G-PROTEIN MEDIATED SIGNAL TRANSDUCTION IN SACCHAROMYCES CEREOVISIAE

BY

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DECLARATION

The work presented in this report was carried out by me at the Department of Biochemistry, University of Ghana, Legon and at the Department of Biological Chemistry, University of California, Los Angeles, USA under the supervision of Professors F.N. Gyang and John Colicelli.

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SEPTEMBER, 1995
DEDICATION

To my father,

Mr. Avedezi Dzudzor

and

to the memory of my beloved son,

Frederick Kwasi Selom Dzudzor
ACKNOWLEDGEMENTS

I would like to express my sincere gratitude to all who contributed in making this work a success.

I am especially grateful to my supervisors, Prof. F.N. Gyang and Prof. J. Colicelli for their patience, invaluable suggestions and relentless effort in seeing to the successful completion of this work. I am also greatly indebted to Prof. M.E. Addy, Head of Department and a member of the supervisory committee for her criticism, suggestions and always keeping me on my toes. I also wish to thank Drs. R.A. Acquah, N.A. Adamafio, S.K. Gbewornyoh, J.P. Adjimani and Prof. K.K. Oduro, all of the Department of Biochemistry Legon, for their criticisms and support.

A special note of thanks is due Meenakshi Ramakrisman, who did the original suppressor cloning and Brian Spain for the continuous work on YGC1 and MCM1. Both Meena and Brian were students under Prof. J. Colicelli at the Department of Biological Chemistry, UCLA, Los Angels. My sincere thanks also goes to Limin Han, Raji Pillai, both students at Colicell’s laboratory for their support. I also owe a debt of gratitude to Prof. E. Neufield, Head, Department of Biological Chemistry, UCLA and Mrs. Antoinette Green for their support and generosity to me when I was undertaking this research. I thank the entire staff of Biological Chemistry Department, UCLA for their co-operation.
Last, but not the least, I will like to thank Miss Christine Kamasah, my wife for her moral support. I have also not forgotten my colleagues, Charles Brown, Lambert Faabuluom and Ernest Agbovi (a dedicated and devoted friend) for their support. Finally I wish to thank the entire technical staff at the Biochemistry Department for their assistance.

The manuscript was typed by Ms. H.P. Agbesi of ISSER, Legon. I deeply appreciate her help.

I am, however, wholly responsible for any shortcoming with regard to this work.
Yeast mating type locus gene alpha2 (MATα2), Yeast G protein complementing gene (YGC1) and minichromosome maintenance gene (MCM1) have been identified by isolation of plasmids that are able to complement or suppress a *gpa1::HIS3* mutation. MATα2 and YGC1 rescue both MATα and MATα-gpa1::HIS3 haploid cell types whereas MCM1 complements only MATα gpa1::HIS3 cell type. MATα2 is known to be a general repressor and a determinant of both haploid and diploid cell types. MCM1 is known to be a general transcriptional activator. YGC1 has not been characterised, hence its function or mode of action is not previously known.

G protein alpha subunit (GPA1) is a yeast G protein α subunit that negatively controls the budding yeast pheromone signal transduction pathway. Disruption of GPA1 results in constitutive arrest of the signal pathway that leads to cell cycle arrest at the early G1 phase of the cell cycle.

Both Southern analysis and sequencing showed that MATα2, YGC1, MCM1 have no homology to GPA1. Disruption of MATα2 (that is *mata2::URA3*) leads to constitutive arrest of the cell cycle at the G1 phase. MATα2 also has no sequence homology to GPA2, the other G protein α subunit in yeast, known to be involved in cAMP pathway in yeast. It has been shown here that MATα2 rescues *gpa1::HIS3* cells even in single copy, centromere plasmids. Mating efficiency is largely reduced in cells kept alive with MATα2. MATα2 does not have
the pheromone response elements (PREs) common to the STE genes (whose disruption leads to insensitivity to mating factors).

The plasmid TGC was also constructed and used in creation of the yeast haploid strains LG1 and LG2. This was an attempt to screen a mammalian cDNA library for possible analogs of GPA1. These strains were used to isolate two mammalian analogs that complement the gpa1::HIS3 mutation.

The results indicate that MATa2, YGC1 and MCM1 are components or modulate component(s) of the signaling pathway. It also showed that MATa2 is even a more potent negative regulator of the signaling pathway than GPA1, since overexpression is not a prerequisite for negatively regulating the pathway. MATa2 does not belong to the G protein family since it has no GTP/GDP binding and/or exchange domains.
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<td><strong>G protein</strong></td>
<td>Guanine nucleotide binding protein</td>
</tr>
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<td><strong>FUS</strong></td>
<td>Genes whose activation leads to the Fusion of the Cell</td>
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<td><strong>GPA1</strong></td>
<td>Yeast G protein alpha subunit</td>
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<td><strong>MATa2</strong></td>
<td>Mating type locus alpha 2 gene</td>
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<td><strong>YGC</strong></td>
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<td><strong>MCM1</strong></td>
<td>Minichromosome maintenance gene</td>
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<td><strong>STE</strong></td>
<td>Genes whose inactivation leads to sterility of the cell</td>
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<tr>
<td><strong>CDC</strong></td>
<td>Cell division cycle genes</td>
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<tr>
<td><strong>ßME</strong></td>
<td>ß-mecarptoethanol</td>
</tr>
<tr>
<td><strong>SDS</strong></td>
<td>Sodium dodecyl sulfate</td>
</tr>
<tr>
<td><strong>LB</strong></td>
<td>Luria-Bertani medium</td>
</tr>
<tr>
<td><strong>MES</strong></td>
<td>2(-N-morpholino) ethanesulfonic acid</td>
</tr>
<tr>
<td><strong>YPD</strong></td>
<td>Yeast peptone dextrose media</td>
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| **SCG1**          | *Saccharomyces cerevisiae*  
                     | G protein alpha subunit |
CHAPTER ONE

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

The fundamental goal of Molecular Biology is to understand the metabolic processes that govern growth and development, differentiation and diseases in plants and animals. To achieve this end, the enzymatic and structural functions of proteins must be recreated and characterised in vitro. A comprehensive understanding of a protein’s function requires that the gene encoding the protein be cloned for further manipulation and characterization. Cloning a gene allows one to:

(a) Sequence it and determine if the encoded protein (or RNA) contains particular motifs which will help us understand its function.

(b) Use mutagenesis to introduce nucleotides substitutions, insertions or deletions into the gene. The effect of these mutations on the activity of the protein reveal important insights into its mechanism of action.

(c) Express the protein at high levels so that it can be purified and characterised further to;

(i) Determine its structure by X-ray crystallography or Two-Dimensional Nuclear Magnetic Resonance (2-D NMR).

(ii) Use as a therapeutic agent to cure or control a particular disease brought about by defective gene and hence gene product.
iii) Perform extensive biochemical studies.

Molecular biologists employ plasmids as vectors for numerous purposes, hence construction of plasmid vectors is important tasks for the geneticist. Construction of plasmid vectors has involved the incorporation of ancillary sequences that are used for a variety of purposes, including visual identification of recombinant clones by histochemical tests, generation of single-stranded DNA templates for DNA sequencing, transcription of foreign DNA sequences in vitro, direct selection of recombinant clones, and expression of large amounts of foreign proteins. Bacteria contain certain mechanisms to control the copy number of the plasmid to a level that affords them protection from the antibiotic but not at the expense of cellular functions. The control of plasmid copy number resides in a region of the plasmid DNA that includes the origin of DNA replication. For example, (2|jm) and centromere (CEN) gene plasmids are multi and single copy plasmids respectively which were constructed and used in this work.

All biological systems have the ability to process and respond to enormous amount of information. Much of this information is provided to individual cells in the form of changes in concentration of hormones, growth factors, neuromodulators, or other molecules. These ligands interact with transmembrane receptors, and this binding event is transduced into an intracellular signal. Several families of cell surface receptors and ligands that are coupled to different mechanisms of signal transduction have been characterized.
Response to pheromones during the process of yeast mating provides an opportunity to study signal transcription in a unicellular eukaryote. Haploid a and a cells of the budding yeast, *Saccharomyces cerevisiae*, are able to grow vegetatively or can mate to form a diploid a/a cell. The process of mating is mediated by extracellular peptide mating pheromones and integral membrane protein receptors. This programme of signaling and response leads to cellular differentiation in preparation for mating, which is manifested by transcriptional induction of numerous genes, by morphological changes, and by arrest of the cell cycle in the G1 phase. The study of the cell cycle or its mutants has increased the understanding of how individual cell cycle steps (such as DNA synthesis and mitosis) are coordinated so that the events occur in the right order. The analysis of cell cycle mutants has also revealed how cells maintain a constant average size over many cell divisions. This size regulation requires that the continuous events of the cell cycle collectively referred to as cell growth, are coordinated with the cycle of stepwise events that includes DNA synthesis, centrosome duplication, and mitosis. If there is no coordination between growth and the stepwise events, the average cell size can only be maintained if the doubling time for cell mass is exactly equal to the length of the cycle of stepwise events (Murray and Kirschner, 1989).

This signal transduction pathway leading eventually to the arrest of the cell cycle at the G1 phase is very crucial in our understanding of cellular division and growth control, because we
sometimes think of tumor cells as uniform, completely undifferentiated and fast growing but this is not really true. There are in fact many kinds of tumors arising from many tissues and they may retain some of the characteristics from their tissue of origin. In addition, they need not grow at a rapid rate. They have simply exited from a no growth (cell cycle arrest) or controlled growth (i.e stem cell) state or they have escaped controlled cell death (apoptosis). In other words, tumor grows because fewer cells exit the cell cycle, whereas in normal tissue, fewer cells are cycling; more cells exit the cell cycle. In cancers, a great number of progeny cells continue to cycle, because they have lost the growth control mechanisms. In the yeast Saccharomyces cerevisiae, cells of a mating type produce α-factor and respond to α-factor, and cells of a-mating type produce α-factor and respond to α-factor. Because the ability to produce mating factors and respond to them is required for mating, it has been possible to identify many of the genes and proteins that play roles in this signaling process by isolation of mutants that are defective in mating. Attention has been given to the negative growth factors such as TGF-β (Moses et al., 1990) that trigger differentiation and cell-cycle arrest. Thus the yeast signaling pathway provides an experimental model to study pathways in mammalian signaling systems with the techniques of manipulative molecular genetics. For example, some of the components of the yeast pathway are also found in mammalian signaling systems – G proteins with their distinctive receptors that have seven
membrane-spanning regions as well as several serine/threonine protein kinases. For yeast, we now outline the complete pathway beginning with ligand binding to the receptor at the cell surface and culminating in events within and affecting the nucleus, that is differentiation and cell-cycle arrest.

