

Mutations in three other genes are known to depress the efficiency of karyogamy, \textit{cdc4}, \textit{28}, and \textit{37} (Dutcher and Hartwell, 1982; 1983). It has been suggested that each of these gene products acts on the nucleus to prepare it for karyogamy prior to cell fusion. Consistent with this idea is the fact that pretreatment of \textit{a} cells with \(\alpha\)-factor greatly increases the frequency of karyogamy during spheroplast fusion (Rose et al, 1986).

Overall, analysis of mutants defective for pheromone-induced cell fusion and karyogamy provides a glimpse into the complicated processes involved in the last step execution of the conjugation program.

\textbf{Other Mutations Affecting Pheromone Response Pathway}

Along with \textit{STE} mutants, many other mutations were isolated that cause an unresponsive or a non-mating phenotype in \textit{a} and (or) \(\alpha\) cells. These mutants were isolated by selecting for different properties which in some cases exhibited pleiotropic defects in mating as well.

A number of non-mating mutants are required for the production of pheromones. \textit{RAM1}, \textit{STE6} and \textit{STE14} gene products are involved in \(\alpha\)-factor modification and secretion (Chan et al, 1983; Wilson and Herskowitz, 1987; Kuchler et al, 1989; Powers et al, 1986). \textit{KEX2} and \textit{STE13} gene products are the \(\alpha\)-pheromone
processing endopeptidase and aminodipeptidase, respectively (Chan et al., 1983; Julius et al., 1983, 1984; Dmochowska et al., 1987).

The spt3 mutation was isolated as a suppressor of a solo δ element (product of Ty transposon recombination) insertion upstream of HIS4 gene (Hirschhorn and Winston, 1988). Transcription of several mating-specific genes is decreased in a spt3 mutant, suggesting its indirect involvement in mating.

Isolation of Constitutive Mutants in the Pheromone Response Pathway

The mating signal transduction pathway can be subdivided, tentatively, into two types of elements: positive and negative regulatory elements. Positive regulatory elements activate the pathway in response to pheromones and negative regulatory elements inhibit the response in the absence of pheromones. The predicted phenotype for a loss of function (recessive) mutation in a positive element would be loss of pheromone responsiveness, whereas a null mutation in a negative regulatory element would give constitutive activation of the pathway. Special alleles of each element can be also envisioned. Change of function (probably dominant) mutations in a positive element could produce constitutive activation of the pathway, whereas a special allele of a negative element could give an unresponsive phenotype.

As described above, mutations in the positive regulatory elements have been identified by the isolation of unresponsive
mutants (MacKay and Manney, 1974a, b; Hartwell, 1980). Several important components of the signal transduction pathway, such as pheromone receptors and $\beta\gamma$ subunits of G protein among others, were characterized as positive elements. No direct search for negative regulatory elements has been attempted. However, one such element has been isolated in various indirect ways. The $SCG1$ ($Saccharomyces cerevisiae$ G protein) gene was isolated in an attempt to clone the $SST2$ gene by suppression of the $sst2$ supersensitive phenotype (Dietzel and Kurjan, 1987b), suggesting $SCG1$ involvement in the mating pathway. The same gene was cloned independently as $GPA1$ (G protein $\alpha$ subunit) based on its homology to the $\alpha$ subunit of the mammalian G protein, and as $cdc70$, an allele which confers mating competence to a strain lacking $\alpha$-factor receptor (Miyajima et al, 1987; Jahng et al, 1988). Deletion of $SCG1(GPA1)$ shows characteristic pheromone-like cell-cycle arrest with subsequent formation of projections ("shmoos") (Dietzel and Kurjan, 1987a; Miyajima et al, 1987). Overexpression of the $\alpha$ subunit of the G protein in a cells causes an $\alpha$-factor unresponsive phenotype (D. Tipper and J. Kurjan, unpublished observations). The $dac1$ mutation which causes a pheromone unresponsive phenotype, maps to the $SCG1/GPA1$ locus, and is apparently a recessive, special allele of this negative regulatory element (Fujimura, 1989). Overexpression of $SCG1/GPA1$ also suppresses constitutive activation of the pathway caused by overexpression of the $\beta$ subunit of the G protein ($STE4$ gene product) consistent with its negative role in the pheromone

To expand the scope of available mutants, we developed a screen designed to identify more constitutive mutations exhibiting pheromone-independent cell-cycle arrest. The benefit from such a search is two-fold. First, new complementation groups might be identified which are involved in signal transduction. Second, a collection of mutants showing two different phenotypes (constitutive and unresponsive) permits the use of double mutant analysis to order the temporal sequence of events in this pathway. The work presented in this thesis will concentrate on the isolation and characterization of constitutive mutants in the pheromone response pathway, double mutant analysis between constitutive and sterile mutants, and the ability of constitutive mutants to recover from post-receptor induced cell cycle arrest.

Yeast as a Model System for Understanding Signal Transduction

The genetic analysis presented above suggests that in yeast, as in higher eucaryotic organisms, the response to external stimuli is mediated by a trimeric G protein coupled to a receptor with seven membrane spanning domains. This arrangement is common to rhodopsin and β-adrenergic receptor systems which have been studied and characterized extensively.
Rhodopsin is a light activated receptor that is located in the plasma membrane of rod outer segments. Rhodopsin has seven membrane-spanning domains, with its amino-terminus on the outside and carboxy-terminus inside the cell. The receptor is coupled to the trimeric G protein, transducin, consisting of α, β, and γ subunits. Photoexcited rhodopsin, formed by photoisomerization, catalyzes GTP GDP exchange in transducin. Transducin-GTP, the activated form of transducin, then stimulates cGMP phosphodiesterase, which rapidly hydrolyses cGMP to close sodium channels in the plasma membrane (reviewed by Stryer and Bourne, 1986; Gilman, 1987).

The return to the dark state requires that the receptor be deactivated so that it does not continue to catalyze the activation of transducin. Deactivation is accomplished by the action of two proteins, rhodopsin kinase and arrestin. The rhodopsin kinase binds to the activated receptor and catalyzes phosphorylation of several serines and threonines on the carboxy-terminal tail. Arrestin binds to the activated and phosphorylated receptor. This binding is competitive with respect to transducin. Once arrestin has bound, it acts as a cap, preventing rhodopsin from catalyzing the activation of transducin (reviewed by Stryer and Bourne, 1986). The interactions in this cycle are likely to be common to the actions of other G proteins.

Another example of a mammalian signal transduction system is the adrenergic receptors which mediate the effects of catecholamines. The β-adrenergic receptor is a plasma membrane
protein that possesses seven potential membrane spanning domains and is coupled to a guanine nucleotide regulatory protein (G protein). Binding of epinephrine to the receptor in vitro cause guanine nucleotide exchange in the G protein which in turn activates the adenylyl cyclase system (reviewed by Benovic et al, 1988). Two major patterns of desensitization have been distinguished in adrenergic receptors, homologous and heterologous (Lefkowitz et al, 1980). In homologous desensitization, only the subsequent response to the desensitizing hormone is attenuated, whereas, in heterologous desensitization attenuation occurs when response to one agonist diminishes response to multiple agonists. In the case of homologous desensitization, the major pathways are: receptor sequestration in a compartment distinct from the plasma membrane, and receptor down-regulation as well as other less defined desensitization pathways. Multiple mechanisms operate in case of heterologous desensitization pattern such as receptor alteration (phosphorylation), alterations in the G protein and adenylyl cyclase (Benovic et al, 1988).

It is interesting to note the large number of structural similarities between the two mammalian systems and the yeast signal transduction system. All possess receptors with seven potential membrane spanning domains and a receptor-coupled trimeric guanine nucleotide binding protein. All systems also have complicated recovery mechanisms that allow cells to desensitize to a prolonged stimulus.
Tremendous progress has been achieved in understanding the mechanisms of signal transduction in all systems. However, the yeast pheromone response system provides a number of advantages, the most important of which is sophisticated genetic analysis. In addition, transformation of yeast cells with altered genes presents an opportunity to study many biological processes in vivo. The work in this thesis presents an example of such analysis with the aim of furthering our understanding of general signal transduction mechanisms.
CHAPTER I

ISOLATION AND CHARACTERIZATION OF CONSTITUTIVE MUTANTS

Introduction

This chapter describes the isolation and behavior of constitutive mutants in the pheromone response pathway. The rationale for isolation of mutants with constitutive activation of the pathway phenotype is summarized below. Consider two types of elements that control the pheromone response pathway: positive regulatory elements, which promote the response when pheromone is present, and negative regulatory elements, which prevent the response in the absence of pheromone. Positive regulatory elements have been identified by isolating recessive pheromone-unresponsive mutants (MacKay and Manney, 1974a, b; Hartwell, 1980). Loss-of-function mutations in the negative regulatory elements should cause the opposite phenotype, constitutive activation of the pathway. One negative regulatory element has so far been identified, the \textit{SCG1} locus \textit{(GPA1; CDC70)} (Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Jahng et al, 1988). In addition to the loss-of-function mutations, it
should be possible to isolate dominant gain-of-function mutations in both positive and negative regulatory elements. Dominant alleles of the positive element should cause constitutive activation of the pathway whereas dominant alleles of the negative regulatory elements should be unresponsive.

The screen was designed for isolation of constitutive mutants in the pheromone response pathway. These mutants should exhibit pheromone-independent arrest of the cell cycle. Ideally, this screen should identify both recessive mutations in the negative regulatory elements and dominant mutations in the positive elements. Dominance tests, map positions, and complementation analysis are also described.

Materials and Methods

Strains, Media and Genetic Methods

Strains used in this study are listed in Table 5. All strains are congenic to strain 381G (Hartwell, 1980). YM-1 is a liquid rich medium (Hartwell, 1967). YEPD solid medium (Mortimer and Hawthorne, 1969) was supplemented with 40 mg/l adenine except for the sectoring assays, which used YEPD plates without adenine supplement. The 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 a carbon
source; the minimal medium was supplemented with amino acids (40 μg/ml) or adenine (20 μg/ml) as needed. Standard genetic methods were used for all strain constructions (Mortimer and Hawthorne, 1969).

**Plasmids**

Plasmid pDK243 (Koshland et al, 1985) is a centromere plasmid containing *ADE3* and *LEU2*. This *ADE3* allele was only partially functional which allows transformants containing one or two copies of the plasmid to be distinguished. Plasmid pDJ117 was constructed by inserting the *MATα* (SalI-XhoI) fragment from plasmid αX124 (Tatchell et al, 1981) into the SalI site of pDK243. Plasmid pDJ119 was constructed by ligating together the SalI-SmaI fragment from pDJ117 containing *MATα ADE3* and the SalI-SmaI fragment from YEp24 containing the 2μm circle origin and *URA3*. Plasmid YCp50-C3 (Dietzel and Kurjan, 1987a) contains the *SCG1* gene inserted into the EcoRI site of YCp50. Plasmid YEpDT-PGK-Gs is a high-copy-number plasmid that contains the replication origin from 2μm circle, the *URA3* gene and the bovine Gsα gene fused to the promoter of the yeast *PGK* gene (D. Tipper, personal communication).

**Mutant Isolation**

Overnight cultures of parent strain DJ602-136 containing plasmid pDJ117 (*MATα ADE3 LEU2 CEN3*) grown in minimal medium without leucine were mutagenized with ethylmethane sulfonate
(Prakash and Sherman, 1973) to 50%-80% survival. Cells were plated on YEPD (without adenine supplementation) at about 100 colonies per plate. Plates were incubated at 30°C for 3 days and then transferred to room temperature to permit full color development (about 3 days). Colonies (10⁵) were screened representing 40 independent populations of mutagenized cells. Potential nonsectoring colonies were picked and restreaked twice on the same medium. Eighty-seven nonsectoring colonies were crossed to strain DJ603-5-3. The hpl mutant cells that lose plasmid pDJ117 appear to be fertile even though they cannot divide. Diploids were selected on supplemented minimal medium and induced to sporulate, and the asci were dissected. Only five independent mutants gave the expected 2:2 segregation pattern (i.e., each tetrad gave 2 normal segregants and 2 segregants resembling α-factor arrested cells) and were designated DB1, DB4, DB6, DB7, and DB8 (Table 5). Three nonsectoring mutants (DB2, DB3, and DB5) also gave 2 normal: 2 inviable segregation, however they were dropped from further analysis since the 2 inviable segregants in each tetrad did not resemble α-factor arrested cells. In addition, DB2 and DB5 mutants were not suppressed by a sir3⁴ths mutation, and DB3 isolate's nonsectoring phenotype was dependent on the presence of ADE3 gene (D. Blinder and D. Jenness, unpublished observation). The remaining seventy-nine mutants gave very few viable segregants and were not considered further. Mutagenesis was also performed with the DJ603-136 strain containing the control plasmid pDK243; no
nonsectoring colonies were detected among the $10^3$ colonies that were screened.

Dominance and Recessiveness Test

The nonsectoring mutants containing the pDJ117 plasmid were crossed to the DJ603-6-4/pDJ119 strain, and diploids containing plasmid pDJ119 were selected on supplemented minimal medium. Diploids ($MAT\alpha cry1^r/MAT\alpha CRY1^s$) were made homozygous ($MAT\alpha cry1^r/MAT\alpha cry1^r$) at the mating type locus by selecting for cryptopleurine-resistant recombinants (Hartwell, 1980). Purified diploid colonies were cultured in YM-1 and then plated on the YEPD + cryptopleurine (1 μg/ml). Resistant colonies were tested for mating type and streaked on the YEPD plates (without adenine supplementation) to check for sectoring. Diploid strains containing recessive $hpl$ mutations ($MAT\alpha/MAT\alpha hpl+/)$ showed a sectoring phenotype due to loss of plasmid pDJ119, whereas the isolate containing the dominant $HPL-6$ (DB6) mutation ($MAT\alpha/MAT\alpha HPL-6+/)$ was nonsectoring. The nonsectoring isolate was confirmed to be a heterozygote by performing tetrad analysis.

Complementation Analysis

Nonsectoring mutants were transformed by the lithium acetate procedure (Sherman et al, 1986) with plasmid YCp50-C3 (SCG1) or YCp50 (vector) (Dietzel and Kurjan, 1987a). Transformants were selected on the minimal medium (without uracil) supplemented with
casamino acids (Difco Laboratories) (1%). Transformants were restreaked on the same medium and scored for sectoring.

Results

A screen was designed that would allow the isolation of constitutive mutants in the pheromone response pathway. This procedure relies on the fact that the genes encoding the α-factor receptor (STE2) and other components of this pathway (STE4, STE5) are expressed only in haploid a cells but not in diploid a/α cells (Nakayama et al, 1985; Hartig et al, 1986; Miller et al, 1985; Freedman et al, 1985). Mutations that lead to constitutive activation of the pheromone response pathway should lead to constitutive arrest of cell division in a cells; however, no phenotype should be apparent in a/α diploids since they are deficient at least in some elements of the response pathway. This phenotype is designated Hpl for haploid-lethal. Dominant and recessive mutations that give rise to the Hpl phenotype are designated HPL and hpl, respectively.

Potential haploid-lethal mutants were identified by using a colony-sectoring assay. The parent strain was partially diploid in that it was a haploid MATa strain that contained the MATα allele on a plasmid. Presence of a1-α2 regulatory proteins in the same cell should repress all haploid specific transcription, thus making the cell phenotypically "diploid-like" (reviewed by Herskowitz, 1988). The
haploid-lethal mutants that arise in this strain should divide
normally if they contain the plasmid, however, those cells that lose
the plasmid should stop dividing. To monitor plasmid loss, the MATα
locus was introduced into the plasmid pDK243 (Koshland et al, 1985).
This plasmid contains the ADE3 gene and therefore causes red
pigment to form in certain host cells (ade2 ade3). Occasional loss of
the plasmid during the colony formation results in red colonies with
white sectors (Koshland et al, 1985). Since haploid-lethal mutants are
unable to survive when they lose plasmid pDJ117 (MATα ADE3), they
should be unable to form white sectors.

Nonsectoring MATα hpl mutants were tested for haploid
lethality by performing a cross with a MATα HPL+ strain. Mutant
cells that lose the MATα plasmid should, presumably, activate the
pheromone response pathway constitutively and thus become
competent to mate with α cells (Nakayama et al, 1988). The resulting
MATα/MATα hpl-//HPL+ diploids which did not contain plasmid
pDJ117 (see Materials and Methods) were induced to sporulate and
were subjected to tetrad analysis. If two spores in each tetrad
produced normal colonies and the other two spores produced large
pear-shaped cells, then the mutant was designated hpl (or HPL) for
haploid-lethal (Figure 1A).