An important backdrop for the research studies described here is the extensive knowledge of the molecular basis for cell specialization in yeast. a and a cells produce different receptors and different mating factors. a/a cells lack these specialized products and others involved in response (and several more). These differences result from cell-type-specific regulation of gene expression by identified transcriptional regulatory proteins and have been the subject of several reviews (Herskowitz, 1989; Dolan & Fields, 1990). Other reviews focus on aspects of the signaling system (Kurjan, 1990; Dohlman et al., 1991; Marsh, 1991) and the mating process itself (Cross et al., 1988).

The purpose of this literature review is to describe the yeast signal transduction pathway at our present state of knowledge indicating both what is known and what is less certain or unknown. Beginning with an overview of our current view of signaling in the pheromone response pathway of yeast, a brief mention is made of responses and assays for signaling. These are followed by detailed description of receptors, the G protein, other signal transduction components, MATa2 and MCM1 genes all of which are involved in the pathway. Mention is also made of transcriptional activation and
genetic and/or biochemical evidence for the specific roles of the above mentioned genes or gene products when known. Description of how the pathway culminates in cell-cycle arrest concludes the literature review.
1.2 LITERATURE REVIEW

Overview of Pheromone Response

Pheromone response in yeast starts with extracellular peptide mating factors (α-factor and α-factor) binding to integral membrane protein receptors, and cells of α-mating type produce an α-factor receptor. Both receptors are coupled to the same heterotrimeric G protein, Gaβγ. The α subunit has GDP bound in one state and GTP bound in the other state. Stimulation of the receptor causes a switch to the GTP-bound state of Ga, which leads to release of the βγ subunit of the G protein. Gaβγ then activates downstream components of the signaling pathway (refer to figure 1). Proteins required for further signaling include several serine/threonine protein kinases and other products. Ultimately a transcriptional activator, STE12, is activated, which leads to differentiation, that is, increased transcription of several genes (figure 1) including those encoding cell-surface proteins involved in cell-cell interaction and fusion (figure 1). G1 cyclins are inactivated, leading to cell cycle arrest. The signaling pathway is similar in α and α cells except for the receptors.

1.2.2 Responses and Assays for Signaling

The ability to mate exemplified by the formation of zygotes or prototrophic colonies (Sprague, 1991), serves as one assay for ability to carry out signal transduction in response to pheromones,
since production of mating pheromones and the ability to respond to them are essential for mating. There are also several single assays for different steps in the mating process. In particular, haploid cells respond to purified or synthetic pheromones of the opposite mating type. Both a cells and a cells arrest in the G1 phase of the cell cycle as unbudded cells, undergo morphological changes (from an ovoid cell to a pear-shaped shmoo (figure 2), and exhibit transcriptional induction of several genes (Cross et al., 1988). The $FUS1$ gene (or a $FUS1$-lacZ hybrid gene) provides a particularly convenient assay for this process since its expression is increased several hundredfold by mating factors (Trueheart et al., 1987). Several different assays for pheromone production or response examine growth arrest (Sprague, 1991). One of these is the zone-of-inhibition assay in which the ability of a purified mating factor to inhibit growth when spotted on a lawn of test cells is determined. Another assay involves formation of cells of aberrant morphology in response to α-factor (shmoo formation). Both zone-of-inhibition and aberrant morphology assays are used in this study.

Many of the genes involved in the pheromone response pathway were identified because mutations in these genes confer resistance to the growth-inhibitory effect of the mating factors.
Figure 1: Differentiation and cell cycle arrest in response to pheromone.

Binding of pheromone (hatched circle) alters the conformation of the receptor. As a result of an attendant conformational change Ga·GTP replaces GDP, and Gαγ is released. Activated Gαγ then initiates a signal that passes through STE5 to four protein kinases (STE11, STE7, FUS3 and KSS1). The transcriptional activator STE12 is rapidly phosphorylated and transcription of target genes is stimulated. Some target genes are directly involved in differentiation (FUS1, KAR3), others in cell arrest (FUS3, FAR1). As a result of the action of FUS3, FAR1, and other hypothetical protein(s) (X), CLN products do not accumulate and cell cycle arrest in G1 ensues. Arrow heads indicate stimulation and terminal bars inhibition of the signal. The parallel lines represent the plasma membrane, the serpentine line represents a pheromone receptor and the three boxes represent the α, β and γ subunits of the G protein.
Binding of mating factors to cognate receptors induces cellular and morphological differentiation. As a result, the cells mate and form an a/α zygote. The arrow heads show the cycle of haploid and diploid cell types formation. Double circles indicate the budding of yeast and the ovoid shows the shmoo formation in response to the mating signal.

### 1.2.3 The \( \alpha \)-Factor Receptor

\( \alpha \)-Factor is an unmodified peptide of thirteen amino acid residues (Duntze \textit{et al}., 1970) and is necessary for mating by \( \alpha \) cells (Kurjan, 1985). It activates \( \alpha \) cells via the \( \alpha \)-factor receptor encoded by the \textit{STE2} gene (Nakayama \textit{et al}., 1985) and is degraded by a specific extracellular peptidase encoded by the \textit{BAR1} gene (Sprague & Herskowitz, 1981). Both of these genes are expressed only in \( \alpha \) cells (Kronstad \textit{et al}., 1987). Mutations in \textit{STE2} affect pheromone response only of \( \alpha \) cells. The main structural features of the receptor are seven hydrophobic domains that are thought to span the membrane, leaving the N-terminus outside the cell and the C-terminus inside the cell (Nakayama \textit{et al}., 1985). A diverse family of integral membrane protein receptors coupled to G protein has seven hydrophobic, potential membrane-spanning domains (Ross, 1989; Dohlman \textit{et al}., 1991). Supporting the notion that these hydrophobic domains are important for \( \alpha \)-factor receptor function, their size and position is conserved in the \textit{Saccharomyces kluyveri} \( \alpha \)-factor receptor, which is only 50% identical to the S.
*cerevisiae* product (Marsh & Herskowitz, 1988). Despite structural conservation, the α-factor receptor has no obvious sequence identity with known mammalian receptors.

α-Factor binds specifically to a mating-type cells with an equilibrium dissociation constant of $6 \times 10^{-3}$ (Jenness et al., 1986) to $2 \times 10^{-8}$ (Raths et al., 1988). The receptor is localized to the cell surface as shown by indirect immunofluorescence of a STE2-lacZ fusion protein that retains receptor activity (Marsh & Herskowitz, 1988). Genetic evidence shows that a cells have only one receptor gene, and Scatchard analysis indicates that all detected receptors have a single affinity (Marsh et al., 1991). There are roughly $10^4$ binding sites per cell, as determined by binding of $^{35}$S- or $^3$H-labelled α-factor, or by binding competition studies (Raths et al., 1988). Temperature-sensitive mutations in the STE2 gene lead to temperature-sensitive binding of α-factor (Jenness et al., 1983). The α-factor has also been cross-linked to the STE2 gene product, albeit with low efficiency (Blumer et al., 1988).

α-factor is internalized and degraded by a cells in a process that is dependent on the presence of the α-factor receptor (Jenness & Spatrick, 1986). Surface α-factor binding sites are also down-regulated after exposure to α-factor in a process that does not require G proteins (Jenness & Spatrick, 1986). The receptor is the only protein in yeast currently known to be subject to specific endocytosis (Marsh et al., 1991).
1.2.4 **The α-Factor Receptor**

α-Factor is a farnesylated and carboxy-methylated peptide of twelve amino acid residues, unrelated to α-factor (Xue et al., 1989; Schafer et al., 1990) and is necessary for mating by α cells (Michaelis & Herskowitz, 1988). Its synthesis and secretion follow a strikingly different route from α-factor (Kuchler et al., 1989). The α-factor receptor is encoded by STE3 and expressed only in α cells (Nakayama et al., 1985). Mutations in STE3 block mating and mating-factor response only in α cells. Binding studies have not been performed with α-factor since it is hydrophobic and exhibits a high level of nonspecific binding. Synthetic α-factor is active in inducing cellular responses at nanomolar concentrations, thus suggesting that affinity of the α-factor receptor for its ligand is in the same range as that of the α-factor receptor for α-factor (Xue et al., 1989).

The STE3 gene encodes a hydrophobic protein with a predicted molecular weight of 54kd (Nakayama et al., 1985). The α-factor receptor, like the α-factor receptor, has seven potential membrane-spanning domains and a long hydrophilic carboxyl-terminus. A variety of experimental data are consistent with a receptor topology similar to that of α-factor receptor (Clark et al., 1988). Despite the potentially similar structure, the α-factor and α-factor receptors have very little amino acid sequence homology; some similarity is however observed between position 222-268 in STE2 and 117-163 in STE3 (Marsh et al., 1991).
1.2.5 Receptor Structure and Function

A working model for the α-factor receptor and other G protein-coupled receptors is that they consist of a central core made up of a bundle of seven membrane-spanning helices that contacts ligand towards its outer face and G protein on its inner face (Dohlman et al., 1991). Ligand specificity determinants for distinguishing *S. cerevisiae* and *S. kluyveri* α-factors, as revealed by studies with receptor hybrids, lie in the central region that includes receptor hydrophobic domains (Marsh et al., 1991). Studies of receptor mutants show that residues that control receptor activation also lie in hydrophobic domains. These studies suggest that the α-factor peptide may activate the receptor in a manner analogous to the activation of the β-adrenergic receptor by epinephrine, where it has been shown that ligand binding and receptor activation involve the membrane-spanning domains (Ross, 1989). Most of the large hydrophilic, carboxy-terminal domain of the α-factor receptor is not required for ligand binding or G protein activation. Cells lacking this domain are hypersensitive to α-factor, which suggests a role in desensitization (Reneke et al., 1988; Konopka et al., 1988). Some residues immediately following the seventh hydrophobic domain (beyond residue 295) may be required for signaling, since truncation at this point reduces mating efficiency (Marsh et al., 1991).

1.2.6 Receptors Control the Ability to Respond to Specific Factors

In theory, the ability of each mating type cell to respond
only to the pheromone of the opposite cell type could be determined
in at least two ways: the receptor for a given pheromone could be
expressed only by the appropriate cell type, or receptors for both
types of pheromone could be expressed, in which case the necessary
coupling proteins could be cell-type specific. Yeast uses the
first scheme - each cell transcribes only one receptor gene.
Further analysis demonstrates that both receptors are coupled to
the same intracellular machinery and downstream responses. An a
cell engineered to produce the a-factor receptor instead of the
usual a-factor receptor responds to a-factor- instead of a-factor
(Nakayama et al., 1987; Bender & Sprague, 1989). An a cell
engineered to produce the a-factor receptor undergoes autocrine
arrest (Nakayama et al., 1987).

Other receptors expressed in cells of S. cerevisiae are also
able to function. S. kluyveri is a yeast with a mating system
resembling that of S. cerevisiae. Each of these yeasts responds
to its own a-factor than to the heterologous a-factor
(McCullough & Herskowitz, 1979). When STE2 in S. cerevisiae was
replaced with the STE2 homologue from S. kluyveri, the resulting
strain responded preferentially to S. kluyveri a-factor (Marsh &
Henskowitz, 1988), thereby demonstrating that the STE2 protein is
the specificity determinant for a-factor. The ability of the S.
kluyveri receptor to function in S. cerevisiae indicates that the
S. cerevisiae G protein is compatible with the receptor from S.
kluyveri.
Mammalian receptors can also function in yeast. A β-adrenergic receptor expressed in yeast displayed the binding characteristics of the receptor found in mammalian cells (King et al., 1990). Agonist-induced activation of the yeast pheromone response pathway (measured by induction of FUS1-lacZ expression) required co-expression of mammalian GqS. The yeast system may prove useful for genetic analysis of mammalian receptors, since mutant receptors with altered signaling properties are easily identifiable by plate assay.

1.2.7 Yeast G Protein

The α-factor and α-factor receptors appear to be coupled to a single heterotrimeric yeast G protein (guanine nucleotide-binding protein) present in both cell types (Kurjan, 1990). In the mammalian systems that have been studied, activation of receptor leads to replacement of GDP with GTP on the α subunit of G protein and separation of Gq subunit from the Gqγ subunit (Stryer & Bourne, 1986; Kaziro et al., 1991). A similar coupling process is thought to occur in yeast, although biochemical studies have not yet confirmed it in yeast. In budding yeast, unlike some other well-studied mammalian systems, it is the free Gqγ subunit rather than the Ga subunit that is responsible for activating signaling targets (Dietzel & Kurjan, 1987; Whiteway et al., 1987). Activation of phospholipase A2 by Gqγ has been reported to occur in bovine rod outer segments (Jelsema & Axelrod, 1987). Also Gqγ derived from Gs, Gi and Go stimulates protein kinase dependent phosphorylation of
both muscarinic acetylcholine receptor and rhodopsin (Haga & Haga, 1992).

Association of receptor and G protein increases the ligand affinity of many G protein-coupled receptors (Stryer & Bourne, 1986). The yeast α-factor receptor apparently behaves similarly: alteration in affinity of the α-factor receptor for its ligand therefore provides an assay for interactions between the G protein and receptor. Mutants defective in Gᵦ or Gᵧ show reduced α-factor binding (Jenness et al., 1987; Blumer & Thorner, 1990) which suggest that Gᵦᵧ is necessary for proper association of Gᵦ with the receptor. Yeast membranes exposed to the non-hydrolyzable GTP analogue, GTPγS, which locks G protein into the GTP-bound state, have a ninefold lower affinity for α-factor (Blumer & Thorner, 1990). This observation supports the model that in yeast, as in mammalian cells, the receptor is not associated with the GTP bound form of G protein.

1.2.8 Ga Subunit of Yeast G protein
Yeast Gᵦ genes were identified by cross-hybridization, to a rat Gᵦi DNA probe (Nakafuku et al., 1987, 1988) and by selecting for genes whose overexpression confers resistance to mating factors (Dietzel & Kurjan, 1987). One of the Gᵦ genes of yeast, GPA1 (also known as SCG1), is involved in mating factor response (Miyajima et al., 1987). On the other hand, genetic and biochemical analyses have suggested that GPA2, the other gene coding for Gᵦ may participate in regulation of the intracellular levels of cAMP. The first clue
for solving the function of GPA1 in yeast cells was obtained by the analysis of its expression. Northern blot analysis indicated that GPA1 was expressed only in haploid cells (Miyajima et al., 1987), whereas GPA2 was expressed both in haploid and diploid cells (Nakafuku et al., 1988). Later, it was found that the level of GPA1 transcript was increased several fold in response to mating factors as in the case of other haploid-specific genes (Jahng et al., 1988). The apparent differences of the expression pattern of the GPA1 and GPA2 genes strongly suggest differential function for these two genes. GPA1 (SCG1) gene product would be referred to as Ga in this study.

The yeast Ga subunit contains 472 amino acid residues and is 45% identical to rat Gai (Miyajima et al., 1987). Like the mammalian Ga, the yeast protein is membrane associated (Blumer & Thorner, 1990) and has also been shown to be myristoylated (Marsh et al., 1991).

The regions of strongest identity between the GPA1 (SCG1) product and other Ga subunits include the guanine-nucleotide-binding consensus region and GTP-hydrolysis region. Similarity in other regions is also generally high, although the yeast protein has 110 extra amino acid residues (126-235) not found in the mammalian Gα subunits (Marsh et al., 1991). The role, if any of this extra domain is not known.

Deletion of the GPA1(SCG1) gene results in constitutive activation of all mating pheromone responses: induction of FUS1-lacZ and cell-cycle arrest is observed in the absence of pheromone
and receptor (Miyajima et al., 1987). Since absence of Ga causes the pathway to become activated, Ga is obviously not required to propagate signal for activation. Rather, Ga is formally a negative regulator of signaling necessary to maintain the pathway in a quiescent state, apparently by binding to Gβγ.

Studies of mammalian Ga and receptors in yeast demonstrate specific interactions between components involved in signaling. Yeast cells lacking yeast Ga but expressing mammalian Ga are not constitutively activated (Dietzel & Kurjan, 1987). It thus appears that the mammalian Gαs can interact with the yeast Gβγ. These cells, however, are not inducible by α-factor, which indicates that the activated receptor cannot interact appropriately with the mammalian Gαs subunit. This finding is complementary to the observation that function of the β-adrenergic receptor expressed in yeast requires coexpression of Gαs (King et al., 1990). Hybrid studies with yeast and mammalian Ga suggest that the C-terminus of Ga may be required for interaction with the receptor (Kang et al., 1990). Some point mutations in the C-terminus of Ga also block signaling (Stone & Reed, 1990; Hirsch et al., 1991). Of special interest are two substitutions in this region that exhibit different phenotypes in different cell types and suggest that these residues may be specially involved in receptor-Gα interactions (Hirsch et al., 1991). The Pro467 mutant exhibits a much more severe defect in α cells than in β cells, which suggest that it interacts less well with the α-factor receptor than with the α-factor receptor. In contrast, the Pro466 mutant exhibits a somewhat
more severe defect in a cells (Marsh et al., 1991). Such mutations may lead to a further understanding of how a single yeast Ga can interact with two receptors that lack obvious sequence homology. It may also be possible to identify determinants on the receptor that interact with Ga by exchanging regions of STE2 and STE3 and determining their ability to function with these Ga mutants. Also, again using genetic approaches, several kinds of "activated" mutations of the GPA1 gene have been characterized. A GPA1\textsuperscript{val-50} mutation, which has a substitution of Gly-50 with valine, was introduced by site-directed mutagenesis based on the analogy with the val-12 mutation of Ras (Miyajima et al., 1989). This mutation of Ras decreases GTPase activity and increases transformation activity (Seeburg et al., 1984). The other mutations, GPA1\textsuperscript{lys-355} and GPA1\textsuperscript{lys-364}, were selected from a pool of mutants based on phenotypic changes (Stone and Reed, 1990). The alignment of Ga primary structures shows that the mutations correspond to Val-49 mutation of Gas proteins. According to the model described above, constitutive activation of GP1a protein would cause phenotypes supersensitive to the mating factors. This has turned out to be the case in short-term responses: i.e growth arrest and gene inductions of cells carrying these GPA1 mutations were elicited by a 100-fold lower concentration of mating factors than required for wild-type cells. More interestingly, however, these mutations also enhanced recovery from factor-induced growth arrest, and after long-term incubation with factors, mutant cells finally showed phenotypes of factor-resistant growth (Stone & Reed, 1990). One possible
explanation is that independent of growth arrest and gene-induction pathways driven by the $\beta y$-subunit, GP1α can turn on another signaling pathway which leads to a recovery from mating factor responses. All of the evidence described above has relied on genetic studies. Biochemical studies are necessary to elucidate the precise molecular mechanisms of G protein function in mating factor signal transduction.

1.2.9 Cyclic-AMP Pathway and GPA2

In addition to the mating factor signaling system, *S. cerevisiae* has another signal transduction pathway, which operates in the early G1 phase of the cell cycle. This is mediated by nutrients such as glucose, which serves as an extracellular signal for the activation of adenylate cyclase, and cAMP plays a crucial role in cell cycle progression at this stage (Matsumoto *et al.*, 1985). It is well known that GTP-binding proteins encoded by *RAS1* and *RAS2*, which are yeast counterparts of mammalian Ras, participate in the control of the activity of adenylate cyclase. In contrast to yeast adenylate cyclase, mammalian adenylate cyclase activity is regulated by two G proteins, Gs and Gi. A recent study reports that a yeast G protein GPA2, in addition to Ras protein, is involved in the regulation of cAMP levels in the cell.