Mutagenized parental strain DJ603-136 which contains the
plasmid, pDJ117 was screened for appearance of nonsectoring
colonies. Eighty-seven nonsectoring isolates were obtained from
about 10^5 colonies; only five independent isolates satisfied our
criteria for the presence of an hpl (or HPL) mutation. Results of the crosses are presented in Table 1.

There was an apparent phenotypic difference between arrested hpl cells. The MATa hpl cells generally divided several times, producing a small, 10-50 cell colony of severely distended ("shmoo") cells. In contrast, MATα hpl mutants formed larger colonies of 200-2,000 cells most of which were large and round. The HPL-6 mutant cells failed to divide upon germination at 22°C and exhibited first cycle arrest in both MATa and MATα cells.

Suppression of the hpl phenotype

First, it was important to determine that the nonsectoring phenotype was not related to the LEU2 or CEN3 markers on the pDJ117 plasmid. Potential nonsectoring mutants (DB1, DB4, DB6, DB7 and DB8) were transformed with plasmid pDJ119 which carries the URA3 gene for selection and the 2μm origin of replication. All Ura+ transformants were able to lose pDJ117 plasmid, as judged by their Leu- phenotype, and remain nonsectoring (data not shown).

Next, it was important to establish whether the suppression of the growth defect in these mutants was dependent on the simultaneous expression of the MATa and MATα loci and that the phenotype was not a consequence of plasmid per se. Representative mutants (DB1, DB6, and DB7) were crossed to strain DJ146-16 which contains the temperature-sensitive mutation sir3ts. The sir3ts mutation causes defects, at the non-permissive temperature (34°C),
for repression of silent MAT\(a\) and MAT\(\alpha\) genes located at the HML and HMR loci (Rine and Herskowitz, 1987). The expected result was that sir3\(^{ts}\) mutation would suppress the haploid-lethal phenotype at the non-permissive temperature (34° C). Indeed, the crosses \((hpl^- SIR3^+ X HPL^+ sir3^-)\) gave significantly more viable spores at 34° C than those seen in control crosses \((hpl^- SIR3^+ X HPL^+ SIR3^+)\) (Figure 1), consistent with suppression of \(hpl^-\) by \(sir3^{ts}\), Table 2. In addition, the expected number of double mutants \((hpl^- sir3^{ts})\) were identified in each cross, as progeny that showed growth defects at 22° C but grew normally at 34° C (Table 2; Figure 1B).

**Dominance-Recessiveness Test**

A sectoring assay was used to determine dominance or recessiveness of the mutants showing the Hpl phenotype. The nonsectoring mutants containing pDJ117 (YCp MAT\(\alpha\) ADE3 LEU2) plasmid were crossed to the DJ603-6-4 strain carrying pDJ119 plasmid (YEp MAT\(\alpha\) ADE3 URA3), and diploids containing pDJ119 plasmid were selected on supplemented minimal medium. Diploids \((MAT\alpha\ cryI^+/MAT\alpha\ CRYI^+)\) were made homozygous \((MAT\alpha\ cryI^+/MAT\alpha\ cryI^+)\) at the mating-type locus by selecting cryptopleurine-resistant recombinants (Hartwell, 1980). Mating type homozygous strains could mate as \(a\) cells, and therefore were expressing the mating pathway. Diploid strains containing recessive \(hpl\) mutations \((MAT\alpha/MAT\alpha, hpl/+\) showed a sectoring phenotype due to loss of plasmid pDJ119, whereas the isolate containing the
dominant $HPL-6$ mutation ($MATa/MATa, HPL-6/+)$ was nonsectoring. The nonsectoring isolate was confirmed to be a heterozygote by performing tetrad analysis. Results of the dominance test are summarized in Table 3. The recovery of both dominant and recessive mutants is consistent with the existence of both positive and negative regulatory elements.

**Complementation Analysis**

All recessive $hpl$ mutations ($hpl-1$, $hpl-4$, $hpl-7$, and $hpl-8$) were found to be alleles of the $SCG1$ gene, which is thought to encode the $\alpha$ subunit of a G protein (Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Jahng et al, 1988). The nonsectoring phenotype of these $hpl$ mutants (DB1, DB4, DB7, and DB8) was reversed by transforming them with plasmid YCp50-C3 containing the $SCG1$ gene (Dietzel and Kurjan, 1987a), whereas control plasmid YCp50 had no effect (Table 3). Furthermore, all four $hpl$ mutations mapped to chromosome VIII between $arg4$ and the centromere (Table 3), as expected for alleles of $scg1$ (Miyajima et al, 1987). We have reassigned all recessive $hpl$ mutations ($hpl-1$, $hpl-4$, $hpl-7$, and $hpl-8$) as $scg1-5$, $scg1-4$, $scg1-7$, and $scg1-8$, respectively, to be consistent with the nomenclature of Dietzel and Kurjan (1987a).

The dominant $HPL-6$ mutation mapped to a position at or near the $STE4$ locus. A cross between $HPL-6$ and $ste4-3$ (Table 4) gave 41 tetrads all of which were parental ditypes. This corresponds to a distance of less than 1 cM. In addition, the cross between $HPL-6$ and
his3 showed expected linkage (17 cM). The tight linkage between the 
HPL-6 and ste4-3 presumably reflects allelism of these mutations. 
The HPL-6 was therefore reassigned as STE4Hp1. These results 
provide strong evidence that the STE4 gene encodes a positive 
regulatory element since it can give dominant alleles that are 
haploid-lethal and recessive alleles that are unresponsive to 
pheromones.

Discussion

Both positive and negative regulatory elements appear to 
control pheromone response. Positive regulatory elements cause 
activation of the pheromone response pathway when pheromone is 
present, and negative regulatory elements inhibit the pathway when 
pheromone is absent. Mutants affecting the positive regulatory 
elements have been identified by isolating pheromone unresponsive 
mutants which continue to divide in the presence of α-factor 
(Manney and Woods, 1976; Hartwell, 1980). In the present study, a 
new screen was developed for isolation of constitutive mutants in the 
pheromone response pathway. This constitutive phenotype is 
predicted for mutants that lack a negative regulatory element or for 
mutants that contain an altered positive element that promotes the 
signal in the absence of pheromones.
Several criteria were used to establish *bona fide* hpl mutants. Nonsectoring phenotype was required for hpl mutants carrying either the pDJ117 or pDJ119 plasmid, as well as suppression of the hpl lethality by the sir31s mutation. Only five mutants from a total of eighty-seven nonsectoring candidates were selected for further analysis. Recessive mutations were found to be alleles of SCG1 (also designated GPAI or CDC70), which had been previously described (Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Jahng et al, 1988). One of our mutations was dominant and mapped to the STE4 locus; this observation suggested that the STE4 gene encodes a positive regulatory element.

In general, the screen described here, for isolation of constitutive mutants could be useful in isolation of lethal mutations in other essential genes. For example, diploid-lethal as well as haploid-lethal mutants in the pheromone pathway could be identified by placing the DAF1-1 (WHI1-1) mutant on the color-marked plasmid pDK243 (Koshland et al, 1985). The DAF1 (WHI1-1) is a dominant mutation in the cyclin gene which shortens the G1 phase of the cell cycle, bypassing the pheromone arrest point (Cross, 1989; Nash et al, 1988). Since, this mutation affects equally diploid and haploid cells, mutations which cause constitutive activation of the pheromone response pathway in both haploids (*MATa* or *MATα*) and diploids (*MATa/MATα*), could be identified in such screen due to their dependence on the DAF1 (WHI1-1) suppressor for growth.
Many other uses of such analysis can be envisioned, including the identification of essential genes in yeast (Riles and Olson, 1988).
Figure 1. Suppression of the Haploid-lethal Phenotype by \textit{sir3^{ts}} Mutation

The non-sectoring \textit{hpl-1} mutant (DB1) was crossed to the \textit{sir3^{ts}} (DJ146-16) strain (B) and to the \textit{SIR3}^{+} strain (DJ603-5-3) (A). The diploids were sporulated the asci were dissected at 34\textdegree C (Mortimer and Hawthorne, 1967). (A) The \textit{hpl-1} x \textit{SIR3} cross. All tetrads show 2 (w.t.): 2 (mutant) segregation. (B) The \textit{hpl-1} x \textit{sir3^{ts}} cross. Most of the
Figure 1 (continued).
tetrads show 3 (w.t.): 1 (mutant) segregation which is consistent with
sir3ts suppression of the hpl-1 mutation. The expected number of
double mutants hpl-1 sir3ts were recovered and were shown to
exhibit temperature-dependent phenotype (see Text). Dissection
slabs were photographed with Polaroid Type 55 film.
Table 1. *MATa hpl*<sup>-</sup> (pDJ117) X *MATα HPL*<sup>+</sup> Crosses

<table>
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<th>0:4</th>
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<th>2:2</th>
<th>3:1</th>
<th>4:0</th>
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</tr>
<tr>
<td>DB7 <em>hpl</em>-7</td>
<td>1</td>
<td>0</td>
<td>8</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>DB8 <em>hpl</em>-8</td>
<td>0</td>
<td>1</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td><em>HPL</em>&lt;sup&gt;+&lt;/sup&gt;</td>
<td>0</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>3</td>
</tr>
</tbody>
</table>

<sup>a</sup> Mutant isolates.

<sup>b</sup> Entries are the number of tetrads showing the wild-type: mutant ratio indicated. Mutants included cells with the Hpl phenotype and all non-germinating spores.
Table 2. *MATα hpl- SIR3+* (pDJ117) X *MATα HPL+ sir3ts* Crosses

<table>
<thead>
<tr>
<th>Mutant</th>
<th>0:4</th>
<th>1:3</th>
<th>2:2</th>
<th>3:1</th>
<th>4:0</th>
<th># C/S</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>hpl-1</em></td>
<td>0</td>
<td>2</td>
<td>8</td>
<td>20</td>
<td>7</td>
<td>34</td>
</tr>
<tr>
<td><em>HPL-6</em></td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>8</td>
<td>4</td>
<td>16</td>
</tr>
<tr>
<td><em>hpl-7</em></td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>8</td>
<td>0</td>
<td>8</td>
</tr>
<tr>
<td><em>HPL</em>+</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1</td>
<td>9</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Strains containing mutant alleles were crossed to the strain DJ146-16 by standard genetic methods (Mortimer and Hawthorne, 1967).

b. Entries are the number of tetrads showing the wild-type: mutant ratio indicated. Mutants included cells with the Hpl phenotype and all non-germinating spores.

c. # C/S - Total number of double mutant (*hpl sir3ts*) segregants showing cold-sensitive phenotype, i.e., abberant, shmoo, cell morphology at 22°C and wild-type cells at 34°C.
Table 3. Dominance Test and Complementation Analysis

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Mutant</th>
<th>Dominant/ Recessive</th>
<th>pDJ117 Alone</th>
<th>YCp50</th>
<th>YCp50-C3</th>
<th>arg4</th>
<th>trp1</th>
<th>Linkage</th>
</tr>
</thead>
<tbody>
<tr>
<td>DB1</td>
<td>hpl-1</td>
<td>recessive</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>7.5</td>
<td>5</td>
<td></td>
</tr>
<tr>
<td>DB4</td>
<td>hpl-4</td>
<td>recessive</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>34.2</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td>DB6</td>
<td>HPL-6</td>
<td>dominant</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>NL</td>
<td>NL</td>
<td></td>
</tr>
<tr>
<td>DB7</td>
<td>hpl-7</td>
<td>recessive</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>15.9</td>
<td>2.27</td>
<td></td>
</tr>
<tr>
<td>DB8</td>
<td>hpl-8</td>
<td>recessive</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>16.6</td>
<td>2.7</td>
<td></td>
</tr>
<tr>
<td>DJ603-136</td>
<td></td>
<td>-</td>
<td>+</td>
<td>NT</td>
<td>NT</td>
<td>-</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

a. The dominance test is described in Materials and Methods.
b. Nonsectoring mutants containing plasmid pDJ117 were transformed with YCp50 or YCp50-C3 (SCGl) and assayed for sectoring (see Materials and Methods). (-)-signifies nonsectoring phenotype and (+)-sectoring phenotype.
c. Tetrad segregation was 2 mostly (wt) : 2 (mutant); only the two wild-type colonies were analyzed for each tetrad. In the hpl X arg4 crosses and in the hpl X trp1 crosses the arg4 and trp1 markers were assumed to give 2:2 segregation. Genetic distance is given in centimorgans (cM) and was calculated according to the formula: 100(T + 6NPD)/(2(PD + NPD + T)).

NT-Not tested. NL-No linkage.
Table 4. Mapping of *HPL-6*

<table>
<thead>
<tr>
<th>Cross</th>
<th>Tetrad types&lt;sup&gt;b&lt;/sup&gt;</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PD</td>
<td>NPD</td>
<td>T</td>
<td>Linkage&lt;sup&gt;c&lt;/sup&gt;</td>
<td></td>
</tr>
<tr>
<td><em>HPL-6 X ste4</em></td>
<td>41</td>
<td>0</td>
<td>0</td>
<td>&lt;1 cM</td>
<td></td>
</tr>
<tr>
<td><em>HPL-6 X his3</em></td>
<td>19</td>
<td>0</td>
<td>10</td>
<td>17 cM</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Haploid strains containing the markers indicated were mated and subjected to tetrad analysis. The strains were DB6 (*HPL-6*), DJ242-1-1 (*ste4*), and 5680-4 (*his3*).

<sup>b</sup> Tetrad segregation was 2 mostly (wt) : 2 (mutant); only the two wild-type colonies were analysed for each tetrad. The *ste4* and *his3* markers were assumed to give 2:2 segregation. PD is the number of parental ditype, NPD is the number of nonparental ditype, and T is the number of tetratype asci.

<sup>c</sup> Genetic distance is given in centimorgans (cM) and was calculated according to the formula: 100(T + 6NPD)/2(PD + NPD + T).
### Table 5. Strain List

<table>
<thead>
<tr>
<th>Strain</th>
<th>Genotype</th>
</tr>
</thead>
<tbody>
<tr>
<td>381G</td>
<td>MATα cryl ade2-1 his4-580 lys2 trp1 tyr1 SUP3-4&lt;sup&gt;ts&lt;/sup&gt;</td>
</tr>
<tr>
<td>DJ602-136</td>
<td>381G MATα ade3 TYR1 ura3 leu2</td>
</tr>
<tr>
<td>DB1</td>
<td>381G MATα ade3 TYR1 ura3 leu2 scg1-5 with pDJ117</td>
</tr>
<tr>
<td>DB2</td>
<td>381G MATα ade3 TYR1 ura3 leu2 hpl-2 with pDJ117</td>
</tr>
<tr>
<td>DB3</td>
<td>381G MATα ade3 TYR1 ura3 leu2 hpl-3 with pDJ117</td>
</tr>
<tr>
<td>DB4</td>
<td>381G MATα ade3 TYR1 ura3 leu2 scg1-4 with pDJ117</td>
</tr>
<tr>
<td>DB5</td>
<td>381G MATα ade3 TYR1 ura3 leu2 hpl-5 with pDJ117</td>
</tr>
<tr>
<td>DB6</td>
<td>381G MATα ade3 TYR1 ura3 leu2 STE4&lt;sup&gt;Hpl&lt;/sup&gt; with pDJ117</td>
</tr>
<tr>
<td>DB7</td>
<td>381G MATα ade3 TYR1 ura3 leu2 scg1-7 with pDJ117</td>
</tr>
<tr>
<td>DB8</td>
<td>381G MATα ade3 TYR1 ura3 leu2 scg1-8 with pDJ117</td>
</tr>
<tr>
<td>DJ603-5-3</td>
<td>381G MATα CRY1 ade3 leu2</td>
</tr>
<tr>
<td>DJ146-16</td>
<td>381G MATα CRY1 sir3&lt;sup&gt;ts&lt;/sup&gt;</td>
</tr>
<tr>
<td>DJ603-6-4</td>
<td>381G MATα CRY1 ade3 ura3 leu2 with pDJ119</td>
</tr>
<tr>
<td>DJ709-1-2</td>
<td>381G MATα ADE2 HIS4 lys2 TYR1 TRP1 ade6 arg4</td>
</tr>
<tr>
<td>5680-4</td>
<td>381G MATα ADE2 HIS4 TRP1 TYR1 his3 arg1 tcml</td>
</tr>
<tr>
<td>DJ242-1-1</td>
<td>381G MATα ste4-3&lt;sup&gt;ts&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>a</sup>. All strains are congenic to 381G (Hartwell, 1980).