Yeast cells cultured under starvation conditions transiently accumulate cAMP in response to glucose (Eraso *et al.*, 1985). Introduction of YEpGPA2 (a multicopy plasmid carrying the GPA2 gene) in wild type cells was found to enhance glucose-induced cAMP
accumulation remarkably (Nakafuku et al., 1988). In addition, YEpGPA2 suppressed the growth defect by a temperature-sensitive (ts) mutation of the RAS2 gene[ras2-101(ts)]. In ras2-101(ts) cells, mutant RAS2 proteins would not support the activation of adenylate cyclase at nonpermissive temperature and therefore glucose could not induce cAMP formation. Introduction of YEpGPA2 restored the cAMP response in the mutant cells at high temperature. These results suggest that GPA2, in addition to Ras proteins, is involved in the regulation of cAMP levels in S. cerevisiae.

Since GPA2 could not restore gpa1 phenotypes (Kaziro et al., 1991), STE4-and STE18-encoded β- and γ- subunits respectively cannot interact with GPA2. This implies that an additional set of genes that code for β- and γ-subunits interacting with GPA2 must be present in yeast cells.

1.2.10 GBγ Subunits of Yeast G protein

The β and γ subunits of yeast G proteins can be considered as a unit since they function together: null mutations in the genes encoding these proteins lead to similar phenotypes (Whiteway et al., 1989, 1990). Mammalian G protein β and γ subunits copurify as a tight complex and likewise function as a unit (Stryer & Bourne, 1986). The STE4 gene encodes a product with similarity to mammalian GB (Whiteway et al., 1989). The yeast analogue to the Gγ subunit is encoded by the STE18 gene, which has only weak sequence similarity to the mammalian Gγ, but is of similar size (Whiteway et al., 1989).
The *STE4* gene product is predicted to be a protein of 423 amino acid residues. *STE18* encodes a predicted product of 110 amino acid residues and shares an important feature with mammalian Gγ subunits (Whiteway *et al.*, 1989): both end with a consensus amino acid sequence (Cys-aliphatic-aliphatic-X amino acids) for isoprenylation, a lipid modification that may localise the subunit to the membrane (Whiteway *et al.*, 1989). Isoprenylation may be required for Gβγ to function in signaling (Schafer *et al.*, 1989). Mammalian Gγ subunits have a related lipid modification (Mumby *et al.*, 1990). Both *STE4* and *STE18* are required for response to pheromones (Whiteway *et al.*, 1989): mutants defective in these genes are unresponsive to mating factors. As noted above, inactivation of the *GPA1 (SCG1)* gene causes constitutive response of the pathway, presumably because an activator of the response pathway is liberated. The behaviour of mutants defective in both *GPA1* and in the *STE4* or *STE18* gene indicates that it is the *STE4* and *STE18* products that are responsible for activating the pathway: *gpa1 (scg1) ste4*, and *gpa1 (scg1) ste18* mutants exhibit the phenotype of *ste4* and *ste18* mutants - the response pathway is not activated (Nakayama *et al.*, 1988; Whiteway *et al.*, 1989). The role of Gα is apparently to prevent signaling by Gβγ in the absence of receptor/ligand interaction (Whiteway *et al.*, 1989).

Blinder *et al.* (1989) identified mutations of *STE4* that lead to constitutive expression of the signaling pathway. These mutations, termed haploid-specific lethals (*STE4*<sup>hapl</sup>), cause lethality only in a and α haploid cells but not in a/a diploids in
which several essential components of the signaling pathway (such as STE5, STE12, and FUS3) are turned off.

The \textit{STE4} mutations may cause synthesis of a \textit{G\beta} subunit that is insensitive to inhibition by the \textit{Ga} subunit, but which preserves its ability to interact with downstream components of the pathway.

The importance of the balance between \textit{Ga} and \textit{G\beta\gamma} has also been demonstrated by studies in which different subunits are overproduced. Overexpression of \textit{G\beta (STE4)} alone, or with \textit{G\gamma (STE18)} leads to constitutive mating-factor responses (Whiteway \textit{et al.}, 1990; Cole \textit{et al.}, 1990). This induction is overcome by overproduction of \textit{Ga}, (Whiteway \textit{et al.}, 1990; Cole \textit{et al.}, 1990), presumably by converting free \textit{G\beta\gamma} subunits back to \textit{G\alpha\beta\gamma} heterotrimers and restoring the normal ratio of the subunits. Overexpression of \textit{G\alpha (SCG7)} has also been observed to counter the growth inhibition of certain \textit{a} strains (mutants defective in the \textit{SST2} gene) exposed to \textit{a}-factor (Kang \textit{et al.}, 1990; Cole \textit{et al.}, 1990).

At the C-terminus of Ste18 proteins, a cys-A-A-X sequence (A represents aliphatic amino acid and X is the last amino acid) common to all the \textit{G\gamma}-subunits is found. In Rasp21, this motif signals posttranslational modification of the C-terminus, which is required for membrane association and biological activity (Willumsen \textit{et al.}, 1984). Studies have demonstrated that the conserved cysteine in the motif is the site of polyisoprenylation of both Ras (Casey \textit{et al.}, 1989) and \textit{G\gamma} proteins (Mumby \textit{et al.}, 1990). It was also shown that a mutational change of the cysteine (Cys-107) to serine resulted in the loss of function of Ste18
Furthermore, a yeast dpr1/ram1 mutation, which was originally isolated as a defective mutation in postranslational processing of yeast Ras proteins, was found to affect the membrane association and biological activity of Ste18 protein (Kaziro et al., 1991). These results indicate that G protein γ-subunits and ras proteins may share a set of the same modification process.

1.2.11a Additional Components Involved in G Protein and Receptor Function

Although in vitro studies with mammalian receptors and G proteins suggest that pheromone, receptor, and $G_\text{q} \beta \gamma$ are sufficient to permit GDP/GTP exchange and coupling to downstream responses, it is possible that other components are involved in vivo in modulating the signaling response. In yeast, genetic screens have identified a number of genes whose products may modulate activity of the G protein. Inactivation of these genes leads to activation of the signaling pathway; hence, these products can be formally considered as negative components of the pathway. The cell division cycle (CDC) genes with this behaviour are CDC36 and CDC39 (Neiman et al., 1990; de Barros Lopes et al., 1990), CDC72 and CDC73 (Reed et al., 1988), and SRM1 (Clark & Sprague, 1989) (See table 1). Mutants with temperature-sensitive defects in any of these genes exhibit cell-cycle arrest in G1 and induction of $FUS1$-lacZ expression at non-permissive temperature. Activation of the response pathway in these mutants is blocked by inactivation of the
STE4 gene, as observed for mutants defective in G1 itself. Thus these proteins might be regulators of G protein activity, receptor-G protein adapters, new G protein subunits or modifiers of the G proteins.

CDC72 has been shown to be identical to the NMT1 gene, which codes for N-myristoyl transferase (Duronio et al., 1989). The yeast G1 polypeptide is myristoylated in wild-type strains, but not in the cdc72 mutants (Marsh et al., 1991). CDC36 may also control synthesis of a functional G1 subunit (Neiman et al., 1990). Transcription of GPA1(SCG1) is normal in these strains (de Baros Lopes et al., 1990); hence CDC36 might be involved in post-translational modification of G1. Physiological analysis of a cdc39-ts mutant indicates that CDC39 does not control synthesis of a functional Ga subunit, but rather raises the intriguing possibility that it might play a role in communication between the activated receptor and Ga, or be involved in stabilizing the GDP-bound form of Ga (Neiman et al., 1990).

The SRM1 gene (Clark & Sprague, 1989) shares extensive similarity with the mammalian gene, RCC1 (Uchida et al., 1990), and is identical to the PRP20 gene, which is involved in messenger RNA metabolism (Aebi et al., 1990). The relationship between the SRM1 product and the response pathway is obscure and might be very indirect (Marsh et al., 1991).

It should be noted that the genes CDC36, CDC39, CDC72 and SRM1 are known to play roles above and beyond their roles in the signal transduction pathway. This is in contrast to the GPA1(SCG1) gene,
which is essential only for the signal transduction pathway. This difference can be readily discerned by the observation that a/a strains defective in GPA1(SCG1) are viable whereas a/a strains defective in CDC36 etc are inviable (Marsh et al., 1991).

Several other genes have been identified that may represent other components involved in early steps in the signal transduction pathway. The DAF2 product may be involved in STE4 function (Cross, 1990). Certain mutations in the RAM1 gene (also known as DPR1, SGP2, or STE16), which is necessary for farnesylation of RAS and the a-factor precursor, also cause defects in the response pathway (Nakayama et al., 1988; Matsumoto et al., 1988), perhaps because of a failure to modify STE18 or some other product.
<table>
<thead>
<tr>
<th>Gene</th>
<th>Function</th>
<th>Null mutation phenotype</th>
<th>Cell-type expression</th>
</tr>
</thead>
<tbody>
<tr>
<td>STE2</td>
<td>α-factor receptor</td>
<td>Unresponsive</td>
<td>α</td>
</tr>
<tr>
<td>STE3</td>
<td>α-factor receptor</td>
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<td>α</td>
</tr>
<tr>
<td>GPA1(SCG1)</td>
<td>Gα subunit</td>
<td>Constitutive (lethal)</td>
<td>α,α</td>
</tr>
<tr>
<td>STE4</td>
<td>Gβ subunit</td>
<td>Unresponsive</td>
<td>α,α</td>
</tr>
<tr>
<td>STE18</td>
<td>Gγ subunit</td>
<td>Unresponsive</td>
<td>α,α</td>
</tr>
<tr>
<td>STE20</td>
<td>Protein Kinase</td>
<td>Unresponsive</td>
<td>All</td>
</tr>
<tr>
<td>STE5</td>
<td>Unknown</td>
<td>Unresponsive</td>
<td>α,α</td>
</tr>
<tr>
<td>FUS3</td>
<td>Protein Kinase</td>
<td>Unresponsive</td>
<td>a,α</td>
</tr>
<tr>
<td>STE7</td>
<td>Protein Kinase</td>
<td>Unresponsive</td>
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</tr>
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<td>Unresponsive</td>
<td>All</td>
</tr>
<tr>
<td>KSS1</td>
<td>Protein Kinase</td>
<td>See text</td>
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</tr>
<tr>
<td>STE12</td>
<td>Transcriptional activator</td>
<td>Unresponsive</td>
<td>a,α</td>
</tr>
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</tr>
<tr>
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</tr>
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</tr>
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</tr>
<tr>
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<td>Constitutive (lethal)</td>
<td>All</td>
</tr>
<tr>
<td>SRI1</td>
<td>Unknown</td>
<td>Constitutive (lethal)</td>
<td>All</td>
</tr>
</tbody>
</table>