<sup>b</sup>. The cryl and CRY1 alleles determine resistance and sensitivity to the fungicide cryptopleurine. The temperature-sensitive allele of ste4-3 (Hartwell, 1980) leads to sterility and α-factor resistance at
the non-permissive temperature (34°C). The sir3<sup>ts</sup> mutation was originally designated ste8-5 (Hartwell, 1980). scgl-5, scgl-4, scgl-7, scgl-8, and STE4<sup>Hpl</sup> are the same as hpl-1, hpl-4, hpl-7, hpl-8, and HPL-6, respectively (see text).
CHAPTER II

DOUBLE MUTANT ANALYSIS

Introduction

The yeast pheromone response provides a model genetic system for studying signal transduction. Two types of mutations have been identified in this pathway; pheromone unresponsive mutants and pheromone-independent constitutive mutants (Hartwell, 1980; Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Jahng et al, 1988; Blinder et al, 1989). Null mutants which exhibit an unresponsive phenotype \(ste\), presumably affect positive regulatory elements whereas null constitutive mutants \(hpl\) are likely to affect negative regulatory elements. The dominant constitutive mutation \(HPL-6\) is a special allele of \(STE4\), a positive regulatory element. When two of these mutants showing opposite phenotypic traits are crossed, the phenotype of the double mutant can infer the order in which the two gene products act in the pathway.

Hypothetically, there are five possible relationships between two gene products affecting the pheromone pathway (Figure 1). If each of the two genes can mutate to give either an unresponsive or
consitutive phenotype (dominant or recessive), then the double mutant phenotype can be predicted in all possible arrangements. If the $X$ gene product functions before the $Y$ gene product in a sequential pathway, double mutants containing a constitutive allele of $X$ and an unresponsive allele of $Y$ will exhibit an unresponsive phenotype, and a double mutant containing a constitutive allele of $Y$ and an unresponsive allele of $X$ will show a constitutive phenotype. Reversing the order of the genes will reverse the phenotypes (Figure 1, lines 1 and 2). It is also possible that the two genes are interdependent and affect the same regulatory element (either directly or indirectly). For example, the two gene products may have a direct relationship if they are different subunits of the same regulatory element. They may indirectly control the same step if one gene product regulates the synthesis or processing of the other. In the case of the interdependent genes, both double mutants would exhibit the same null phenotype, i.e., both will be unresponsive if they control a positive element or constitutive for a negative element (Figure 1, lane 3). If, however, two independent parallel pathways operate in cells (Figure 1, lines 4 and 5), then two possibilities can be envisioned. The first possibility assumes that both steps in parallel pathways are required for a response (and), the other possibility assumes that only one step in either pathway is required (or). If both steps are required for a response, it would be unlikely to isolate a mutant which causes a constitutive activation phenotype. In the case where either one of the two steps is required for a response, it
would be unlikely to obtain an unresponsive mutant. In this chapter, the order of genes in the pheromone response pathway was determined and a comprehensive genetic model proposed.

Materials and Methods

Strains, Media, and Genetic Methods

Strains used in this study are listed in Table 3. All strains are congenic to strain 381G (Hartwell, 1980). Standard genetic methods were used for all strain constructions (Mortimer and Hawthorne, 1967).

Plasmids

Plasmid YEpDT-PGK-Gs is a high-copy-number plasmid that contains the replication origin from 2μm circle, the URA3 gene, and the bovine Gsα gene fused to the promoter of the yeast PGK gene (D. Tipper, personal communication).

Results

The order in which two elements act in a pathway can be inferred from the phenotype of the double mutant, when the two single mutants give opposite phenotypes. This approach was used to
determine the order of function for several regulatory elements that constitute the pheromone response pathway.

Results presented in Table 1, reveal that the constitutive mutations, scgl and STE4Hpl, cause activation of the signalling pathway at post-receptor steps. Crosses of scgl X ste2 (ste2-3ts or ste2-10::LEU2) produced tetrads with only two viable spores. Since scgl is unlinked to ste2, the two mutations should segregate independently. Consequently, if ste2 suppressed scgl there should have been an excess of viable colonies. This result suggests that the SCG1 gene product affects a post-receptor step in the pheromone response pathway. The failure of ste2 to suppress scgl has been previously established by others (Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Nakayama et al, 1988). The cross between STE4Hpl and ste2-10::LEU2 gave also, at most, two viable spores suggesting that STE4 gene product activates a post-receptor step as well (Table 1). This is consistent with an earlier observation that ste4 mutants bind the pheromone but fail to respond to it (Jenness et al, 1987).

The temperature-sensitive, unresponsive mutation ste5-3ts was found to suppress the constitutive phenotype of scgl and STE4Hpl mutants at the non-permissive temperature (34°C). Crosses between scgl and ste5-3ts, and STE4Hpl and ste5-3ts gave tetrads with more than two viable spores at 34°C. The expected number of double mutants (scgl ste5-3ts and STE4Hpl ste5-3ts) were recovered in each cross. The double mutants were sterile at 34°C but became
large and pear-shaped ("shmoo-like") when cultured at 22° C (Figure 2). Figures 2C and 2D show the morphology of the double mutant after the cells had been cultured at 22° C for 16 hours. These mutants resembled in their size and shape the α-factor-arrested cells (Figure 2B). Taken together, these results indicate that the STE5 gene product acts at a post-receptor step, "downstream" from the SCG1 and STE4, since mutations in STE5 can block scg1 and STE4<sub>Hpl</sub> generated signal.

The order between SCG1 and STE4 was determined by two experiments. In the first experiment, the scg1-7 constitutive mutation was suppressed by the ste4-3<sup>ts</sup> unresponsive mutation (Table 1). This observation suggests that SCG1 can act before STE4. Others have reported that unresponsive mutations in ste4 and ste5 suppress constitutive mutations in scg1 (Nakayama et al, 1988). The second experiment depended on the observation that overexpression of the mammalian G protein α subunit will suppress the scg1 mutation and cause a dominant pheromone-unresponsive phenotype (Dietzel and Kurjan, 1987a; D. Tipper, personal communication). As expected, when the plasmid containing the bovine G<sub>Sα</sub> gene expressed from the yeast PGK promoter (YEpDT-PGK-G<sub>S</sub>), was introduced into the MATα scg1-7 /pDJ117 strain (DB7), it was able to restore the sectoring phenotype. The scg1 mutant transformants were therefore no longer dependent on the MATα ADE3 (pDJ117) plasmid for growth. However, the YEpDT-PGK-G<sub>S</sub> was not able to restore sectoring to the STE4<sub>Hpl</sub> strain (DB6). Thus, the G<sub>Sα</sub> protein
was unable to block the $\text{STE4}^{\text{Hp1}}$ generated signal. In addition, two other strains were transformed with the YEpdT-PGK-Gs plasmid, scgl $\text{ste5-3}^{\text{ts}}$ (DJ707-10-3) and scgl $\text{STE4}^{\text{Hp1}}$ $\text{ste5-3}^{\text{ts}}$ (DJ761-4-4). The transformants could be grown at 34° C due to suppression of constitutive phenotype by $\text{ste5-3}^{\text{ts}}$ mutation. However, at 22° C only the scgl $\text{ste5-3}^{\text{ts}}$ (DJ707-10-3) transformant could grow and scgl $\text{STE4}^{\text{Hp1}}$ $\text{ste5-3}^{\text{ts}}$ (DJ761-4-4) exhibited no growth. The above experiments suggest that the $\text{SCG1}$ gene product acts before the $\text{STE4}$ gene product in the pheromone response pathway.

It is interesting to note that the $\text{ste12-2}^{\text{ts}}$ unresponsive mutation suppressed the $\text{STE4}^{\text{Hp1}}$ constitutive arrest (Table 1). This result indicated that $\text{STE12}$ gene product probably acts after the $\text{STE4}$ controlled step or that $\text{STE12}$ and $\text{STE4}$ may act together at the same step.

A mutation in the yeast cyclin homolog, $\text{WHI1/DAF1/CLN1}$ ($\text{WHI1-1}$) suppressed both constitutive mutations in scgl and $\text{STE4}^{\text{Hp1}}$. The $\text{WHI1-1}$ mutation causes small cell size phenotype, presumably by shortening the G1 phase of the cell cycle (Nash et al, 1988; Cross, 1989). Crosses scgl $\times$ $\text{WHI1-1}$ and $\text{STE4}^{\text{Hp1}}$ $\times$ $\text{WHI1-1}$ revealed an excess of viable spores as compared to the control crosses scgl $\times$ $\text{WHI}^{+}$ and $\text{STE4}^{\text{Hp1}}$ $\times$ $\text{WHI}^{+}$ (Table 2). The anticipated number of double mutants were recovered from each cross, and all growing colonies exhibited wild-type cell morphology. Therefore, mutations which bypass the pheromone arrest point will suppress the post-receptor constitutive signal. This result supports the notion
that pheromones cause cell cycle arrest though the action of cyclins (Richardson et al, 1989), and that the action of α-factor on cyclins is mediated by the SCG1 and STE4 gene products.

**Discussion**

Double mutant analysis is often used to determine the order in which gene products function in a pathway. This approach requires that the elements controlling separate steps in the pathway can mutate to give different phenotypes. The isolation of unresponsive and constitutive mutations that affect the pheromone response pathway allowed such analysis. Assuming that each of the genes analysed controls a separate step in a linear pathway, the results from Tables 1 and 2, and results of others (Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Nakayama et al, 1988) indicate that the gene products function in the following order: α-factor, STE2, SCG1, STE4, STE5, cell cycle arrest (Figure 3). The site of action for STE12 and WHI1 gene products can not be predicted at this time, except that they must act after the STE4-controlled step. This order does not provide evidence for direct interactions between any of the gene products since the various mutations may only indirectly affect a particular step in the response pathway. It is likely that yet unidentified components may be involved in signal transduction between steps.
Based on DNA sequence homology, predictions can be made to account for the mode of action for several elements. The *SCG1* gene product shows homology to the α subunit of mammalian G proteins, whereas, the *STE4* gene product is homologous to the β subunit (Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Whiteway et al, 1989). Based on the genetic model (Figure 3), it is the β subunit that activates the pheromone response pathway rather than the α subunit, unlike the well-studied mammalian systems. Even though examples exist in mammalian systems that suggest pathway activation by the βγ subunits, the issue is highly debated (Logothetis et al, 1987; Cerbai et al, 1988). Genetic evidence presented in this chapter suggests that such activation occurs in yeast, making the subject even more interesting.
Phenotype of Double Mutants

<table>
<thead>
<tr>
<th>Order of function</th>
<th>( X^{Hpl} Y^{Unresp} )</th>
<th>( X^{Unresp} Y^{Hpl} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>( X ) before ( Y )</td>
<td>Unresponsive</td>
<td>Haploid Lethal</td>
</tr>
<tr>
<td>( Y ) before ( X )</td>
<td>Haploid Lethal</td>
<td>Unresponsive</td>
</tr>
<tr>
<td>( X \rightarrow Y )</td>
<td>Both double mutants show null phenotype</td>
<td></td>
</tr>
<tr>
<td>( X \rightarrow ) or ( Y \rightarrow )</td>
<td>Inconsistent with unresponsive mutation</td>
<td></td>
</tr>
<tr>
<td>( X \rightarrow ) and ( Y \rightarrow )</td>
<td>Inconsistent with constitutive mutation</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Predicted Phenotypes for Double Mutants.
Figure 2. Mutant Phenotypes after the Shift to 22°C.

Cultures growing exponentially at 34°C (between 10^6 and 2 x 10^7 cells/ml) were diluted to 10^5 or 10^6 cells/ml and shifted to 22°C. After 16 hours, the cells were fixed in formaldehyde (3.7%) and photographed with a Nikon phase-contrast microscope.

(A) DJ164-7-1 (MATa barl ste5-3ts); (B) DJ164-7-1 (MATa barl ste5-3ts) with 5 x 10^{-9} M α-factor; (C) DJ728-9-3 (MATa scgl1-7 ste5-3ts); (D) DJ736-34-1 (MATa STE4H^1 ste5-3ts). All four panels are the same magnification.
Possible Mechanism for Pheromone Response.

\[
\text{STE2} \rightarrow \text{SCG1} \rightarrow \text{STE4} \rightarrow \text{STE5} \rightarrow \text{Arrest}
\]

\[
\alpha\text{-Factor} \rightarrow \text{STE2} \rightarrow \text{SCG1} \rightarrow \text{STE4} \rightarrow \text{STE5} \rightarrow \text{Arrest}
\]

Figure 3. Genetic Model for the Pheromone Response Pathway.
Table 1. Suppression of Haploid-Lethal mutants by ste Mutants

<table>
<thead>
<tr>
<th>Crossa</th>
<th>Segregation of Mutant Phenotypeb</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>( T(C) )</td>
</tr>
<tr>
<td>( scg1-4 \times ste2-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( SCG1^+ \times ste2-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( scg1-5 \times ste2-10^{ts}:LEU2 )</td>
<td>30(^{o}) C</td>
</tr>
<tr>
<td>( scg1-4 \times ste2-10^{ts}:LEU2 )</td>
<td>30(^{o}) C</td>
</tr>
<tr>
<td>( STE4^{Hpl} \times ste2-10^{ts}:LEU2 )</td>
<td>30(^{o}) C</td>
</tr>
<tr>
<td>( STE4^{Hpl} \times STE^+ )</td>
<td>30(^{o}) C</td>
</tr>
<tr>
<td>( scg1-5 \times ste5-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( scg1-4 \times ste5-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( scg1-7 \times ste5-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( STE4^{Hpl} \times ste5-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( STE4^{Hpl} \times ste5-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( scg1-7 \times ste4-3^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
<tr>
<td>( STE4^{Hpl} \times ste12-2^{ts} )</td>
<td>34(^{o}) C</td>
</tr>
</tbody>
</table>

NT, not tested
Table 1 (continued)

a. Haploid strains containing the mutations indicated were mated and subjected to tetrad analysis. Strains were DB1 (scg1-5), DB4 (scg1-4), DB6 (STE4H^pl), DB7 (scg1-7), 4264-4-2 (ste2-3^ts), DJ112-4-3 (ste2-10::LEU2), 5561-9-3 (ste5-3^ts), DJ242-1-1 (ste4-3^ts), DJ602-136/pDJ117 (MATa SCG1^+ STE4^+), 381G-2-1 (MATa STE^+), 6333-13-1 (ste12-2^ts).

b. Entries are the number of tetrads showing the wild-type: mutant ratio indicated. Mutants included cells with the Hpl phenotype and all nongerminating spores.

c. # C/S- Entries indicate the number of cold-sensitive segregants per total number of segregants tested. The segregants that showed a normal cellular phenotype at 34°C were tested at 22°C.
Table 2. Suppression of the constitutive mutants by WHI1-1 mutation.

<table>
<thead>
<tr>
<th>Cross</th>
<th>0:4</th>
<th>1:3</th>
<th>2:2</th>
<th>3:1</th>
<th>4:0</th>
</tr>
</thead>
<tbody>
<tr>
<td>scgl-7 X WHI1-1</td>
<td>0</td>
<td>1</td>
<td>5</td>
<td>8</td>
<td>3</td>
</tr>
<tr>
<td>scgl-7 X WHI+</td>
<td>0</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>STE4Hpl X WHI1-1</td>
<td>0</td>
<td>1</td>
<td>4</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>STE4Hpl X WHI+</td>
<td>0</td>
<td>3</td>
<td>7</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

a. Strains containing mutant alleles were crossed by standard genetic methods (Mortimer and Hawthorne, 1967).

b. Entries are the number of tetrads showing the wild-type: mutant ratio indicated. Mutants included cells with Hpl phenotype and all non-germinating spores. The total number of double mutants (scgl-7 WHI1-1, STE4Hpl WHI1-1) is consistent with suppression of constitutive mutants by an unlinked mutation.
Table 3. Strain List

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<tr>
<th>Strain</th>
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<td>381G</td>
<td>MATa cryl ade2-1 his4-580 lys2 trpl tyr1 SUP4-3ts</td>
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Table 3 (continued)

a. All strains except 328-4b and 328-4d are congenic to 381G (Hartwell, 1980). Strains 328-4b and 328-4d were a gift from B. Futcher (Cold Spring Harbor Laboratory).

b. The cry1 and CRY1 alleles determine the resistance and sensitivity to the fungicide cryptopleurine. The temperature-sensitive alleles of ste2-3, ste4-3, ste5-3, and ste12-2 (Hartwell, 1980) lead to sterility and α-factor resistance at the nonpermissive temperature (34° C).

ste2-10::LEU2 is a genetic substitution that removes the entire STE2 gene. The bar1 mutation (Sprague and Herskowitz, 1981) inhibits α-factor degradation in a cell cultures. The WH11-1 is a dominant allele of WH11 gene which causes α-factor insensitivity and decreased cell size (Cross, 1989; Nash et al, 1988). metx is a spontaneous methionine requirement (B. Futcher, personal communication).
CHAPTER III

RECOVERY OF CELLS FROM POSTRECEPTOR-INDUCED SIGNAL

Introduction

This chapter will examine the elements of the pheromone response pathway which are involved in recovery of cells from constitutive cell cycle arrest. The preceding chapter assessed the order in which several elements affect signal transduction. The temperature-sensitive allele, ste5-3ts, suppresses constitutive mutations in SCG1 and STE4 in a temperature-dependent manner. The ste5-3ts scg1 and ste5-3ts STE4HP1 double mutants exhibited normal cell morphology and grew at the wild-type rates at 34° C, but cultures arrested cell division when shifted to 22° C. This observation provided a convenient, temperature-dependent way to handle these constitutive mutants. It therefore became possible to determine the effect of constitutive activation of the pheromone response pathway and the subsequent ability of cells to adapt to this signal.

In the absence of mating, yeast cells will eventually recover from the effects of pheromones and will resume cell division cycle (Chan, 1977; Moore, 1984). Recovery of a cells results from two
different properties: the extracellular protease that degrades α-factor (Ciejk and Thorner, 1979) and the intrinsic ability to adapt to the undegraded pheromone (Moore, 1984). The adaptive response could involve either modification of the cell surface receptors or attenuation of the postreceptor signal. There are known modifications of the STE2 receptor, such as α-factor induced phosphorylation and internalization of α-factor/receptor complexes (Reneke et al, 1988; Jenness and Spatrick, 1986; Chvatchko et al, 1986). Mutations in three genes are known to increase sensitivity to pheromones and, therefore, impare the recovery process. The bar1 mutation (also designated sst1) eliminates α-factor degradation (Chan and Otte, 1982a, b; Sprague and Herskowitz, 1981). The ability of a cells to adapt to α-factor is blocked by the sst2 mutation and by carboxy-terminal deletions of the α-factor receptor gene (STE2) (Konopka et al, 1988; Reneke et al, 1988). The sst2 and ste2 supersensitive mutations probably affect different processes since the double mutant exhibits an even more extreme phenotype than either of the single mutants (Konopka et al, 1988; Reneke et al, 1988).

In this chapter, mutants constitutive for the pheromone response were used to demonstrate the existence of at least two postreceptor mechanisms that control adaptation. Both mechanisms are induced after the STE5-controlled step. One of these mechanisms requires the SST2 gene product. This was the first evidence that the SST2 gene affects postreceptor functions. The effect of constitutive
mutations on the maintenance of receptors on the cell surface was also investigated.

Materials and Methods

Strains, Media and Genetic Methods

The strains used in this study are listed in Table 1. All strains are congenic to 381G (Hartwell, 1980). Inhibitor medium was YM-1 containing 10 mM NaN₃ and 10 mM KF. Standard genetic methods were used for all strain constructions (Mortimer and Hawthorne, 1967).

Growth Curves

Cultures were inoculated in YM-1 and incubated overnight at 34°C with shaking. Exponentially growing cultures (less than 2x10^7 cells/ml) were diluted to 10^5 or 10^6 cells per ml in fresh YM-1 and transferred to 22°C. Samples of 1 ml were withdrawn at the specified times and fixed in 3.7% formaldehyde. For cell number determinations, the samples were lightly sonicated (to disperse cell aggregates) and counted in a particle counter (Coulter Electronics, Inc).

Binding Assays

The ³H-α-factor-binding assay was adapted from a previous procedure (Jenness and Spatrick, 1986). Cultures were grown overnight in YM-1 at 34°C to final density of no more then 2x10^7
cells per ml. Cells were diluted to 5x10^6 cells per ml and shifted to 22°C in a shaking water bath. α-Factor (10^-8 M) and cycloheximide (10 μg/ml) were added to the specified cultures at the time of the shift. At the various time points, 25-ml samples were collected on a nitrocellulose filter (type HA; 0.45-μm pore size; Millipore Corp.). Filters were rinsed twice with 2 ml of inhibitor medium, resuspended in 10 ml of inhibitor medium, and incubated for 2 hours at 25°C to dissociate reversibly bound α-factor (Jenness et al, 1983). The cells were then centrifuged and resuspended in 0.5 ml of inhibitor medium. Samples (90 μl) of the cell suspension were incubated for 30 minutes (25°C) with either 10 μl of ^3H-α-factor (2x10^-7 M; active ligand) or 10 μl solution containing ^3H-α-factor (2x10^-7 M) and unlabeled α-factor (2x10^-5 M). A 90-μl sample of each reaction mixture was diluted to 2 ml. The cells were then collected on a glass fiber filter (GF/C; Whatman, Inc) and rinsed twice with 2 ml of inhibitor medium. Filters were dried and counted in a liquid scintillation counter. Specific binding was determined as the counts per minute bound to the experimental filter (without unlabeled α-factor) minus the counts per minute bound to the control filter (with unlabeled α-factor). All measurements were performed in duplicate. ^3H-α-factor was described previously (Jenness et al, 1986) except that active ligand represented only 33% of the total radioactivity.

Results
Recovery of cells from division arrest

MATa cells are able to overcome α-factor cell cycle arrest and resume division cycle (Chan, 1977; Moore, 1984). α-Factor causes a cells to arrest only transiently. The duration of the arrest is proportional to the α-factor concentration. The scg1-7 and STE4Hpl mutants, which show postreceptor constitutive activity of the pheromone response pathway (Blinder et al, 1989), were used to identify elements of the pathway that control the process of adaptation. The temperature-sensitive unresponsive mutation ste5-3ts blocks the constitutive signal generated in these mutants at 34°C but not at 22°C. The double mutants scg1-7 ste5-3ts and STE4Hpl ste5-3ts divide normally at 34°C but arrest cell division when cultures are shifted to 22°C. Figure 1B and 1C show results for the MATa scg1-7 ste5-3ts and MATa STE4Hpl ste5-3ts mutants, respectively, that had been cultured at 34°C and then shifted to 22°C. Both double mutants arrested cell division transiently with kinetics similar to those observed for the control cells (MATa ste5-3ts) that had been treated with α-factor (Figure 1A).

The scg1-7 mutant (Figure 1B) began dividing again after about 20 hours similar to results obtained for the control cells treated with 5x10^-9 M α-factor (Figure 1A). At the end of the time course (30 hours at 22°C), the double mutant cells were cultured overnight at 34°C and then returned to 22°C. Cultures showed arrest and
recovery kinetics indistinguishable from Figure 1B (Figure 2A), therefore, initial recovery at 22° C did not result from accumulation of revertants in the culture. Similar results were obtained for MATα scg1-7 ste5-3ts, MATα scg1-4 ste5-3ts, MATα scg1-4 ste5-3ts, strains except that α cells recovered more readily than a cells (Figure 2B, 2C, and 2D, respectively). No recovery was detectable for the null mutant scg1::lacZ6 (J. Hirschman and D. Jenness, unpublished observations), suggesting that cells are unable to recover from stronger signal or that partially functional SCG1 product is required for recovery. The SCG1 role in recovery from cell cycle arrest has been suggested by others (Miyajima et al, 1989).

The recovery of MATa STE4Hpl ste5-3ts double mutant from cell cycle arrest is shown in Figure 1C. Cells divided two or three generations before they reduced their division rate and passed through an inflection point after which the division rate steadily increased as cells adapted to the post-receptor signal. The STE4Hpl mutant showed a delay in the cell cycle arrest compared to scg1-7 arrest kinetics. This delay probably reflected the instability of the STE4Hpl gene product at 34° C, followed by slow accumulation of the gene product after the shift to 22° C. In addition, STE4Hpl mutants exhibited improved growth and reduced pheromone responsiveness at temperatures above 34° C, which is consistent with instability of the STE4Hpl product at 34° C.

Evidence for two post-receptor recovery mechanisms
There are several ways cells are able to regulate their sensitivity to α-factor. The extracellular protease, the \( BAR1 \) gene product, controls degradation of α-factor (Chan and Otte, 1982a, b; Ciejk and Thorner, 1979; Sprague and Herskowitz, 1981). The \( SST2 \) gene product controls a recovery process that is independent of α-factor degradation (Chan and Otte, 1982a, b). The carboxy-terminal domain of the α-factor receptor, the \( STE2 \) gene product, controls yet another recovery mechanism that is independent of \( BAR1 \) and \( SST2 \) (Konopka et al, 1988; Reneke et al, 1988). It was of interest therefore, to determine whether any of these recovery processes also operate on the postreceptor constitutive signals generated in the \( scg1 \) and \( STE4^{Hp1} \) mutants. The following triple mutants were constructed to test this hypothesis: \( sst2-1 \ scg1-7 \ ste5-3^{ts}, \ bar1-1 \ scg1-7 \ ste5-3^{ts}, \ ste2-10::LEU2 \ scg1-7 \ ste5-3^{ts}, \) and \( sst2-1 \ STE4^{Hp1} \ ste5-3^{ts} \). These constructs were then tested for the ability to recover from arrest of cell division.

The \( scg1 \) mutant did not require \( STE2, SST2 \) or \( BAR1 \) gene products to recover from division arrest. When \( scg1-7 \ ste2-10::LEU2 \ ste5-3^{ts} \) was shifted from 34°C to 22°C (Figure 3A), the kinetics of cell division arrest and recovery were similar to the \( scg1-7 \ ste5-3^{ts} \) double mutant (Figure 1B). Similar results were obtained with \( scg1-7 \ bar1 \ ste5-3^{ts} \) (not shown). The \( scg1-7 \) recovery was independent of the α-factor receptor and α-factor proteolysis. The \( scg1-7 \ sst2 \ ste5-3^{ts} \) mutant recovered from division arrest at the same point in time (20 hours) after the temperature shift (Figure 3B), however, the
division rate of the recovered cells was somewhat slower. It is therefore possible that SST2 gene product, although unnecessary for recovery, does play an accessory role in the recovery of scg1-7 from division arrest. This SST2-independent mechanism was also independent of the receptor, since scg1-7 sst2-1 ste5-3ts cells and scg1-7 sst2-1 ste2-10::LEU2 ste5-3ts cells showed similar arrest and recovery profiles (Figure 4B and 4C). The recovery of these mutants was not due to accumulation of α-factor resistant cells, since all cells in a population showed the same behavior. Cultures were grown at 34o C and shifted to 22o C as described in the legend to Figure 6 (data not shown).

The STE4Hpl mutant depended on the SST2 gene product for recovery from division arrest. The STE4Hpl sst2-1 ste5-3ts mutants did not recover by 30 hours after the shift from 34o C to 22o C (Figure 3C). In contrast, STE4Hpl ste5-3ts mutants readily recovered by 20 hours at 22o C (Figure 1C). These results indicate that SST2 gene product is required for recovery from the postreceptor signal which is generated in STE4Hpl mutants. Cells that contained both scg1-7 and STE4Hpl mutations showed no recovery during the time course examined. When the scg1-7 STE4Hpl ste5-3ts mutant was shifted from 34o C to 22o C, cells divided very slowly after growing rapidly for about three generations (Figure 3D). This result suggests that either cells cannot recover from a stronger signal or that SCG1 product is required for STE4Hpl recovery.
The properties of the individual cells were monitored during the recovery process. Liquid cultures growing at 34°C were spread on solid medium and cultured at 22°C. Figure 5 shows results for cells cultured at 22°C for 16 hours. The scgl-7 ste5-3ts and STE4Hpl ste5-3ts double mutants (Figure 5C and 5D, respectively) arrested cell division and became large and pear shaped. These mutants resembled control ste5-3ts cells which had been treated with α-factor for the same amount of time (16 hours at 22°C) (Figure 5B). After 48 hours at 22°C, the scgl-7 ste5-3ts and STE4Hpl ste5-3ts double mutants (Figure 6C and 6D) had recovered from division arrest as judged by appearance of small budding cells at the edge of the microcolony. In contrast, the STE4Hpl sst2-1 ste5-3ts produced microcolonies which consisted of about 10 large, severely distended cells (Figure 6E). The scgl-7 STE4Hpl ste5-3ts cells also were mostly large and unbudded after 48 hours at 22°C (Figure 6F). In summary, these results indicate that STE4Hpl mutant depends on the SST2 gene product for recovery and that the scgl-7 mutant can recover from division arrest by a different mechanism.

Receptor down-regulation is independent of SCG1

The α-factor induced down-regulation of the α-factor receptors can potentially regulate pheromone response pathway. Binding of α-factor causes rapid loss of receptor sites from the cell surface (Jenness and Spatrick, 1986) and subsequent reaccumulation of the receptors due to de novo receptor synthesis. It was, therefore, of
interest to determine whether the constitutive signal generated in \textit{scg1} mutants affects the accumulation of the receptor sites. The \textit{scg1-7 ste5-3} and \textit{ste5-3} mutants were grown at 34\textdegree\ C and shifted to 22\textdegree\ C. The binding capacity of cells was determined at regular intervals (Figure 7A). Increased binding capacity of the control \textit{ste5-3} cells was expected, since the number of cells in the culture increased during the time-course of the experiment, and since \textit{STE5}+ cells contain at least twice as many binding sites as \textit{ste5-3} at 34\textdegree\ C (Jenness et al, 1987). The \textit{scg1-7 ste5-3} double mutant exhibited similar behavior, however, the somewhat greater rate of receptor reaccumulation was probably due to the postreceptor constitutive signal. Both cultures showed normal down-regulation and reaccumulation of the receptors when \textit{\alpha}-factor was added at the time of the shift to 22\textdegree\ C (Figure 7A).

The receptor turnover rates were also monitored in the \textit{scg1-7 ste5-3} and \textit{ste5-3} strains in the absence of the new receptor synthesis (Figure 7B). Both cultures were grown at 34\textdegree\ C and shifted to 22\textdegree\ C for 4 hours to allow expression of the \textit{scg1} phenotype. Cycloheximide was added to block new receptor synthesis. The \textit{scg1-7 ste5-3} and \textit{ste5-3} strains both showed slow receptor loss from the cell surface which was accelerated by addition of \textit{\alpha}-factor. Results from these experiments suggest that the \textit{scg1} mutation does not cause a higher constitutive rate of receptor turnover. The signal for down-regulation of the receptors is presumably induced before the \textit{SCG1}-controlled step.
Discussion

The ability of cells to adapt to a prolonged stimulus is inherent in many signal transduction systems. These adaptation mechanisms are often very complex and affect different steps of the signal pathway (Pugh and Altman, 1988; Sibley et al, 1987; Stock et al, 1985). The a cell's ability to adapt to α-factor pheromone is regulated by several genes. One of them is the secreted, α-factor specific protease, BAR1, which degrades pheromone in culture (Chan, 1977; Chan and Otte, 1982a; Ciejek and Thorner, 1979; Moore, 1983). The expression of this protease is constitutive but it is induced several fold in the presence of α-factor (Kronstad et al, 1987). The other element involved in adaptation to the signal is the C-terminus of the STE2 receptor. The carboxy-terminal deletions of the receptor that are missing 105 amino acids show supersensitivity to the pheromone and exhibit a defect in adaptation (Konopka et al, 1988; Reneke et al, 1988). Yet another component which is involved in adaptation is the SST2 gene product. Expression of the SST2 gene product is induced by pheromone treatment (Dietzel and Kurjan, 1987b). Mutations in SST2 cause increased pheromone sensitivity and defects in adaptation (Chan and Otte, 1982b). The above three elements apparently act in independent pathways since combinations of any two results in sensitivity to the α-factor which is
more extreme than either of the single mutants (Konopka et al, 1988; Reneke et al, 1988). Results presented in this chapter suggest that \( SST2 \) affects postreceptor events in adaptation and that another \( SST2 \)-independent adaptation mechanism may operate in these cells.