Phenotype is with regard to expression of pheromone-induced responses. Constitutive activation of these responses leads to cell-cycle arrest and death. See text for references.
1.2.11b. Adaptive Response to the Mating Factors

Yeast cells exposed to the mating-factors recover from G1 arrest after a period of time and resume growth. Thus arrest is transient. There are several different mechanisms for adapting to the mating factors. As noted above, the α-factor receptor is subject to internalization (Jenness & Spatrick, 1986). In addition, α-cells produce an extracellular protease, coded by the BAR1 gene, that inactivates α-factor by degradation, thus allowing enhanced recovery to α-factor (Sprague & Herskowitz, 1981; Mackay et al., 1988). It was discovered that α-cells inactivate α-factor (Marcus et al., 1991).

Yeast cells are able to adapt even in the absence of α-factor inactivatin (Moore, 1984). This adaptive response appears to function by several independent pathways and to involve the receptor, Gq, Gq and the SST2 gene. Receptor mutants lacking the C-terminal segment are supersensitive to mating pheromones (Konopka et al., 1988; Reneke et al., 1988). The C-terminal segment is rich in serine and threonine residues and is hyperphosphorylated in response to α-factor (Marsh et al., 1991).

Mutants of Gq with a Gly to Val substitution at position 50 (analogous to the rasV12 mutation that reduces the intrinsic GTPase activity of ras) exhibit a complex phenotype that appears to indicate that Gq plays a role in adaptation (Cole et al., 1990). Another interpretation of such mutants is given by Kurjan et al., (1991). These strains are partially constitutive for the pathway and supersensitive to growth arrest by α-factor. However, they also
appear to exhibit a stronger ability to recover from arrest than do wild-type cells. Miyajima et al., (1989) have proposed that the activated Ga subunit (Ga-GTP) provokes a recovery process. Irie et al., (1991) identified a gene SGV1, which encodes for a protein kinase related to CDC28 (42% identical), that may play a role in this Ga-stimulated adaptive response.

Cole & Reed (, 1991) reported that the STE4 protein (Gβ) is rapidly phosphorylated after α-factor treatment of a cells. This phosphorylation appears to play a role in adaptation, based on the observation that deletion of a segment of STE4 eliminates pheromone-induced phosphorylation and causes cells to become hypersensitive to mating factors (Marsh et al., 1991). It has been further shown that an intact Ga subunit is required for this phosphorylation. These observations lead to the hypothesis that the Gα- and SGV1- dependent recovery process proposed by Miyajima et al., (1989) may function by stimulating phosphorylation of Gβ (Irie et al., 1991).

The product encoded by the SST2 gene is involved in desensitization or recovery from signaling. Mutations in SST2 cause strains of either mating type to be hypersensitive to mating factor and to have prolonged responses (Chan & Otte , 1982). Transcription of SST2 is highly pheromone-inducible and thus serves to turn off response to pheromone and promote recovery (Dietzel & Kurjan , 1987). The target of SST2 does not appear to be the receptor C-terminus since hypersensitivity resulting from receptor truncation and sst2 mutations is additive (Konopka et al., 1988; Roneke et
al., 1988). SST2 may act on Ga or some other G protein subunit since certain Ga mutant alleles are epistatic to sst2 mutations (Kurjan et al., 1991). Also, overexpression of Ga partially overcomes an sst2 mutation (Dietzel & Kurjan, 1987). The SST2 product may stimulate the intrinsic GTPase activity of Ga and thus be analogous to GAP acting on RAS in mammalian cells (McCormick et al., 1988), or to IRA1 and IRA2 acting in yeast (Tanaka et al., 1990).

The recovery processes mediated by SST2, GB, and the C-terminus of the receptor appear to be independent of each other. This conclusion is drawn from the observation that mutants doubly defective in these components exhibit more severe defects than do mutants with mutations in individual components (Marsh et al., 1991). For example, the extremely hypersensitive phenotype of strains with a C-terminal deletion of STE2 and a mutation in SST2 makes it unlikely that SST2 function by phosphorylating or otherwise affecting the C-terminus of the receptor (Konopka et al., 1988; Reneke et al., 1988).

1.2.12 Downstream From the G Proteins

Six genes (STE5, STE7, STE11, STE12, STE20 and FUS3) that have been identified as necessary for signal transduction appear to function after or at the level of the G protein. This placement is based on the behaviour of double mutant strains with alterations in Gq and GB and STE5 or other STE genes. As noted earlier, null mutations in GPA1(SCG1) cause constitutive behaviour of the
pathway, whereas null mutations in the STE genes are nonresponsive to α-factor. The important result (Nakayama et al., 1988) is that strains defective in both GPA1 and any of the STE genes or FUS3 (Elion et al., 1990) are nonresponsive for example, a gpa1 ste11 strain exhibits the properties of the ste11 mutant. These observations are interpreted to indicate that the STE11 protein acts after Ga in a simple linear pathway. Another interpretation is that GPA1 and STE11 function in separate pathways that intersect downstream from GPA1 (Elion et al., 1990). One cannot distinguish between these two possibilities at present, but for simplicity, we consider these STE genes products as functioning downstream of the G protein. Analogous epistasis results have been obtained using the special mutation of the GB subunit, encoded by the STE7 \textsuperscript{Boi} mutation described earlier, which leads to constitutive expression of the pathway (Blinder et al., 1989). These findings place the STE products and FUS3 downstream of GB (or functioning at the same step in the pathway as GB). Additional epistasis tests with mutants defective in CDC36 and CDC39 genes are consistent with these findings (Neiman et al., 1990; de Barros Lopes et al., 1990). 

After activation of the G protein, the liberated GB\textsubscript{β} subunit is presumed to interact with one or more proteins to propagate the signal. The gene product that lie immediately downstream of GB\textsubscript{β} - STE20, STE5, STE7, STE11 and FUS3 are candidates for this target. Leberer et al.,(1992) demonstrated through epistasis relationship that STE20 could lie closest to the GB\textsubscript{β} subunits. This supposed interaction between the GB\textsubscript{β} and STE20 product is however not clear.
and must be substantiated by further genetic and biochemical studies. Thus, identification of the target for Gβγ is one of the most important challenges in understanding the yeast signaling pathway.

In several mammalian systems, synthesis or degradation of a second messenger molecule such as cAMP, cGMP or IP₃ is controlled by activation of the G protein (Iyengar & Birnbaumer, 1990). No such second messenger molecule has been identified in yeast. An early contention that cAMP was the second messenger for this pathway (Thorner, 1982) has not been substantiated (Casperson et al., 1983). None of the genes in the signaling pathway has features of known enzymes involved in second messenger production or regulation. Studies of the genes that lie downstream of the G protein have, however, provided some information as to their function: STE7, STE11, STE20 and FUS3 appear to code for protein kinases and STE12 code for a transcription factor that is subject to phosphorylation (see table 1).

STE7, STE11, FUS3 and STE20 all contain significant similarity to the catalytic domains of serine/threonine protein kinases (Teague et al., 1986; Rhodes et al., 1990; Elion et al., 1990; Leberer et al., 1992). They contain all of the 15 amino acid residues conserved in most protein kinases (Rhodes et al., 1990). STE7, STE11 and STE20 share 25-30% identity with each other (Leberer et al., 1992) and 22-27% identity with FUS3 (Rhodes et al., 1990). FUS3 has 34-36% identify to the CDC28/cdc2 kinase family, which plays important roles in cell-cycle regulation. FUS3
bears even more similarity (54% identity) to another putative yeast protein kinase, which is encoded by the KSS1 gene (Courchesne et al., 1989). A mammalian protein kinase with particular similarity to FUS3 and KSS1 has been identified (Boulton et al., 1990). This protein, the insulin-stimulated protein kinase (ERK1, extracellular signal-regulated protein kinase), is 50-52% identical to FUS3 and KSS1 and shares a C-terminal extension that is not present in the CDC28/cdc2 family. It thus appears that these protein kinases identify a new structurally related group of protein kinases, which appear to play roles in response of cells to extracellular signals.

The FUS3 and KSS1 genes are functionally redundant in some respects and not in others (Elion et al., 1990, , 1991). Strains deleted for either KSS1 or FUS3 respond to α-factor (exhibiting induction of FUS1-lacZ), whereas strains defective in both genes do not (Elion et al., 1991). Thus it appears that doubly defective strains are unable to propagate the signal needed to activate transcription (which appears to be activation of STE12). In contrast, FUS3 and KSS1 are both able to propagate the signal leading to transcriptional activation. FUS3 and KSS1, however, are not functionally interchangeable in all respects. This difference can be seen from the observation that FUS3+kss1 strains arrest in response to α-factor, whereas fus3’KSS1+ strains do not (Elion et al., 1990, , 1991). An important clue as to how this occurs comes from the observation (Elion et al., 1991) that the ability of fus3 strains to undergo cell-cycle arrest is restored if they are also defective in the CLN3 gene, which codes for a G1 cyclin. It has
been proposed that the normal role of \textit{FUS3} is to inactivate the \textit{CLN3} protein perhaps by phosphorylating it (Elion et al., 1990). It is not possible to know from these observations if \textit{CLN3} is a direct substrate of \textit{FUS3}. These studies of \textit{FUS3} and \textit{KSS1} reveal some of the complexities likely to be encountered in studying protein kinases with multiple substrates and overlapping specificities; it appears that \textit{FUS3} has at least two substrates, only one of which is a substrate for \textit{KSS1} (Marsh et al., 1991).