The constitutive mutants in the pheromone response pathway provided a convenient model for investigation of the post-receptor adaptive mechanisms. There were several reasons to suspect that adaptation mechanisms affect \( SCG1 \) gene product or later steps in the pathway. The spores containing the \( scgl \) mutation continue to divide slowly upon germination (Dietzel and Kurjan, 1987a), prolonged expression of the \( scgl \) phenotype leads to decreased mating efficiency (Nakayama et al, 1988), and expression of the \( SCG1 \) gene is enhanced in the presence of pheromones (Jahng et al, 1988).

The strains used for this study contained temperature-sensitive mutation \( ste5-3^{ts} \), which blocked constitutive signals at the non-permissive temperature. This block at the non-permissive temperature \( (34^\circ C) \) appears to affect steps in signal transduction which are executed after the \( SCG1 \) and \( STE4 \) controlled steps. At the permissive temperature \( (22^\circ C) \) \( ste5-3^{ts} \) mutants showed \( \alpha \)-factor response and recovery behavior (Figure 1A) similar to that observed for \( STE^+ \) cells (Jenness and Spatrick, 1986; Moore, 1984). The arrest and adaptation of the \( scgl-7 \) \( ste5-3^{ts} \) and \( STE4^{Hpl} \) \( ste5-3^{ts} \) cultures after the shift to \( 22^\circ C \) indicated that the recovery mechanisms were induced after the \( STE5 \)-controlled step.
The results of this study suggest that at least two post-receptor adaptive mechanisms operate in a cells. The $STE4^{Hpt}$ mutant adapted to the constitutive signal by an $SST2$-dependent mechanism. This finding indicates that $SST2$ gene product is involved in post-receptor adaptation at or after the $STE4$-controlled step. In contrast, the $scg1$ mutant recovered from the constitutive arrest partially by an $SST2$-independent mechanism, however, $scg1\; stt2\; ste5-3^{ts}$ slower growth as compared to $scg1\; SST2\; ste5-3^{ts}$ suggests a role for $SST2$ product in the recovery of $scg1$ mutants.

The pheromone receptor ($STE2$) is not involved in adaptation of the $scg1$ mutants. The recovery of $scg1$ mutants was unaffected by deletion of the $STE2$ gene (Figures 1A and 3B). In addition the number of the receptor sites on the cell surface and their down-regulation rate in response to $\alpha$-factor was largely unaffected by the $scg1$ mutation (Figure 7).
Figure 1. Recovery of \textit{scgl} and \textit{STE4Hpl} Mutants from Division Arrest.

Growth rates of mutant strains were examined after the cultures had been shifted from 34°C to 22°C. (A) Affect of \(\alpha\)-factor on strain DJ164-7-1 (\textit{MATa bar1-1 ste5-3ts}). \(\alpha\)-Factor was either not added \(\square\), or added at the time of temperature shift at the concentration of \(10^{-9}\) \(\square\), \(5\times10^{-9}\) \(\triangle\), or \(10^{-8}\) \(\blacktriangle\) M. (B) Comparison of strain DJ728-9-3 (\textit{MATa ste5-3ts scgl-7}) \(\square\) with strain 5561-10-2 (\textit{MATa ste5-3ts}) \(\square\). (C) Comparison of strain DJ736-34-1 (\textit{MATa ste5-3ts STE4Hpl}) \(\square\) with strain 5561-10-2 \(\square\).
Figure 2. Recovery of \textit{scg}\textsubscript{1-7} and \textit{scg}\textsubscript{1-4} in MAT\textit{a} and MAT\textit{\alpha} Strains.

Cultures were treated as described in the legend to Figure 1. (A) The cultures from Figure 1B that had been growing at 22\textdegree C for 30 hours were shifted to 34\textdegree C and grown overnight at 34\textdegree C. Cultures were diluted to 10\textsuperscript{5} cells per ml and shifted back to 22\textdegree C. Time points were taken as described in Materials and Methods. Comparison of
Figure 2 (continued)

strain DJ728-9-3 (MAT\(\alpha\) ste5-3\(^{ts}\) scgl-7) \(\square\) with strain 5561-10-2 (MAT\(\alpha\) ste5-3\(^{ts}\)) \(\square\). (B) Comparison of strain DJ728-12-1 (MAT\(\alpha\) ste5-3\(^{ts}\) scgl-7) \(\square\) with strain 5561-9-3 (MAT\(\alpha\) ste5-3\(^{ts}\)) \(\square\). (C) Comparison of strain DJ733-9-3 (MAT\(\alpha\) ste5-3\(^{ts}\) scgl-4) \(\square\) with strain 5561-10-2 (MAT\(\alpha\) ste5-3\(^{ts}\)) \(\square\). (D) Comparison of strain DJ733-9-2 (MAT\(\alpha\) ste5-3\(^{ts}\) scgl-4) \(\square\) with strain 5561-9-3 (MAT\(\alpha\) ste5-3\(^{ts}\)) \(\square\).
Figure 3. Recovery of Double Mutants from Division Arrest.
Cultures were treated as described in the legend to Figure 1. (A)
Comparison of strain DJ751-12-2 (MATa ste5-3\textsuperscript{ts} ste2-10::LEU2 scg1-7) (■) with strain DJ231-2-2 (MATa ste5-3\textsuperscript{ts} ste2-10::LEU2) (□).
Recovery of scgl mutant was independent of the receptor. (B)
Comparison of strain DJ750-8-1 (MATa ste5-3\textsuperscript{ts} sst2-1 scgl-7) with strain 5528-10-3 (MATa ste5-3\textsuperscript{ts} sst2-1). Recovery of the scgl mutant was partially dependent on the SST2 gene. (C)
Comparison of strain DJ760-1-1 (MATa ste5-3\textsuperscript{ts} sst2-1 STE4\textsuperscript{Hpl}) with strain 5528-10-3. Recovery of the STE4\textsuperscript{Hpl} mutant required SST2. (D) Comparison of strain DJ761-5-3 (MATa ste5-3\textsuperscript{ts} scgl-7 STE4\textsuperscript{Hpl}) with strain 5561-10-2 (MATa ste5-3\textsuperscript{ts}). Double mutant scgl-7 STE4\textsuperscript{Hpl} recovered worse than either the scgl-7 or the STE4\textsuperscript{Hpl} mutants alone.
Figure 4. Recovery of the scgl-7 sst2 ste2 Mutant Cells

Cultures were treated as described in legend to Figure 1. (A) Comparison of strain DJ728-9-3 (MATa ste5-3ts scgl-7) ■ with strain 5561-10-2 (MATa ste5-3ts) □. The scgl-7 cells recover from cell cycle arrest. (B) Comparison of strain DJ750-8-1 (MATa ste5-3ts sst2-1 scgl-7) ■ with strain 5561-10-2 (MATa ste5-3ts) □. Recovery of scgl-7 cells is partially dependent on the SST2 gene product. (C) Comparison of strain DJ781-2-1 (MATa ste5-3ts ste2-10::LEU2 sst2-1 scgl-7) ■ with strain 5561-10-2 (MATa ste5-3ts)
Figure 4 (continued)

Recovery of scgl-7 cells is partially dependent on SST2 gene product.
Figure 5. Morphologies of Mutant and α-Factor Arrested Cells

YM-1 liquid cultures growing exponentially at 34°C (10^6 to 2×10^7 cells per ml) were spread on YEPD plates at 22°C (about 10^6 cells per plate). Microcolonies were photographed after 16 hours at 22°C. (A) Strain DJ164-7-1 (*MATa ste5-3ts*); (B) strain DJ164-7-1 spread on YEPD containing 5×10^-9 M α-factor; (C) strain DJ728-9-3 (*MATa ste5-3ts scg1-7*); (D) strain DJ736-34-1 (*MATa ste5-3ts STE4Hpl*). The four panels are phase-contrast micrographs at the same magnification.

Figure 6. Cell Morphologies after Recovery from Division Arrest.

Cells were treated as described for Figure 3 except that the plates were incubated for 48 hours at 22°C. (A) Strain 5561-10-2 (*MATa ste5-3ts*); (B) strain 5528-10-3 (*MATa ste5-3ts sst2*); (C) strain DJ728-9-3 (*MATa ste5-3ts scg1-7*); (D) strain DJ736-34-1 (*MATa ste5-3ts STE4Hpl*); (E) strain DJ760-1-1 (*MATa ste5-3ts STE4Hpl sst2*); (F) strain DJ761-5-3 (*MATa ste5-3ts scg1-7 STE4Hpl*). All four panels are at the same magnification. Bar, 50 μm.
Figure 7. Down-regulation of the Receptor Sites.

(A) Down-regulation and reaccumulation of receptor sites in the scg1-7 ste5-3<sup>ts</sup> mutant DJ730-2-3 (△,▲) and the ste5-3<sup>ts</sup> control strain DJ164-7-1 (□,■). Cultures growing exponentially at the 34<sup>o</sup>C were shifted to 22<sup>o</sup>C at time zero. Cultures received no α-factor (△,□) or 10<sup>-8</sup> M α-factor (▲,■). Samples were withdrawn at the times indicated, and the <sup>3</sup>H-α-factor-binding capacity was determined. (B) The rate of the receptor loss is unaffected by the scg1-7 mutation. Exponentially growing cultures of the scg1 ste5-3<sup>ts</sup> mutant DJ730-2-3 (△,▲) and the ste5-3<sup>ts</sup> control strain DJ164-7-1 (□,■) were diluted to
Figure 7 (continued)

2.5x10^6 cells per ml and shifted to 22^\circ C. After 4 hours at 22^\circ C (time zero), the cultures received cycloheximide (10 \mu g/ml) and either no \alpha-factor (\triangle, \square) or 10^{-8} M \alpha-factor (\triangle, \blacksquare). Binding capacity is expressed as the percentage of the initial value in the absence of \alpha-factor.
### Table 1. Strain List

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<td>DJ164-7-1</td>
<td>381G ste5-3(^{ts}) bar1-1</td>
</tr>
<tr>
<td>DJ728-9-3</td>
<td>381G ste5-3(^{ts}) scgl-7</td>
</tr>
<tr>
<td>DJ728-12-1</td>
<td>381G MATα ste5-3(^{ts}) scgl-7</td>
</tr>
<tr>
<td>DJ733-9-3</td>
<td>381G ste5-3(^{ts}) scgl-7</td>
</tr>
<tr>
<td>DJ733-9-2</td>
<td>381G MATα ste5-3(^{ts}) scgl-7</td>
</tr>
<tr>
<td>DJ781-2-1</td>
<td>381G ste5-3(^{ts}) scgl-7 ste2-10::LEU2 sst2-1</td>
</tr>
<tr>
<td>DJ730-2-3</td>
<td>381G ste5-3(^{ts}) bar1-1 scgl-7</td>
</tr>
<tr>
<td>DJ736-34-1</td>
<td>381G ste5-3(^{ts}) STE4(^{Hp})</td>
</tr>
<tr>
<td>DJ231-2-2</td>
<td>381G ste5-3(^{ts}) bar1-1 ste2-10::LEU2 leu2 ura3</td>
</tr>
<tr>
<td>DJ751-12-2</td>
<td>381G ste5-3(^{ts}) scgl-7 ste2-10::LEU2 leu2 ura3</td>
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<td>381G ste5-3(^{ts}) sst2-1 scgl-7</td>
</tr>
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<td>381G MATα ste5-3(^{ts})</td>
</tr>
<tr>
<td>DJ761-5-3</td>
<td>381G ste5-3(^{ts}) TYR1 scgl-7 STE4(^{Hp})</td>
</tr>
<tr>
<td>DJ760-1-1</td>
<td>381G ste5-3(^{ts}) sst2-1 STE4(^{Hp})</td>
</tr>
</tbody>
</table>

\(^a\) All strains are congenic to strain 381G (Hartwell, 1980).

\(^b\) ste2-10::LEU2 is a genetic substitution that removes the entire STE2 gene. The bar1-1 mutation (Sprague and Herskowitz, 1981)
Table 1 (continued).

inhibits α-factor degradation in a cell cultures. The *scgl*-7 and *
STE4Hp* mutations result in constitutive activity of the pheromone
response pathway (Blinder et al, 1989).
CHAPTER IV

ISOLATION AND CHARACTERIZATION OF CONSTITUTIVE MUTATIONS IN STE5

Introduction

This chapter describes the isolation and analysis of constitutive mutations in the STE5 gene. The STE5 gene product is a positive element in the pheromone response pathway, since recessive mutations that inactivate its function are unresponsive to pheromones. The analysis of double mutants containing constitutive and unresponsive mutations presented in Chapter II suggested the functional order of genes in the pheromone pathway. The STE5 gene product was proposed to affect the pheromone response after the STE4 step, since the temperature-sensitive mutation ste5-3ts was able to suppress the constitutive mutation STE4Hp1. However, the reciprocal experiment between ste4-3ts and STE5Hp1 was not possible, and therefore the possibility remained that STE4 and STE5 products act at the same step in the pathway, i.e., the STE5 product may control transcription, processing or the stability of the STE4 gene product. To resolve this question, it was important to isolate
mutations in \textit{STE5} (\textit{STE5}^{Hpl}) which cause constitutive activation of the pheromone response pathway. If \textit{STE5}^{Hpl} mutants are able to activate the pheromone response pathway in the absence of the \textit{STE4} gene product, i.e., a \textit{ste4 \textit{STE5}^{Hpl}} double mutant is constitutive, then the \textit{STE5} gene product acts after the \textit{STE4} controlled step.

Isolation of the \textit{STE5}^{Hpl} mutants was performed in the \textit{sir3}^{ts} genetic background. The \textit{sir3}^{ts} mutation allows expression of mating-type information from the silent cassettes (\textit{HML} and \textit{HMR}) at 34\degree C, but not at 22\degree C. Thus, the \textit{sir3}^{ts} cells are phenotypically diploid at 34\degree C and haploid at 22\degree C (Hartwell, 1980; Rine and Herskowitz, 1987). A \textit{sir3}^{ts} recipient strain was transformed with an \textit{in vitro} mutagenized \textit{STE5} plasmid and transformants were selected at 34\degree C. The \textit{STE5}^{Hpl} mutant candidates were identified by growth deficiency and the presence of aberrant cell morphology ("shmoos") at 22\degree C. Potential \textit{STE5}^{Hpl} mutant plasmids were transformed into the \textit{ste2}, \textit{ste4}, \textit{ste12} and \textit{ste18} strains to investigate the effects of constitutive activation of the pheromone response pathway at the \textit{STE5} step. A possible model for \textit{STE5} gene action is discussed.

Materials and Methods

Strains, Media and Genetic Methods

Strains used in this study are listed in Table 4. YM-1 is a rich liquid medium (Hartwell, 1967). The 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; the minimal medium was supplemented with amino acids (50 μg/ml) or adenine as (20 μg/ml) as needed. Minimal medium lacking uracil was supplemented with casamino acids (Difco) (-Ura+CAA) (0.1%). Standard genetic methods were used for all strain constructions (Mortimer and Hawthorne, 1967).

**Plasmid Constructions**

All yeast transformations utilized the Lithium Acetate procedure (Ito et al, 1983). Plasmid pHB30 (YEp13 that carries the STE5 genomic fragment) was kindly provided by M. Hasson and J. Thorner. YEpSTE5 and YIpSTE5 were constructed by ligating the 4.5 kb Bam HI-PvuII fragment containing STE5 from pHB30 with the Bam HI-SmaI digested YEp352 or YIp352 vectors, respectively (Hill et al, 1986). Genetic disruption of the STE4 gene (DJ656-2) was achieved by transforming the diploid strain DJ656 with the PstI-XbaI fragment of STE4 with the LEU2 gene inserted at the BglII site. Deletion of the STE18 gene (DJ656-4) was achieved by transforming DJ656 with the SphI-HindIII fragment containing a LEU2 substitution of the STE18 gene between the the NsiI sites. The M81p12 (STE4) and M65p1 (ste18::LEU2) plasmids were kindly provided by M. Whiteway. The deletion of the STE12 gene (DJ656-3) was constructed by transforming DJ656 with the SacI-SphI fragment
containing the *ste12::LEU2* deletion. The pSUL16 (*ste12::LEU2*) plasmid was kindly provided by B. Errede. The Leu+ transformants were confirmed to contain genetic disruptions or deletions by Southern blot analysis (Sambrook et al, 1989). The integration of YIpSTE5Hpl and YIpSTE5 plasmids was targeted to the chromosomal *URA3* locus by digestion with ApaI.

**Mutant Isolation**

Hydroxylamine mutagenesis was performed essentially as described by Rose and Fink (1987). A mix of 350 mg of hydroxylamine hydrochloride and 90 mg of NaOH was dissolved in water to a final volume of 5 ml. A 0.5 ml aliquot of the solution was added to 10 µg of the plasmid DNA (YEpSTE5) in each of 10 tubes. The tubes were incubated in a 34°C waterbath for 24 hours. At the end of the incubation period 10 µl of 5 M NaCl were added, followed by 1 ml of ethanol to precipitate the DNA. The DNA was pelleted by centrifugation in the microfuge for 10 minutes, washed with ethanol, air dried, and resuspended in 50 µl of 10 mM Tris and 1 mM EDTA (pH 8.0). The DNA was used directly for yeast transformation. The level of mutagenesis was established by loss of function of the nutritional marker on the plasmid. To check for loss of *URA3* function on the plasmid YEpSTE5, the *PyrF* deficient strain of *E. coli* (MH6) was used. Since the yeast *URA3* gene complements the *pyrF* mutation, loss of *URA3* function resulted in uracil auxotrophy among a fraction of the mutagenized plasmid. The ampicillin-resistant
transformants yielded 2.7% of Ura⁻ colonies (11 Ura⁻ Amp⁺/414 Amp²).

Yeast strain DJ676-2-2 (MATα 381G leu2 ura3 sir3ts) was transformed with the mutagenized plasmid YEpSTE5. Transformants were selected on minimal medium lacking uracil (-Ura) at 34° C. Colonies were replica-plated onto two -Ura plates, which were incubated at 34° C and 22° C. Colonies that exhibited normal growth at 34° C but were growth deficient at 22° C, were considered for further analysis. Out of 231 Ura⁺ transformants 12 exhibited cold-sensitive growth. The plasmid DNA was extracted from these candidates by the method of Hoffman and Winston, (1987) and used for transformation of DJ676-2-2 strain. Only four out of twelve transformants confirmed the growth-defective phenotype at 22° C, and one transformant in addition to the growth defect, contained a significant number of aberrant ("shmoo") cells on the 22° C plate. Presence of shmoos was expected for constitutive activation of the pheromone response pathway. This mutant plasmid was designated YEpSTE5Hpl9-1.

The YEpSTE5Hpl9-1 and YEpSTE5 unmutagenized plasmids were used to transform the DJ603-136 strain that carries pDJ117 (MATα ADE3) sectoring plasmid (see Chapter 1). Transformants were selected on -Ura+CAA plates and scored for spontaneous loss of the pDJ117 plasmid. Cells transformed with the mutant plasmid failed to sector, i.e., they were not able to grow without pDJ117 plasmid,
whereas cells containing the unmutagenized YEpSTE5 plasmid were able to form sectors.

**Quantitative Mating Experiments**

The quantitative mating experiments were performed essentially as described by (Hartwell, 1980). Cells were grown overnight in minimal medium (-Ura) at 34° C to a density between 10⁶ and 2x10⁷ cells per ml, diluted to 10⁶ cells per ml, and shifted to 22° C for 3 hours (to allow expression of the Hpl phenotype). The MATα cells to be tested, 2x10⁶ cells, were mixed with 2x10⁶ of MATα cells (EMS63); for testing the MATα cell mating, the 3224-4 MATα strain was used. Cells were collected on a type HA filter (0.45-μm pore size), rinsed with 10 ml of minimal medium (without ammonium sulfate or glucose), and filter was transferred to a -Ura plate at 22° C. Filters were incubated on plates for 6 hours at 22° C. Cells were then suspended in 2 ml of minimal medium, dispersed by vortexing for 1 minute, diluted and plated on unsupplemented minimal medium at 30° C selecting for diploids. For mating at 34° C, the cultures were grown overnight at 34° C, diluted to 2x10⁶ cells per ml and used directly for mating experiments. The mating mix collected on filters was incubated on the prewarmed -Ura plates at 34° C for 6 hours. Following incubation cells were treated exactly as for 22° C mating.

**Plasmid Loss Assays**
Diploid strains containing a gene disruption of $STE2$, $STE4$, $STE12$ or $STE18$ were transformed with either YEp$STE5^{Hp19-1}$ or YEp$STE5$ plasmids and subjected to tetrad analysis. Four spore tetrads that showed the expected segregation of the $LEU2$ marker, which marks the disruption, and $URA3$ marker (YEp plasmid), were used for plasmid loss experiments. Haploid strains carrying either YEp$STE5^{Hp19-1}$ or YEp$STE5$ plasmids were grown overnight in 5 ml of minimal medium lacking uracil (-Ura) at 34° C. Cultures were diluted to $10^5$ cells per ml with 5 ml of liquid rich medium (YM-1) and grown until saturation (4-5 days) at 22° C. Serial tenfold dilutions were made in unsupplemented minimal medium and 10 μl of each dilution were spotted onto a complete minimal plate (C) and plate lacking uracil (-Ura). The percentage of plasmid remaining was calculated by formula: (number of Ura+ colonies X 100)/(Total number of colonies). The "number of colonies" was determined by counting colonies at a dilution where the number can be accurately estimated (>100 cells).

Southern Blot Analysis

The DNA from yeast strains was prepared by method of Hoffman and Winston (1987), subjected to electrophoresis in agarose gels, and transferred to nylon membranes by standard procedures (Sambrook et al, 1989). The radiolabeled probes were made by random primer labeling using a Boehringer Mannheim Biochemicals kit.
Results

Isolation of $STE5^{Hp1}$ mutants

The $STE5$ gene product is a positive element in the pheromone response pathway, and therefore, constitutive mutations in $STE5$ were expected to cause a dominant phenotype. A high-copy-number plasmid carrying the $STE5$ gene (YEp$STE5$) was mutagenized with hydroxylamine as described in Materials and Methods and was used to transform a $sir3^{ts}STE5^+$ strain. A $sir3^{ts}$ mutation allows expression of mating information from the silent cassetts ($HML$ and $HMR$) at $34^\circ$ C but not at $22^\circ$ C. Transformants were selected on minimal plates at $34^\circ$ C and replica-plated to $22^\circ$ C plates. A mutation in $STE5$ that causes constitutive activation of the pheromone response pathway would be repressed in the $sir3^{ts}$ strain at $34^\circ$ C since the cells express both $MATa$ and $MATa$ information, but not at $22^\circ$ C when the $sir3^{ts}$ strain becomes phenotypically haploid. The isolation scheme is depicted in Figure 1. Transformants that exhibited a growth defect and aberrant cell morphology ("shmoos") at $22^\circ$ C and grew normally at $34^\circ$ C were considered for further analysis. From a total of 231 transformants, 12 cold-sensitive candidates were picked and their plasmid DNA was used to retransform the $sir3^{ts}$ strain. Four candidates exhibited a growth defect on a $22^\circ$ C plate, and only one candidate also contained
aberrantly distended cells ("shmoos"). This particular mutant was named YEp\textit{STE5}\textsubscript{Hp1}9-1 and was used for all further experiments.

**Characterization of \textit{STE5}\textsubscript{Hp1} mutant**

First it was important to establish that the\textit{STE5}\textsubscript{Hp1} constitutive phenotype was haploid-specific, i.e., suppressed in a strain that expresses both \textit{MATa} and \textit{MATa} information, and this suppression does not depend on the \textit{sir3}\textsuperscript{ts} mutation \textit{per se}. The strain DJ603-136 that carries pDJ117 (\textit{MATa ADE3 LEU2}) sectoring plasmid was transformed with either the YEp\textit{STE5}\textsubscript{Hp1} or the YEp\textit{STE5} plasmids. Transformants were selected on minimal medium containing leucine but not uracil. The loss of the pDJ117 plasmid in the transformants was monitored by formation of white sectors in a growing red colony. Transformants carrying the YEp\textit{STE5}\textsubscript{Hp1} plasmid failed to sector, whereas the YEp\textit{STE5} transformants sectored readily. This result suggests that the\textit{STE5}\textsubscript{Hp1} mutant causes a growth defect in haploid a cells when they lose the \textit{MATa} plasmid, therefore, the \textit{STE5}\textsubscript{Hp1} mutation is haploid-specific which is consistent with its constitutive activation of the pheromone response pathway. As expected, the \textit{STE5}\textsubscript{Hp1} mutation was dominant, since it caused the constitutive phenotype in both DJ676-2-2 (\textit{sir3}\textsuperscript{ts}) and DJ603-136 strains which carry a wild-type copy of \textit{STE5} gene on the chromosome. This result is in agreement with the prediction (Chapter I) that a positive regulatory element in the pathway can mutate to give either a
recessive unresponsive phenotype, \textit{ste5-3}^{ts} \text{(Hartwell, 1980)}, or a dominant constitutive phenotype, \textit{STE5}_{Hpl}.

Although haploid \textit{sir3}^{ts} cells carrying \textit{YEpSTE5}_{Hpl} plasmid exhibited a growth defect at 22\textdegree C, they did continue to grow slowly. This effect was also observed in haploid segregants of the diploid strain DJ656 (\textit{MATa/MATa}) that has been transformed with the \textit{YEpSTE5}_{Hpl} plasmid (Figure 2). Diploids which were transformed with the mutant plasmid and subjected to tetrad analysis gave rise to segregants that produced either small colonies or single cells arrested at the first cycle (Figure 2A). The occasional formation of wild-type colonies was probably a result of the plasmid loss. The small colonies contained \textit{YEpSTE5}_{Hpl} plasmid as revealed by the tetrad analysis which can explain the slow growth phenotype. The first cycle arrest or formation of the small colony, presumably, depends on the copy number of the mutant plasmid at the time of germination. Diploid cells that carry \textit{YEpSTE5} plasmid gave rise to all viable spores (Figure 2B). In addition a Western blot performed with strains that carry either wild-type or mutant \textit{STE5} plasmid revealed that less \textit{STE5}_{Hpl} product was present relative to the amount of \textit{STE5}^{+} product (M. Hasson and J. Thorner, personal communication). It is possible, therefore, that a high copy of the \textit{STE5}_{Hpl} product is necessary to cause first cycle arrest upon germination, lesser amounts of \textit{STE5}_{Hpl} can cause slow growth of the colony. Also the slow growth phenotype could be the result of the action of several recovery mechanisms activated by the \textit{STE5}_{Hpl} generated signal (see Discussion).
In addition, the $STE5^{Hpl}$ mutation was likely to be somewhat temperature-sensitive. Tetrad dissection of the DJ656/YEp$STE5^{Hpl}$ diploid revealed a more extreme phenotype when the spores were germinated at 22$^\circ$ C, than at 34$^\circ$ C, i.e., they showed a higher percentage of first cycle arrested cells (data not shown). Instability of the mutant protein at 34$^\circ$ C may account for this effect. Consequently, haploid cells that carry the YEp$STE5^{Hpl}$ were propagated at 34$^\circ$ C and were shifted to 22$^\circ$ C for stronger expression of the constitutive phenotype.

**Double Mutant Analysis**

The ability of the $STE5^{Hpl}$ mutation to suppress the sterility phenotype of different ste$^{-}$ strains was determined next. Disruption or deletion mutations of the $STE4$, $STE12$ and $STE18$ genes were constructed as described in Materials and Methods and were confirmed by Southern blot analysis (data not shown). The $STE2$ mutation was described previously (Burkholder and Hartwell, 1985). Diploid strains heterozygous for the $STE$ mutation were transformed with either the YEp$STE5^{Hpl}$ or the YEp$STE5$ plasmids. Transformants were sporulated, dissected and tetrads were analyzed for co-segregation of the ste mutation (marked by the $LEU2$ insertion) and the plasmid (marked by the $URA3$ gene). The ste segregants that carried a mutant or a wild-type $STE5$ plasmid were tested for bypass of the sterility phenotype by quantitative mating as described in Materials and Methods (Hartwell, 1980). Segregants which gave first
cycle arrest were not analysed. Results of these experiments are presented in Table 1. The mating defect of ste2-10::LEU2 deletion was partially suppressed by either the YEpSTEM5Hpl or the YEpSTEM5 plasmids. In contrast, the ste4 and ste18 disruptions were suppressed only by the mutant high-copy-number plasmid. Mating of these strains reached 0.1% of the wild-type mating. The STE5+ plasmid had no effect on mating in these strains. These results suggest that STE5Hpl mutant can bypass the STE4 and STE18 disruptions and activate the pathway at a separate step, after the STE4/STE18 controlled step. As expected, the STE5Hpl mutant did not suppress a deletion of the STE12 gene. The STE12 gene product is thought to control transcription of the haploid specific genes. The STE5Hpl and STE5 genes integrated in a single copy into the URA3 locus had no effect on mating of any of the the disruptions, suggesting that a high-copy number of the STE5Hpl product is necessary for a bypass effect.

The mating defect of the unresponsive temperature-sensitive allele ste4-3ts, was suppressed by STE5Hpl mutant either on a high-copy-number plasmid or as a single copy integrated at the chromosomal URA3 locus (Table 1). The high-copy STE5+ plasmid also partially suppressed the mating defect of ste4-3ts. Suppression of the ste4-3ts mutation by a single copy of STE5Hpl suggests that smaller amounts of mutant STE5 product were needed. This could be due to the presence of a small amount of functional STE4 gene product even at 34°C. Results from the above experiments suggest
that the \textit{STE5} gene product acts at a separate step in the signal transduction pathway, after the \textit{STE4} step, since the \textit{STE5}^{Hpl} mutant can activate the pheromone pathway in the absence of the \textit{STE4} gene product.

\textbf{Plasmid Loss Assays}

Haploid strains that carry the \textit{STE5}^{Hpl} mutant on a plasmid exhibited differential plasmid stabilities when cells were grown in the absence of plasmid selection. The percent of the plasmid remaining was determined as described in Materials and Methods. The results are summarized in Table 2. Diploid (\textit{MATa/MATa}) strains transformed with either the mutant or the wild-type \textit{STE5} plasmid showed no significant difference in the percent of plasmid remaining after several generations of non-selective growth. This result eliminates the possibility that the mutant plasmid has an intrinsic defect which allows it to attain high-copy-number in cells. As expected, the loss of YEp\textit{STE5} unmutagenized plasmid exhibited no difference in either \textit{ste-} or \textit{STE+} background. In contrast, the mutant \textit{STE5}^{Hpl} plasmid was lost at higher frequency from the \textit{STE+} haploid strains. The \textit{ste4}, \textit{ste18}, and \textit{ste12} disruption strains grown in non-selective medium showed a higher percentage of plasmid remaining than the corresponding \textit{STE+} or \textit{ste2} deletion strains (Table 2). In summary, it seems that \textit{STE5} product can activate the pheromone pathway in the absence of \textit{STE2}, \textit{STE4} and \textit{STE18} products, but for a full response it might require the presence of the \textit{STE4}/\textit{STE18}
products in the cell. The \textit{STE12} gene product is also required for \textit{STE5}^{Hpl} generated signal.

\textbf{Affect of the \textit{sst2} mutation on \textit{STE5}^{Hpl}}

The \textit{STE5}^{Hpl} mutation causes slow growth of cells that carry the plasmid. The slow growth defect could arise from the activation of various recovery mechanisms which allow cells to adapt to the \textit{STE5}^{Hpl} generated signal. It was important, therefore to examine the affect of the \textit{sst2} mutation on the growth of the \textit{STE5}^{Hpl} cells. The \textit{SST2} gene product is thought to promote recovery of cells from pheromone arrest (Chan and Otte, 1982a); mutation in \textit{SST2} impairs the cells ability to recover. The \textit{SST2} gene product is necessary for recovery of \textit{STE4}^{Hpl} cells (Chapter III; Blinder and Jenness, 1989). To determine the affect of the \textit{sst2} mutation on growth of the \textit{STE5}^{Hpl} cells, a double mutant strain (\textit{sst2} \textit{Sir3}^{ts}) was constructed and transformed with the YE\textit{pSTE5}^{Hpl} and the YE\textit{pSTE5} plasmids. Growth of the transformants was observed at different temperatures (Figure 3). As expected, at \textit{22^o} C the \textit{SST2} \textit{Sir3}^{ts}/YE\textit{pSTE5}^{Hpl} strain exhibited slow growth compared to \textit{SST2} \textit{Sir3}^{ts}/YE\textit{pSTE5} strain. However, the \textit{sst2} \textit{Sir3}^{ts}/YE\textit{pSTE5}^{Hpl} double mutant showed an even stronger growth defect. This result is consistent with the idea that \textit{SST2} is somehow involved in attenuation of the \textit{STE5}^{Hpl} signal. In addition, the YE\textit{pSTE5} plasmid also caused slow growth in \textit{sst2} \textit{Sir3}^{ts} strain. Possibly overexpression of \textit{STE5}^{+} gene product can lead to partial activation of the signalling pathway. At \textit{34^o} C all strains exhibited
normal growth. Since all of the above experiments were done in the sir3ts background, it was of interest to test the effect of the sst2 mutation in a SIR3+/YEpSTE5Hpl strain. However, in the course of the sst2/YEpSTE5Hpl strain constructions none of the double mutants were recovered, presumably due to an extreme growth defect.