\textit{KSS1} was identified because its expression, when carried on a high copy number plasmid, allows cells defective in the \textit{SST2} gene to become partially resistant to \textalpha-factor (Courchesne et al., 1989). Given that \textit{KSS1} now appears to play a role in propagating the signal through the pathway, it is not clear why a high copy number plasmid carrying \textit{KSS1} should inhibit the functioning of the pathway.

The \textit{STE11} product is the only one of this group of putative protein kinases of yeast that has been shown to have kinase activity. This demonstration has come from an immune-complex phosphorylation assay using an epitome-tagged \textit{STE11} protein (Rhodes et al., 1990). These in vitro studies identify a substrate (p78) of 78kd, which does not correspond to any known gene product, such as \textit{STE4} (47 kd), \textit{STE7} (55kd), \textit{STE12} (112 kd) or \textit{FUS3} (40kd) Rhodes et al., 1990), and neither does it correspond to \textit{STE20} (102kd) Leberer et al., 1992). Whether p78 has a role or not in signal transduction is unknown. There are numerous possible substrates for \textit{STE11} and the putative protein kinases in the response pathway. The following...
gene products have been shown to be phosphorylated. STE2 (Reneke et al., 1988), STE4 (Cole and Reed, 1991), STE5 (Marsh et al., 1991), STE7 (Marsh et al., 1991), STE11 (Rhodes et al., 1990), STE12 (Song et al., 1991) and FAR1 (Marsh et al., 1991).

The steps mediated by STE4, STE7, STE11, STE20 and FUS3 have not been ordered biochemically with respect to each other. However, the behaviour of double mutants carrying these activated alleles and null mutations in other genes suggests that the signaling pathway may not be a simple linear kinase cascade. These protein kinases differ from each other with respect to their regulation. Transcription of the FUS3 gene is activated several-fold after treatment with α-factor (Elion et al., 1990). Although the STE7 gene contains three PRE (pheromone response elements) in its upstream regulatory region, synthesis of STE7 protein is not increased by exposure to α-factor (Marsh et al., 1991). Hyperphosphorylation of STE7 however is rapidly induced by this treatment (Marsh et al., 1991). Neither transcription of STE11 nor activity of its product are increased by treatment of cells with α-factor (Rhodes et al., 1990). The STE7, STE11 and STE20 genes are expressed in all cell types, even in a/a cells which do not respond to mating factors. Their role in a/a cells is not known; mutants defective in STE7, STE11 and STE20 genes do not exhibit any additional phenotypes. In contrast, the FUS3 gene, like many genes in the signal-transduction pathway is expressed only in a and α cells and not in a/a cells. Nothing is known about the STE5 except that it appears to function downstream from G protein and upstream
of STE12 (Marsh et al., 1991).

1.2.13 STE12 and Transcriptional Activation

The target for transcriptional activation of genes by the mating factors is the STE12 product (Dolan et al., 1989; Errede & Ammerer, 1989). Two pieces of information indicate that STE12 is at the end of the pathway for transcriptional activation. First, Ste12 protein binds to the DNA sequence that is responsible for mating-factor inducibility (Dolan et al., 1989; Errede & Ammerer, 1989). Secondly, plasmids that express STE12 at high levels from a pGAL regulatory region bypass the need for various STE genes in the response pathway: substantial expression of FUS1 is observed in strains overexpressing STE12 in ste7 and ste11 mutants (Dolan & Field, 1990), as well as in mutants defective in STE4, STE5, and FUS3 (Marsh et al., 1991). These observations indicate that STE12 is at the end of the signal-transduction pathway for transcriptional induction and lead to the hypothesis that its activity is controlled by phosphorylation, perhaps by STE7, STE11 or FUS3 (Dolan et al., 1989; Errede & Ammerer, 1989).

Transcription of many genes is induced by the mating pheromones (Appeltauer & Achstetter, 1989). These include genes involved in cell fusion and other aspects of cell-cell interactions: FUS1, which is induced more than 100-fold (Trueheart et al., 1987), CHS1 (coding for chitin synthase 1), which is induced 10-fold (Appeltauer & Achstetter, 1989), and AGa1 (coding for α-agglutinin), which is induced more than 20-fold by α-factor.
Lipke et al. (1989), AGA1 (coding for an α-agglutinin subunit); Roy et al. (1991) and KAR3 (coding for a kinesis-like protein necessary for nuclear fusion) (Meluh & Rose, 1990). Genes involved in pheromone biosynthesis, such as structural genes for α-factor (MFa1) and STE13, are induced two to fivefold (Achstetter, 1989). Many of the components of the response pathway are inducible to similar extent; these include STE2, STE3, STE4, STE5, GPA1 and FUS3. The sequence TGAAACA (termed the PRE, pheromone response elements) is present in two to nine copies in the upstream regulatory regions of genes whose transcription is induced by mating pheromones (Trueheart et al., 1987; Konstad et al., 1987) and was shown to be necessary for induction (Kronstad et al., 1987). Studies have also shown that multiple, tandem PRE sequences are sufficient to confer pheromone inducibility to test plasmids (Hagen et al., 1991). Presence of PRE sequences, however, is not always sufficient to confer inducibility to mating factors. Even though they contain multiple PRE sequences in their upstream regions, STE7 and STE12 do not appear to be inducible by α-factor (Marsh et al., 1991).

Although STE12 can bind weakly to individual PRE sequences (Dolan et al., 1989), its binding is greatly enhanced by association with other proteins, such as the general transcription factor, MCM1, and by other as-yet uncharacterized factors (Errede & Ammerer, 1989). PRE sequences are often found in upstream regulatory regions adjacent to MCM1-binding sites (Krostad et al., 1987; Errede & Ammerer, 1989).
**STE12** is essential both for induction of transcription by pheromones and for setting the basal level of transcription of genes in the signal transduction pathway. This can be seen from the observation that transcription of **STE2** and **STE3** is reduced fivefold in a ste12 mutant (Fields et al., 1988; Hagen et al., 1991). Five to twenty-fold decreases in transcription of all α-specific and α-specific genes analysed (**MFα1**, **MFα2**, **MFA1**, **MFA2** and **STE6**) are also observed in ste7 and ste11 mutants (Fields et al., 1988). It appears that the basal level of expression of these genes is due to some spontaneous activity of the pathway (for example, partially active **STE12** protein or free Gβγ) rather than to the presence of a low level of mating factor in cultures (Hagen et al., 1991).

Studies on **STE12** show that it is phosphorylated after pheromone treatment and provide information on its functional domains. For technical reasons (to increase the amount of **STE12**), the phosphorylation studies were carried out with hybrid proteins that contain the DNA-binding domain of **GAL4** (amino acid residues 1-147) attached to all or parts of **STE12**. The **GAL4-STE12** (1-688) hybrid (containing all of **STE12**) is rapidly phosphorylated after addition of α-factor, with kinetics similar to that for induction of pheromone-responsive genes (Song et al., 1991). Both phosphorylation of **STE12** (Song et al., 1991) and transcriptional induction (Achstetter, 1989) can occur in the absence of protein synthesis. The five protein kinases upstream of **STE12** in the response pathway are obvious candidates for being responsible for phosphorylation of **STE12**. The observation that over-production of
STE12 allows induction of FUS1 in ste4, ste7, ste11 and fus3 mutants (Dolan & Fields, 1990) indicates that STE12 must have a basal activity independent of the response pathway.

The GAL4-STE12 hybrids have revealed information on functional domains of STE12. Hybrids containing the entire STE12 polypeptide exhibit some transcriptional activation ability, tenfold above background; this activity is observed only when cells are treated with α-factor. Hybrids containing residues 214-688 (which lack the STE12 DNA-binding domain) or 1-473 (which lack the putative MCM1 interaction domain) exhibit similar induction (Marsh et al., 1991). In contrast, the GAL4-STE12 hybrid containing residues 214-473 behaves quite differently. It exhibits potent, constitutive activation activity, which is 250-fold above background (Marsh et al., 1991). The potent activity of the GAL4-STE12 (214-473) hybrid in comparison with the other hybrids suggests that the 1-214 and 474-788 segments of STE12 contain inhibitory domain. An attractive possibility is that phosphorylation of such an inhibitory domain leads to activation of STE12 (Marsh et al., 1991).

The observation that transcriptional activation by several hybrids is stimulated by α-factor has different possible explanations. If one assumes that the GAL4 domain is sufficient for DNA-binding and localization to the nucleus, these observations indicate that the STE12 activation domain is regulated by the mating response pathway (Song et al., 1991), in particular, by phosphorylation. Other explanations can be envisaged if the GAL4 domain is not sufficient for DNA binding and nuclear localization.
For example, phosphorylation of \textit{STE12} could regulate its entry into the nucleus (Baeuerle & Baltimore, 1988).

1.2.14 \textbf{Interfacing with the Cell Cycle}

One of the responses to mating pheromone is arrest of the cell in the G1 phase of the cell cycle. This is a transient arrest that ensures that nuclear fusion will occur between nuclei containing a 1N complement of chromosomes. The targets for this control appear to be the three G1 cyclins of yeast \textit{CLN1}, \textit{CLN2}, and \textit{CLN3}, which are required for progression from G1 to S and are thought to be required for activity of \textit{CDC28}, the budding yeast homologue of p34\textsuperscript{cdc2} (Wittenberg \textit{et al}., 1990). Arrest in the cell cycle requires that all three G1 cyclins be inactivated (Richardson \textit{et al}., 1989), which may occur through the action of three separate inhibitors (Chang & Herskowitz, 1990). Two of the inhibitors (\textit{FUS3} and \textit{FAR1}) have been identified because mutants defective in these genes have an intact, functioning response pathway but do not undergo growth arrest in response to mating factors (Elion \textit{et al}., 1990; Chang & Herskowitz, 1990). \textit{FUS3} appears to be responsible for inactivating \textit{CLN3} (Elion \textit{et al}., 1990, 1991), and \textit{FAR1} for inactivating \textit{CLN2} (Chang & Herskowitz, 1990). The compound responsible for inhibiting \textit{CLN1} is unknown. The response pathway apparently triggers cell-cycle arrest by enhancing activity of these inhibitors of the G1 cyclins. Transcription of both \textit{FUS3} and \textit{FAR1} is induced several fold by mating factors Elion \textit{et al}., 1990; Chang & Herskowitz, 1990). In addition, \textit{FAR1} is rapidly phosphorylated.
after treatment of α cells with α-factor (Marsh et al., 1991). The existence of mutants such as strains that lack FAR1 (Chang & Herskowitz, 1990) or that carry the CLN3-1 mutation (Cross, 1988) that have an intact signal response system, but that do not arrest in response to mating factors, demonstrates that cell-cycle arrest is not a requirement for differentiation in yeast.