The experiments described here are consistent with the SST2 gene product affecting the pheromone response pathway either before the STE5 step or after it. Previous experiments suggest the former possibility, i.e., the SST2 gene products affects the pathway at STE4 step; the sst2 STE4Hpl double mutants do not recover from constitutive arrest, and ste4-3ts unresponsive mutation can be suppressed by sst2 mutation together with ros1, 2, or 3 (Blinder and Jenness, 1989; Jenness et al, 1987). It could be reasoned that if the SST2 product acts at or through the STE4 gene product then the sst2 mutation will have no effect in the ste4 deletion strain, however, if the SST2 product affects post-STE4 steps then the presence or absence of the STE4 product will have no effect. This possibility was tested by constructing sst2 ste4::LEU2 and SST2+ ste4::LEU2 strains transformed with YEpSTE5Hpl. The rate of the YEpSTE5Hpl plasmid loss in both strains was identical (Table 3). This result is consistent with a prediction that SST2 gene product acts before or at STE4, and therefore, before the STE5 step in the pheromone response pathway.

Discussion
The \textit{STE5} gene product is a positive element in the pheromone response pathway. The recessive-unresponsive mutation in \textit{STE5}, \textit{ste5-3ts}, was able to block the $\alpha$-factor signal (Hartwell, 1980) and constitutive signals generated in \textit{scg1} and \textit{STE4Hpl} mutants (Nakayama et al, 1988; Blinder et al, 1989). The analysis of double mutants containing constitutive and unresponsive mutations suggested that the \textit{STE5} gene product acts after or at the same step as the \textit{STE4} product. To resolve this question, a constitutive allele of the \textit{STE5} gene was isolated which in high copy can cause activation of the pheromone response pathway. The mutation was thought to be localized to the \textit{STE5} gene for three reasons. First, the fragment containing the \textit{STE5} promoter and coding region when introduced into the unmutagenized YIp352 vector was able to cause suppression of the \textit{ste4-3ts} mutation (Table 1). Second, the YEp\textit{STE5Hpl} plasmid and the YEp\textit{STE5+} unmutagenized plasmid exhibited similar frequency of plasmid loss from the \textit{MATa/MATa} diploid strain, suggesting that Hpl phenotype was not a result of high copy number \textit{per se}. Finally, marker rescue experiments localized the \textit{STE5Hpl} mutation to the amino-terminal third of the protein (M. Hasson and J. Thorner, unpublished observations).

The \textit{STE5Hpl} mutant expressed from the YEp352 vector was able to suppress the mating defect of the \textit{ste4::LEU2} deletion and disruption mutants. Disruptions in \textit{STE2} and \textit{STE18} were also suppressed, i.e., the \textit{ste2::LEU2} and the \textit{ste18::LEU2} mutants
transformed with a $STE5^Hpl$ plasmid became fertile, however, disruption of the $STE12$ gene remained sterile. These results suggest that $STE5$ product acts after the $STE4/STE18$ step but before $STE12$ controlled step. A deletion of the $STE2$ receptor as well as a temperature-sensitive mutation in $STE4$ were also suppressed by expressing wild-type $STE5$ product from a high-copy-number plasmid suggesting that overproduction of this positive element can somehow stimulate the pheromone response pathway.

Maintenance of the YEp$STE5^Hpl$ plasmid in various strains was also examined. Surprisingly, the rate of YEp$STE5^Hpl$ plasmid loss was lower in $STE4$ and $STE18$ disruptions as well as in $STE12$ disrupted strains as compared to $STE2$ deletion and $STE^+$ strains. These observations imply that the $STE5^Hpl$-generated signal can be enhanced by a previous step in the pathway.

A "noise" model is proposed to explain the results presented in this chapter. The "noise" model postulates that in the absence of pheromones, cells generate a basal level of signal ("noise") which also allows limited expression of the pheromone inducible genes. This "noise" can be generated at the postreceptor steps, since deletion of the receptor has no effect on the basal expression of the pheromone inducible genes. If $STE5^Hpl$ is a particularly "noisy" mutant which amplifies a previously generated signal, then a high-copy of the $STE5^Hpl$ product will cause a stronger effect. Also the "noise" generated at the $STE5^Hpl$ will depend on the "noise" generated at the preceding steps of the pathway such as the $STE4$ and $STE18$ steps.
The *STE12* gene product presumably controls transcription of several elements in the pathway which can act after the *STE5* step. It was therefore expected that *STE12* disruption would cause suppression of the *STE5^Hp1* generated signal. The "noise" generated in this pathway can then activate several recovery mechanisms including an *SST2*-dependent mechanism, which would be involved in the recovery of *STE5^Hp1* cells from the constitutive signal.
Transform Strain DJ676-2-2

$MATa\ sir3^{ts}\ ura3$

34°C

Replica-plate

34°C 22°C

Pick cold-sensitive colonies as potential candidates bearing $STE5^{Hpl}$

Figure 1. Isolation of $STE5^{Hpl}$
Figure 2. The $STE5^{Hp}$ Mutation Causes Cell Cycle Arrest and Slow Growth of the Haploid Cells.

Diploid strain DJ656 was transformed with either the YE$pSTE5^{Hp}$ or the YE$pSTE5$ plasmids. Transformants were sporulated and dissected. (A) A total of 16 tetrads that carry the YE$pSTE5^{Hp}$ plasmid were dissected. In six tetrads all segregants show cell division cycle arrest. Other tetrads show segregants with growth defect as well as cell
Figure 2 (continued)
cycle arrest. The arrested cells show aberrant cell morphology ("shmoos") consistent with $STE5^{Hpl}$ constitutive activation of the pheromone response pathway. (B) A total of 11 tetrads that carry unmutagenized YEp$STE5$ plasmid exhibit normal growth. Presence of the plasmids in (A) and (B) was confirmed by tetrad analysis.
Figure 3. The \( STE5^{Hpl} \) mutants require \( SST2 \) for growth. Strains DJ676-2-2 (\( sir3^{ts} \)) and DJ788-7-1 (\( sir3^{ts} \) \( sst2 \)) were transformed with either \( YEpSTE5^{Hpl} \) or \( YEpSTE5 \) plamids. Two independent transformants were spotted (10 \( \mu l \) of saturated stock grown at 34° C) on 22° C plate, incubated overnight, and replica-plated twice to 22° C and 34° C. At 22° C the \( sir3^{ts}/YEpSTE5^{Hpl} \) strains, as expected, show a growth defect compared to the
Figure 3 (continued)

$sir3^{ts}/YEpSTE5$ strains, however, the $sst2\ sir3^{ts}/YEpSTE5^{Hpl}$ strain failed to grow. The $sst2\ sir3^{ts}/YEpSTE5$ strain also exhibited a growth defect similar to the $sir3^{ts}/YEpSTE5^{Hpl}$ strain. On $34^\circ C$ plate all strains exhibited normal growth.
Table 1. Quantitative Mating Experiments.a.

<table>
<thead>
<tr>
<th>Strains&lt;sup&gt;c&lt;/sup&gt;</th>
<th>Diploids/filter&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>YEp*STE9</td>
</tr>
<tr>
<td>a</td>
<td>2.5x10&lt;sup&gt;6&lt;/sup&gt;</td>
</tr>
<tr>
<td>a ste2-10::LEU2</td>
<td>4.0x10&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>a ste4::LEU2</td>
<td>&lt;10</td>
</tr>
<tr>
<td>a ste4::LEU2&lt;sup&gt;d&lt;/sup&gt;</td>
<td>&lt;10</td>
</tr>
<tr>
<td>a ste12::LEU2</td>
<td>&lt;10</td>
</tr>
<tr>
<td>a ste18::LEU2</td>
<td>&lt;10</td>
</tr>
<tr>
<td>a ste4-3&lt;sup&gt;ts&lt;/sup&gt; e</td>
<td>2.3x10&lt;sup&gt;3&lt;/sup&gt;</td>
</tr>
<tr>
<td>α ste4-3&lt;sup&gt;ts&lt;/sup&gt;</td>
<td>ND</td>
</tr>
</tbody>
</table>

ND-not done.

a. Quantitative matings were performed as described in Materials and Methods.

b. Diploids/filter were number of colonies growing on the unsupplemented minimal plates multiplied by a dilution factor. An average of the two most significant dilutions was used. In cases where experiments were done twice; an average of the two experiments was used.

c. All mating experiments were done as described in Materials and Methods. Haploid strains used in these experiments were derived as haploid segregants of the diploids in which the disruptions were
Table 1 (continued).

constructed: DJ656 (MATa/MATα), DJ787 (MATa/MATα ste2::LEU2/STE2+), DJ656-2 (MATa/MATα ste4::LEU2/STE4+), DJ656-3 (MATa/MATα ste12::LEU2/STE12+), DJ656-4 (MATa/MATα ste18::LEU2/STE18+).

d. The strain 381GUL (MATa ste4::LEU2) was kindly provided by Gary Cole.

e. The diploid strain DJ783 (MATa/MATα ste4-3ts/ste4-3ts) transformed with the various plasmids, was used to derive haploid strains for mating experiments.
Table 2. Plasmid Loss Experiments<sup>a</sup>.

<table>
<thead>
<tr>
<th>Strains&lt;sup&gt;b&lt;/sup&gt;</th>
<th>% of Plasmid Remaining&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt; STE&lt;sup&gt;+&lt;/sup&gt;</td>
<td>YEpSTE5 46±10</td>
</tr>
<tr>
<td>MATα STE&lt;sup&gt;+&lt;/sup&gt;</td>
<td>YEpSTE5&lt;sup&gt;Hpl&lt;/sup&gt; 2±1</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt; ste2-10::LEU2</td>
<td>40±10</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt; ste4::LEU2</td>
<td>69±4</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt; ste4::LEU2</td>
<td>34±17</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt; ste12::LEU2</td>
<td>35±15</td>
</tr>
<tr>
<td>MATα ste12::LEU2</td>
<td>35±13</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt; ste18::LEU2</td>
<td>75±4</td>
</tr>
<tr>
<td>MATα ste18::LEU2</td>
<td>80±5</td>
</tr>
<tr>
<td>MAT&lt;sup&gt;a&lt;/sup&gt;/MATα STE&lt;sup&gt;+&lt;/sup&gt;/STE&lt;sup&gt;+&lt;/sup&gt;</td>
<td>60±30</td>
</tr>
</tbody>
</table>

<sup>a</sup> Plasmid loss experiments were done as described in Materials and Methods.

<sup>b</sup> Diploids disrupted for the STE gene indicated were transformed with either the YEpSTE5<sup>Hpl</sup> or the YEpSTE5 plasmid. Tetrads from these diploid strains in which the disruption segregated 2 (Leu<sup>+</sup>): 2 (Leu<sup>-</sup>), and plasmid segregated 4 (Ura<sup>+</sup>): 0 (Ura<sup>-</sup>) were used in plasmid loss experiments. In the case of ste2::LEU2 deletion, only MAT<sup>a</sup> strains were used. Diploid strains were: DJ656 (MAT<sup>a</sup>/MATα), DJ787 (MAT<sup>a</sup>/MATα ste2::LEU2/STE2<sup>+</sup>), DJ656-2 (MAT<sup>a</sup>/MATα.
Table 2 (continued).

ste4::LEU2/STE4+), DJ656-3 (MATa/MATα ste12::LEU2/STE12+), DJ656-4 (MATa/MATα ste18::LEU2/STE18+). All four strains in a tetrad were tested for plasmid loss at least twice.

c. Numbers represent mean of at least two experiments with two independent segregants from the same diploid transformant. Standard deviation was calculated for each set of data.
Table 3. Plasmid Loss from ste4 and ste4 sst2 Strains.

<table>
<thead>
<tr>
<th>Strains</th>
<th>% of Plasmid Remaining&lt;sup&gt;b&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>ste4::LEU2</td>
<td>37±2</td>
</tr>
<tr>
<td>ste4::LEU2 sst2-1</td>
<td>36±6</td>
</tr>
</tbody>
</table>

<sup>a</sup> Strains used in these experiments were MATa ste4::LEU2 (DJ656-2-6-2; DJ656-2-10-1) and MATa ste4::LEU2 sst2-1 (DJ789-2-2; DJ789-3-1; DJ789-5-2; DJ789-5-3).

<sup>b</sup> The percent of the plasmid remaining was calculated as described in Materials and Methods. The numbers obtained for several isolates were averaged and standard deviation was determined.
Table 4. Strain List.

<table>
<thead>
<tr>
<th>Strains(^a)</th>
<th>Genotype(^b)</th>
</tr>
</thead>
<tbody>
<tr>
<td>381G</td>
<td>MAT(\alpha) cry1 ade2-1 his4-580 lys2 trp1 tyr1 SUP4-3(^{ts})</td>
</tr>
<tr>
<td>EMS63</td>
<td>MAT(\alpha) his2</td>
</tr>
<tr>
<td>3224-4</td>
<td>MAT(\alpha) his2</td>
</tr>
<tr>
<td>DJ676-2-2</td>
<td>381G MAT(\alpha) sir3-5(^{ts}) leu2 ura3 ade3 TYR1</td>
</tr>
<tr>
<td>DJ603-136</td>
<td>381G MAT(\alpha) leu2 ura3 ade3 TYR1 with pDJ117</td>
</tr>
<tr>
<td>DJ788-7-1</td>
<td>381G MAT(\alpha) sir3-5(^{ts}) leu2 ura3 sst2-1 TYR1</td>
</tr>
<tr>
<td>DJ656</td>
<td>381G MAT(\alpha)/MAT(\alpha) cry1/CRY1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/tyr1</td>
</tr>
<tr>
<td>DJ656-2</td>
<td>381G MAT(\alpha)/MAT(\alpha) cry1/CRY1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/tyr1 ste4::LEU2/STE4</td>
</tr>
<tr>
<td>DJ656-3</td>
<td>381G MAT(\alpha)/MAT(\alpha) cry1/CRY1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/tyr1 ste12::LEU2/STE12</td>
</tr>
<tr>
<td>DJ656-4</td>
<td>381G MAT(\alpha)/MAT(\alpha) cry1/CRY1 leu2/leu2 ura3/ura3 ade3/ade3 TYR1/tyr1 ste18::LEU2/STE18</td>
</tr>
<tr>
<td>DJ787</td>
<td>381G MAT(\alpha)/MAT(\alpha) cry1/CRY1 leu2/leu2 ura3/ura3 ADE3/ade3 TYR1/tyr1 ste2::LEU2/STE2</td>
</tr>
<tr>
<td>DJ783</td>
<td>381G MAT(\alpha)/MAT(\alpha) cry1/CRY1 leu2/LEU2 ura3/ura3 TYR1/tyr1 ste4-3(^{ts})/ste4-3(^{ts})</td>
</tr>
<tr>
<td>DJ656-2-6-2</td>
<td>381G MAT(\alpha) leu2 ura3 TYR1 ste4::LEU2 YEpSTE5(^{Hpl})</td>
</tr>
<tr>
<td>DJ656-2-10-1</td>
<td>381G MAT(\alpha) leu2 ura3 TYR1 ste4::LEU2 YEpSTE5(^{Hpl})</td>
</tr>
<tr>
<td>DJ789-2-2</td>
<td>381G MAT(\alpha) leu2 ura3 sst2 ste4::LEU2 YEpSTE5(^{Hpl})</td>
</tr>
</tbody>
</table>
DJ789-3-1  381G MATa leu2 ura3 sst2 ste4::LEU2 YEpSTES5Hp1  
DJ789-5-2  381G MATa leu2 ura3 sst2 ste4::LEU2 YEpSTES5Hp1  
DJ789-5-3  381G MATa leu2 ura3 sst2 ste4::LEU2 YEpSTES5Hp1

a. All strains are congeneric to strain 381G (Hartwell, 1980) except EMS63 and 3224-4 which were derived from strain S288c.
b. The cryl and CRY1 alleles determine the resistance and sensitivity to the fungicide cryptopleurine. The ste2::LEU2 is a genetic substitution of the entire STE2 gene. The ste4::LEU2, ste12::LEU2 and ste18::LEU2 disruptions were constructed as described in the Materials and Methods. The temperature-sensitive allele ste4-3ts (Hartwell, 1980) lead to sterility and α-factor resistance at the nonpermissive temperature (34°C). The sir3ts mutation was originally designated ste8-5 (Hartwell, 1980).
DISCUSSION

The pheromone response pathway of *Saccharomyces cerevisiae* was used as a model system for understanding mechanisms of signal transduction. A number of similarities exist between yeast and mammalian signal transduction systems. A class of mammalian receptors including the β-adrenergic receptor and rhodopsin possesses seven membrane spanning domains. These receptors respond to an extracellular stimulus and they are coupled to a post-receptor G protein. The α-factor receptor is a member of this class. The ability of signal transduction systems to adapt to the continuous presence of a signal is also common; although, the mechanisms for adaptation appear to vary among the different systems. The pheromone response pathway was used as a genetic model for signal transduction and adaptation in light of the simplicity in laboratory manipulations and advanced genetic analysis that are available for yeast.

Genetic dissection of the pheromone pathway was used to identify elements that control signal transduction and adaptation. Two types of elements were considered: positive regulatory elements, which promote the response when pheromone is present, and negative regulatory elements which inhibit the response when pheromone is absent. Mutations which cause a recessive, pheromone-
unresponsive phenotype presumably affect positive elements in the pathway (Hartwell, 1980). Chapter I of this thesis describes the isolation and analysis of the constitutive mutants in the pheromone response pathway. Two types of the constitutive mutations were identified: recessive mutations in a negative regulatory element, $SCG1$, and a dominant mutation in a positive regulatory element, $STE4$. The $SCG1$ gene, which appears to be a negative element in the pathway, was identified previously as a multicopy suppressor of the $sst2$ mutation as well as a yeast homolog of the $\alpha$ subunit of a mammalian G protein (Dietzel and Kurjan, 1987; Miyajima et al, 1987). The $STE4$ gene product, a positive element of the pathway, was identified as a yeast homolog of the mammalian G protein, $\beta$ subunit (Whiteway et al, 1989). As predicted, the search for constitutive mutants identified both types of elements that affect the pheromone response pathway.

Analysis of the double mutants presented in Chapter II suggested the order in which positive and negative elements affect signal transduction. The current model is that, in the presence of $\alpha$-factor, the $\alpha$-factor receptor somehow negatively regulates the $SCG1$ gene product which then allows the $STE4$ gene product to activate the pathway through the $STE5$ product. This simple model is based on the assumption that these elements act in a dependent linear pathway. The alternative possibility of the two independent parallel pathways is difficult to reconcile with the isolation of both constitutive and unresponsive mutants (see Chapter II).
The recovery of cells from constitutive arrest was analyzed in Chapter III. The temperature-sensitive mutation, *ste5-3ts* suppressed the constitutive phenotype of mutations in *SCG1* and *STE4*. The double mutants grew normally at 34°C but showed growth arrest when shifted at 22°C. The arrest at 22°C was transient, since the double mutants recovered from the arrest and resumed mitotic growth. The effect of *sst2*, *bar1*, or *ste2* deletion mutations on recovery was determined. The *scg1-7 ste2 ste5-3ts* and *scg1-7 bar1 ste5-3ts* triple mutants were able to recover from the cell-cycle arrest. It was concluded that the *STE2* and *BAR1* gene products are unnecessary for recovery of the *scg1-7* mutant cells. In contrast, the *scg1-7 sst2 ste5-3ts* triple mutant showed a partial block in recovery which suggests both *SST2*-dependent and *SST2*-independent recovery mechanisms operate on the *scg1-7* mutants. Both recovery mechanisms apparently require the *SCG1* product, since *scg1*-null mutants do not recover (J. Hirschman and D. Jenness, unpublished observations; Miyajima et al., 1988). The *STE4Hpl* mutant recovered by an *SST2*-dependent mechanism, since *STE4Hpl sst2 ste5-3ts* triple mutants did not recover from the constitutive arrest. This recovery was independent of the *STE2* receptor (data not shown). The *SST2* gene product is, therefore, thought to act at or after the *STE4* step, probably by affecting the *STE4* gene product (see Figure 1).

The isolation of constitutive alleles of *STE5* allowed analysis of double mutants containing *STE5Hpl* and other *ste* mutations (Chapter
IV). The **STE5** gene product was found to act after the **STE4** controlled step but before **STE12** step in the pheromone pathway. The constitutive allele of **STE5** was able to suppress the sterility phenotype of mutations in **STE2**, **STE4**, and **STE18** genes, but failed to suppress a mutation in **STE12**. However, presence of the **STE4** and **STE18** gene products somehow enhanced the effect of the **STE5**\textsuperscript{Hpl} mutation, even though, **STE5**\textsuperscript{Hpl} gene product could bypass the mating deficiency of the **ste4** and **ste18** mutations.

A "noise" model is proposed that would explain the above observations. In the absence of pheromones there is, presumably, a basal level of constitutive signal which is amplified at several postreceptor steps in the pathway, e.g., dissociation of the **SCG1** and the **STE4/STE18** gene products into free $\alpha$ and $\beta\gamma$ subunits may account for such "noise". The **STE5**\textsuperscript{Hpl} mutation may lead to the production of "noise" in addition to amplifying the "noise" which originates from preceding steps in the pathway. This would explain the **STE5**\textsuperscript{Hpl} suppression of the mutations in **STE2**, **STE4**, and **STE18** genes as well as its partial dependence on the **STE4/STE18** gene products.

Several elements of the pheromone pathway possess structural and sequence homologies to the known signal transduction elements. The **STE2** receptor is predicted to possess seven hydrophobic regions each capable of spanning the plasma membrane (Burkholder and Hartwell, 1985; Nakayama et al, 1985). The **SCG1**, **STE4**, and **STE18** genes show homology to the mammalian G protein $\alpha$, $\beta$, and $\gamma$
subunits (Dietzel and Kurjan, 1987a; Miyajima et al, 1987; Jahng et al, 1988; Whiteway et al, 1989), respectively, whereas, the SST2 and STE5 genes do not show apparent homology to any known element at the present time (Dietzel and Kurjan, 1987b; M. Hasson, unpublished observations). Based on these structural and sequence similarities, a model for the pheromone response pathway is proposed (Figure 1).

Binding of pheromones trigger the activation of the pheromone response in yeast cells. The α-factor pheromone binds to the specific receptor, the STE2 gene product, on the surface of a cells (Jenness et al, 1983; 1986; Blumer et al, 1988; Marsh and Herskowitz, 1988)). Upon binding the pheromone-receptor complexes are presumed to be internalized and degraded (Jenness and Spatrick, 1986; Chvatchko et al, 1986). This binding may also cause dissociation of the α and βγ subunits of the G protein as a result of the α subunit undergoing simultaneous GDP to GTP exchange. The free βγ subunits would then activate the pathway through STE5 gene product to cause arrest of the cell division at "START".

Several recovery mechanisms are induced upon exposure to the α-factor. Expression of the Bar1 protease is increased (Kronstad et al, 1987) as well as increased transcription of the SCG1 (Jahng et al, 1988) and the SST2 gene products (Dietzel and Kurjan, 1987b). All of these elements have been implicated in recovery of a cells from the division arrest (Chan and Otte, 1982a, b; Miyajima et al, 1989; Dietzel and Kurjan, 1987b). The induction of at least some recovery mechanisms must occur after the STE5 step in the pathway.
Activation of recovery mechanisms before the STE5 step would have enabled scgl ste5-3ts and STE4Hpl ste5-3ts double mutants to recover at 34°C, and would have resulted in normal growth after the shift to 22°C (Chapter III). Since that was not the case, the activation of recovery depended on the completion of the STE5 step.

The site of action for various recovery mechanisms is poorly defined with exception of the BARI product, which degrades α-factor in the medium (Sprague and Herskowitz, 1982). The SST2 gene product is likely to act at the G protein step, possibly by promoting formation of the inactive, GDP-bound, complex. The supersensitivity of the sst2 mutant may result from an excess of free βγ subunits after the exposure to pheromones. This possibility is supported by several arguments. First, the sst2 supersensitive mutation was suppressed by overexpression of SCG1 gene product (the α subunit) which could have tilted the equilibrium of the G protein subunits toward the formation of the inactive (αβγ) complex. Second, the dependence of the STE4Hpl mutant on the SST2 gene product for recovery indicated that the SST2 product negatively regulates the STE4 product or a subsequent step. Third, the affect of the sst2 mutation on the STE5Hpl mutation was suppressed in the ste4 disruption strain suggesting that SST2 gene product requires STE4 product to elicit its effect in the STE5Hpl mutant. Finally, the sst2 mutation can suppress temperature-sensitive, unresponsive alleles of STE4, (ste4-3, ste4-5, and ste4-6) in combination with either ros1, 2 or 3 mutations, however, sst2 has no effect on ste5-3 allele
suggesting that SST2 product may affect STE4 but not STE5 step in the pathway (Jenness et al., 1987). Together these arguments seem to suggest that SST2 product acts at the STE4 step of the pathway possibly by promoting formation of the inactive complex with the SCG1 gene product.

The SST2-independent mechanism probably affects the pathway before the STE4 step, by regulating the SCG1 gene product. This argument is supported by the observation that STE4Hpl sst2 ste5-3ts triple mutant was unable to recover by an SST2-independent pathway, implying that SST2-independent recovery must act before the STE4-controlled step. In addition, the scg1-7 mutant was able to recover in the absence of the SST2 function, however, the scg1-null mutant did not recover. These results suggest that an SST2-independent recovery mechanism can act in the scg1-7 mutant but that recovery is dependent on the presence of the SCG1 gene product. Consistent with that is the finding that scg1-7 mutation gives rise to a full size protein product (Blumer and Thorner, 1990). It is, therefore, conceivable that the SCG1 gene product is the target of the SST2-independent recovery mechanism. This mechanism might act either by modifying the SCG1 gene product or simply by promoting its synthesis. Both of these possibilities should result in shift of equilibrium toward the formation of inactive (αβγ) G protein complex (Figure 1).

In summary, two postreceptor adaptation mechanisms operate in cells at the level of the G protein. The SST2-dependent pathway
probably targets the *STE4* gene product and an *SST2*-independent mechanism acts at the *SCGl* step. The combination of these two adaptation pathways allows cells to fine-tune their response with respect to the duration and concentration of the stimulus.

Two other recovery mechanisms were proposed to attenuate the \( \alpha \)-factor induced signal (Konopka et al., 1988; Reneke et al., 1988; Miyajima et al., 1989). The carboxy-terminal truncations of the \( \alpha \)-factor receptor result in increased sensitivity to \( \alpha \)-factor (Konopka et al., 1988; Reneke et al., 1988). The combination of the C-terminal truncation with the *sst2* mutation has an even stronger effect than either one of these mutations alone. It is thought, therefore, that the C-terminus of the receptor is responsible for some aspect of cellular adaptation to pheromone that is distinct from adaptation controlled by the *SST2* gene product. Another recovery mechanism is thought to be activated in the *SCGl*Val-50 mutant (Miyajima et al., 1989).

Mutations that substitute valine for glycine at amino acid position 12 of mammalian ras proteins cause a decrease in GTPase activity and an increase in transforming activity (Gibbs et al., 1984; Seeburg et al., 1984). A corresponding mutation in the *SCGl* (*GPA1*) protein, *SCGl*Val-50, in which the Gly-50 residue was replaced by valine, causes two phenotypes besides the ability to complement the *SCGl* deletion mutation. Cells expressing *SCGl*Val-50 protein were supersensitive to the \( \alpha \)-factor, but resumed growth after long-term incubation and became insensitive to subsequent higher doses of the pheromone. The former phenotype is recessive and the latter
phenotype is dominant. One possible explanation for the $SCGI^{Val-50}$ supersensitive phenotype is that the mutant protein has reduced intrinsic GTPase activity. This would be analogous to the $ras^{Val-12}$ mutation (Gibbs et al, 1984; Seeburg et al, 1984) which results in increased levels of the GTP-bound form of this protein. The increased levels of GTP bound to the $SCGI$ product would subsequently result in lower levels of inactive (GDP-bound) $\alpha\beta\gamma$ complex, which in turn might result in responsiveness to lower concentrations of pheromone. This phenotype is recessive. The recovery from the arrest and subsequent unresponsiveness to $\alpha$-factor is dominant. It has been suggested that the $SCGI^{Val-50}$ protein causes the activation of the recovery mechanism. This recovery mechanism is only partially dependent on $SST2$ gene product, since the double mutant $SCGI^{Val-50} sst2$ is able to recover from the $\alpha$-factor arrest (Miyajima et al, 1989).

The model which is proposed to explain the mechanism of pheromone responsiveness and adaptation is based on the similarities to the known mammalian signal transduction systems. The interesting implication from this work is that in yeast as opposed to other systems, the $\beta\gamma$ subunits of the G protein activate the response to pheromones. Several examples of $\beta\gamma$ activation exist in the literature. The $\beta\gamma$ complex has been proposed to activate muscarinic potassium channels in the heart (Logothetis et al, 1987); however, the significance of this finding has been debated (Cerbai et al, 1988). The $\beta$ and $\gamma$ subunits of transducin have been reported to
stimulate phospholipase A\textsubscript{2} in rod outer segments (Jelsema and Axelrod, 1987). However, results presented in this thesis do not exclude a possibility that free $\beta$ and $\gamma$ subunits of G protein bind another $\alpha$ subunit of a different G protein and thus cause the activation of the response. Such a mechanism has a precedent in regulation of the adenylate cyclase system (see Gilman, 1987). The exact mechanism(s) for activation of the pheromone response in yeast will not be known until more direct, biochemical experiments are performed. Only then will it be possible to test directly the model presented in this thesis.
Figure 1. Possible Model for Pheromone Response.

The $STE2$ gene encodes the receptor for $\alpha$-factor; the $SCG1$, $STE4$ and $STE18$ genes are thought to encode $\alpha$, $\beta$, and $\gamma$ subunits of a G protein, respectively. In the absence of $\alpha$-factor, the $\alpha$ subunit of G protein in its GDP form binds the $\beta\gamma$ complex and prevents it from stimulating the subsequent events in the response pathway. In the presence of $\alpha$-factor, occupied receptors promote the exchange of GTP for the GDP bound to the $G\alpha$ subunit. The $G\alpha$ subunit dissociates from the $G\beta\gamma$ subunits. This permits the $G\beta\gamma$ complex to stimulate the events controlled by the $STE5$ gene, which then lead to the arrest of cell division. At least two postreceptor recovery mechanisms are activated after the signal passes the $STE5$ step. The $SST2$ gene product probably acts at the $STE4$ step by influencing formation of the $G\alpha\beta\gamma$ inactive complex ($STE4$ inactivation by modification is not excluded). The other, $SST2$-independent, recovery mechanism probably acts before the $STE4$ ($G\beta\gamma$) step, at the $SCG1$ ($G\alpha$) step by some kind of modification or simply by promoting synthesis of more $SCG1$ product. Both of these mechanisms would stimulate reassociation of the $SCG1$ with the $STE4/STE18$ complex and prevent further stimulation of the pathway.

Solid lines indicate activation of the pathway by the $\alpha$-factor, and dashed lines indicate possible mechanisms for recovery from the pheromone induced arrest.
SCG1 synthesis or modification

Arrest of the Cell Division

SCG1

GTP

STE4

STE18

STE5

SST2

SCG1

GDP

STE4

STE18

STE2
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for a functional modification of \textit{RAS} proteins and for production of mating pheromone \(\alpha\)-factor. \textit{Cell} \textbf{47}: 413-422.