1.2.15 MATα2 and MCM1

The mating type of yeast cell, α or α, is determined by a single locus on chromosome III, called the mating-type locus (MAT), which may contain either of two types of sequences. MATα differs from MATα in the substitution of a 650-bp α-specific sequence for a non-homologous 750-bp α-specific sequence (Nasmyth and Tatchell, 1980). The two genes at MATα, a1 and a2, are transcribed divergently from a central promoter and regulatory region (Johnson & Herskowitz, 1985). The MATα2 gene is required for the determination of both haploid and diploid cell types. In haploid cells, it is necessary for inhibiting the expression of α mating functions, which are otherwise antagonistic to α mating functions (Strathern et al., 1980). In diploid cells, the same inhibition of α mating functions is exerted, but in addition, it acts in conjunction with the a1 gene to regulate MAT transcription, principally the repression of a1 transcription and to allow sporulation (Nasmyth et al., 1981). The MATα2 gene product is therefore necessary for preventing the expression of both α and α mating types in α/α diploids (figure 3), (also Nasmyth et al.,
1981). In vivo, a2 represses transcription of two sets of cell type-specific genes by binding to operator sites together with either the a1 or Mcm1 proteins (figure 3). Each protein targets a2 to a different set of operators (Herskowitz, 1989). The yeast a2 repressor is also a particularly well-characterised member of the homeodomain superfamily of DNA-binding proteins (Wolberger et al., 1991). First identified in a series of Drosophila genes that regulate development, the homeodomain is a conserved sequence of 60 amino acids. Homeodomain-containing proteins have now been found in virtually all eukaryotes examined (Scott et al., 1989).

MCM1 is a yeast transcription factor with homologs throughout the metazoa. MCM1 was first identified as a gene involved in maintenance of artificial minichromosomes in yeast (Elbe and Tye, 1991). It has also been shown to serve as a transcriptional regulator of mating-type-specific genes (Elbe and Tye, 1991). Biochemical data suggests that MCM1 coactivates a-specific genes and corepresses a-specific genes by binding to a 10-base pair dyad symmetry element in their upstream regions (Elbe and Tye, 1991). Intrigued by the cell's capacity to tightly control its growth, and by the consequences of its inability to do so, the objective of this study is to isolate and characterize additional genes of the signal transduction pathway that are involved in this cellular growth control. This goal can only be accomplished by analyzing these genes structurally, genetically and biochemically in other to understand their mode of action. The importance of these growth control points in the cell cannot be overemphasized since they
presumably exist to prevent both replication of a damaged DNA template and segregation of damaged chromosomes. It is thought that the transient delays at these growth control points permits repair of damaged DNA prior to these critical cellular functions and should thus enhance cell survival and limit propagation of heritable genetic errors (Weinert & Hartwell, 1988). Since the yeast provides an experimental opportunity with all of the techniques of manipulative molecular genetics, the yeast pheromone response pathway offers opportunity to explore signal transduction in a genetically tractable organism. This is so because many of the features that have been found to date are remarkably similar to those found in multicellular eukaryotes.

In this study, the focus on the search for possible GPA1(SCG1) homologs was inferred through hybridization studies that there could be at least two additional genes homologous to GPA1(SCG1) in the yeast genome (Dietzel & Kurjan, 1987). This was coupled with the possibility that other gene products are involved in vivo in modulating the signaling response.
Figure 3: Regulation of cell types by MAT locus genes and MCM1

Regulation of α-specific genes (asg), α-specific genes (asg), and haploid specific genes (hsg) in a, a and a/a yeast cells by regulatory proteins encoded at the MAT locus together with MCM1, a constitutive DNA-binding protein. 3a shows transcription of asg by α1-MCM1 proteins and repression of asg by α2-Mcm1 protein complex. 3b shows repression of asg by α2-Mcm1 proteins. 3c shows repression of asg by α2-Mcm1 proteins, hsg by α1-α2 proteins and α1 transcription repression by α1-α2 proteins complex. Solid bars indicate repression of gene transcription whereas arrowheads or wavy arrows denote transcription activation. As a result of this regulation, each diploid or haploid cell type exhibits a distinctive pattern of gene expression.
CHAPTER TWO

MATERIALS AND METHODS

2.1 Materials

Table 2: Yeast Strains Used in this Work

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF1</td>
<td>MATQ leu2, ura3, his3 ade8 trp1, Tyr1, gal1, can1</td>
<td>Colicelli</td>
</tr>
<tr>
<td>FY250</td>
<td>MATa ura3, his3, leu2, trp1</td>
<td>Colicelli</td>
</tr>
<tr>
<td>DC14</td>
<td>MATa his1</td>
<td>Colicelli</td>
</tr>
<tr>
<td>DC17</td>
<td>MATa his1</td>
<td>Colicelli</td>
</tr>
<tr>
<td>DC124</td>
<td>MATa leu2, ura3, his4, trp1 ade8</td>
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</tr>
<tr>
<td>SF1/DC124</td>
<td>MATa/MATa, leu2/leu2, his3/HIS3, HIS4/his4, ura3/ura3, trp1/trp1, ade8/ade8</td>
<td>Colicelli</td>
</tr>
<tr>
<td>GU1</td>
<td>MATa gpa1::HIS3 leu2, ura3, can1 (pTLCG)</td>
<td>Colicelli</td>
</tr>
<tr>
<td>GU2</td>
<td>MATa gpa1::HIS3, leu2, ura3, can1 (pTLCG)</td>
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<tr>
<td>LG1-TC</td>
<td>GU1 with plasmid replacement for TRP1/GPA1/CAN1</td>
<td>This work</td>
</tr>
<tr>
<td>LG2-TC</td>
<td>GU2 with plasmid replacement for TRP1/GPA1/CAN1</td>
<td>This work</td>
</tr>
<tr>
<td>LG1-UG</td>
<td>GU1 with plasmid replacement for URA3/GPA1</td>
<td>This work</td>
</tr>
<tr>
<td>LG2-UC</td>
<td>GU2 with plasmid replacement for URA3/GPA1</td>
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<tr>
<td>GL1-5</td>
<td>GU1 with plasmid replacement for URA3/MCM1/CAN1</td>
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<td>GL1-9</td>
<td>GU1 with plasmid replacement for URA3/MATa3/CAN1</td>
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<td>GL1-12</td>
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<tr>
<td>GL2-5, 9</td>
<td>All created by GU2 replacements for the respective plasmids as in GL1-strains</td>
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<tr>
<td>12, UGC and UC</td>
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Table 3
Plasmids Used in this Study

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<th>Plasmid</th>
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<td>CAN1 Source</td>
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<td>pBluescript vector</td>
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<tr>
<td>pTGC</td>
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<td>This work</td>
</tr>
<tr>
<td>pUC3</td>
<td>URA3/GPA1/CAN</td>
<td>This work</td>
</tr>
<tr>
<td>pUC5</td>
<td>URA3/MCM1/CAN1</td>
<td>This work</td>
</tr>
<tr>
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</tr>
<tr>
<td>pUC</td>
<td>URA3/CAN1</td>
<td>This work</td>
</tr>
<tr>
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<td>LEU2/MATa2</td>
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</tr>
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<tr>
<td></td>
<td>fragment of MATa2</td>
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</tbody>
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The *E. coli* strain DH5α purchased from New England Biolabs, Beverly, MA was used throughout this work for the propagation of the plasmid DNAs.
Table 4

Drop-Out Medium (Synthetic Complete (SC) Medium)

To 100g of Yeast Nitrogen Base without Amino Acids (Difco), were added:

<table>
<thead>
<tr>
<th>Constituent</th>
<th>Amount</th>
</tr>
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<tbody>
<tr>
<td>Adenine</td>
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</tr>
<tr>
<td>Arginine</td>
<td>1.5g</td>
</tr>
<tr>
<td>Asparagine</td>
<td>1.5g</td>
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<td>Aspartic Acid</td>
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<tr>
<td>Histidine</td>
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</tr>
<tr>
<td>Isoleucine</td>
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</tr>
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<td>Leucine</td>
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</tr>
<tr>
<td>Lysine</td>
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<tr>
<td>Methionine</td>
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<td>Phenylalanine</td>
<td>1.5g</td>
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<tr>
<td>Proline</td>
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</tr>
<tr>
<td>Serine</td>
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</tr>
<tr>
<td>Threonine</td>
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<td>Tyrosine</td>
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<td>Tryptophan</td>
<td>1.5g</td>
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<td>Uracil</td>
<td>1.5g</td>
</tr>
<tr>
<td>Valine</td>
<td>1.5g</td>
</tr>
</tbody>
</table>

For the appropriate drop-out recipe, the corresponding constituent was omitted. The constituents were then mixed well. For plates preparations;

9.0g drop-out mix was added to 350ml of water in 1 litre flask (A)

20g glucose was added to 50ml of water in 250ml flask (B)

and 20g of agar was added to 600ml of water in 2 litre flask with stirbar (C).

All flasks were autoclaved for 30 min at 121°C. Contents of flask A and B were mixed, then added to flask C with slow stirring, cooled and poured. YPD
medium contained 1% Bacto-yeast extract, 2% Bacto-peptone, and 2% dextrose (Difco). LB medium contained 1% Bacto-tryptone, 0.5% Bacto-yeast extract and 1% NaCl (pH 7.0)

ENZYMES AND REAGENTS

Restriction endonucleases, DNA modifying enzymes and DNA markers were purchased from New England Biolabs Inc., Stratagene and Boehringer Manhein. Sequencing kits (reagents) were purchased from United States Biochemicals, USA. (α-32P)dATP was obtained from Amersham Corp. Restriction endonuclease linker oligonucleotides were ordered from Stratagene. Bacteriophage T7 and T3 RNA polymerase primers were purchased from New England Biolabs Inc. Other Chemicals and Reagents used were purchased either from Sigma Chemicals Company or from British Drug House Chemicals Ltd (BDH), and were of the highest purity grade commercially available.
2.2 Methods

2.2.1 Construction of the Yeast Clones

Genomic DNA libraries of *Saccharomyces cerevisiae* were constructed by ligating partial Sau3A digests of genomic DNA into the BamH1 sites of a *URA3* marker vector (pUV2). The library was composed of 4 kilobase or larger Sau3A restriction fragments, selected by running the restriction fragments on 0.7% agarose gel alongside a Lambda DNA - Hind III digested marker. Fragments selected were then cut-off from the gel, electroeluted, using dialysis tube and TBE (See appendix) buffer, purified with phenol and chloroform, precipitated with 0.5ml of 100% cold ethanol plus 0.1ml of 3M NaOAc (pH 5.2), washed with 1ml 70% cold ethanol and then randomly ligated to pUV2 cut with *BamH1* using Bacteriophage T4 DNA ligase modifying enzyme.

2.2.2 Selection for the Yeast GPA1 Complementary (YGC) Clones

Screening of the clones or library was based on the complementation of *gpa1*. First, the GU cells were transformed with the *URA3* marker based YGC library. One percent of the transformed cells were then plated on SC-URA (synthetic complete medium without uracil) to test the efficiency of the transformation. The rest, 99% of the transformed GU cells were grown in 1.5 ml SC-URA culture media for 2 days at 30°C with shaking at 250rpm on a rotary platform. This was to enable the GU cells lose the *GPA1/LEU2/CAN1* maintenance plasmid.
Figure 4: Complementation of GPA1

The diagram shows the selection procedure based on the complementation of gpal strains of GU1 and GU2. The strains were maintained by the plasmid GPA1/LEU2/CAN1. Other plasmids (clones) labelled X/URA3 were introduced into the strain and the GPA1/LEU2/CAN1 plasmid destroyed by the uptake of the drug canavanine. Larger circles indicate the GU1 and GU2 strains whereas the smaller inner circles denote the maintenance plasmids. The genotype of the GU cell is underlined. The signaling pathway leading to differentiation and cell arrest is indicated by the arrow. The yeast G protein (αβγ) is also shown. Where the βγ subunit is involved in the signal transduction is denoted by a circle and the signal inhibitory α subunit is indicated with an ovoid. The wavy lines on the largest circle denote the seven transmembrane receptors of the α-or α-factor.
Transformation of GU cells
(*gpa1 leu2 ura3 can1* pGPA1/LEU2/CAN1)
with URA3 based library

Figure 5: Flow Chart describing the selection of high copy suppressors of *gpa1*

Plasmid dependence was checked by streaking single colonies from the SC-URA-ARG+CAN plate onto SC-LEU and SC-URA plates to look for no growth and growth on the respective plates. Plasmids containing the CAN1 gene cannot survive or grow on canavanine plate or media because the CAN1 gene selects against itself. Thus, colonies that survive on the canavanine plate contain the yeast GPA1 complementary clones. No growth on the SC-LEU plate indicates the lost of GPA1/LEU2/CAN1 plasmid since LEU2 gene is its marker.
After 2 days of culturing the cells in SC-URA media, 5μl of the cells were plated on SC-URA-ARG+CAN (SC media without uracil and arginine but has a drug canavanine added). Survivors on this plate lost the GPA1/LEU2/CAN1 maintenance plasmid since CAN1 gene allows the drug canavanine (an arginine analogue) to counter select against itself. Plasmid dependence of the GU cells were determined by streaking single survival colonies from the SC-URA-ARG+CAN plate onto SC-URA and SC-LEU plates. Growth and no growth on these plates respectively confirms the loss of the GPA1/LEU2/CAN1 plasmid as opposed to possible survival due to reversion (Fig.5). This procedure was used to successfully select 8 high copy suppressors of gpa1 from the yeast library. These clones were pYGC5, 6,7,9,11,12,14 and 20 (Fig.12). This method was routinely used to retest and confirm the clones before further analyses were carried out. Figures 4 and 5 show the diagramatic presentation and flow chart of the selection procedure respectively.

2.2.3 Southern Analysis

In order to determine the genes responsible for complementing the gpa1 strain of GU cells, Southern blottings were done as described by Maniatis et. al., (, 1982). Ten micrograms (10μg) of the high copy suppressor plasmids were digested with EcoR1 and HindIII restriction endonucleases. The resulting restriction fragments were separated according to size by electrophoresis on 0.8% agarose gel. The gel was photographed to make sure that the restriction digests were complete and also to note the size of the various DNA fragments. DNA fragments were then capillary transferred from the gel onto a nitrocellulose filter (Maniatis et. al., 1982), using 10x SSC buffer, Whatman 3MM paper, paper towels, plexiglas, Saran wrap, glass plate and a 500g weight. The DNA fragments were denatured by soaking the gel for 45 minutes in 200 ml's mixture of 1.5M Nacl
and 0.5N NaOH with constant and gentle agitation on a rotary platform. The gel
was then rinsed in 250mls of deionized water, and then neutralized by soaking for
35 min in 200 mls of 1M Tris (pH 7.4) containing 1.5M Nacl at room temperature
with constant, gentle agitation. Meanwhile, 1.9Kb EcoR1 fragment of GPAI was
labelled with ³²P using the modifying enzyme T4 polynucleotide kinase reaction.
The GPAI-labelled probe was then denatured by heating at 100°C for 8 min and
rapidly chilled in ice water. The nitrocellulose filter containing the
immobilized single-stranded fragment was then wetted in 6X SSC, slipped into a
heat-sealable bag (Sears Seal-A-Meal bag) and 2.5 ml of prehybridization solution
(see appendix) added after which the bag was sealed. The sealed bag was then
submerged in 68°C water bath for 2 hours with gentle agitation. The bag
containing the filter was then removed from the water bath, opened with scissors
and the ³²P-labelled denatured probe added to the prehybridization solution and
the bag resealed with a heat sealer. The bag was then incubated, for 2 hrs at
68°C. At the end of the incubation period, the filter was washed eight times in
500ml of 2xSSC containing 0.5% SDS, and in 500 ml of 0.1XSSC containing 0.5% SDS,
at room temperature. The filter was incubated for 45 min at 37°C with gentle
agitation and then transferred into a 68°C water bath for another 45 min. The
filter was washed again briefly with 250ml of 0.1XSSC at room temperature. The
excess liquid was removed by placing the filter on a pad of paper towels. The
filter was then covered with Saran wrap and exposed to Kodak (XAR-2)X-ray film
for 24 hrs (Fig 12).

2.2.4 Hybridization of Clone 9 to the Other Clones

This Southern blot was done to classify the various clones. The same
nitrocellulose blot described above (Fig. 12) was stripped in a boiling water
bath. This was done by heating 400ml of 0.05X SSC containing 0.01M EDTA (pH 7.9) (elution buffer) to boiling. The fluid was then removed from the heat and SDS added to a final concentration of 0.1%. This was followed by immersion of the filter in the hot elution buffer for 17 min. The filter was briefly rinsed in 0.01X SSC at room temperature after the immersion step was repeated with a fresh batch of boiling elution buffer. Excess liquid was removed from the filter with paper towels, dried and rehybridized to a $^{32}$P-labeled probe made from Clone 9 (Figure 13).

To determine the uniqueness of clones 5 and 12, the high copy suppressor plasmids 5 and 12 were digested with EcoRI and HindIII, run on 0.8% agarose gel and transferred to a nitrocellulose filter. The filter was then probed with $^{32}$P-labeled clone 12 in a similar way as described above (Figure 14).

2.2.5 Mapping of Clones 5, 9 and 12

The high copy suppressor plasmids were restriction mapped using several restriction endonucleases. This involved the setting up of digests with restriction enzymes that cut only once or not at all in the pUV2 vector. Double enzyme digests were then carried out to locate the restriction sites on the inserts and their respective distances apart by running the restriction fragments alongside vector digests, lambda DNA HindIII digest and X174 DNA-HaeIII digest markers on agarose and polyacrylamide gels respectively. The restriction digests were usually set up in 30µl total volumes comprising the plasmid DNA, enzyme buffer, RNAase (when miniprep DNA was used) and water in eppendorff tubes incubated at 37°C for 1 to 24 hours depending on the activity of the enzyme(s) used. In some cases, the inserts were subcloned into other plasmids such as pKS for further mappings, especially if some restriction sites exist on both the
vector as well as on the insert, more than once (Figures 15 and 16).

2.2.6 Clone Deletions

The clones 5, 9 and 12 were deleted to locate the smallest possible fragment capable of complementing the gpa1. Deletions were done using restriction enzymes based on the restriction sites mapped on the three clones. After these restriction endonuclease site-specific deletions, the fragment ends were either religated in the same plasmid clone using T4 DNA ligase or subcloned into pUV2 and transformed with GU1 and GU2 (MAta and MATa) haploid yeast strains (Figure 20).

2.2.7 Creation of Yeast Strains

First, the yeast strains whose plasmid replacement were to be carried out were transformed with the plasmid which was to replace the original strain maintenance plasmid. The transformed cells were then grown in the media where the replacing plasmid could propagate at the expense of the original strain maintenance plasmid at 30°C for 2½ to 3 days. Then 5μl of the culture media was plated on the SC-plate where the replacing plasmid could grow better for 2½ to 3 days at 30°C. The cells on the "master" plate were then replica-plated onto a velvet and then from the velvet onto an SC-plate containing the introduced plasmid marker gene and another plate containing the marker gene of the original strain maintenance plasmid. The replica plates were then incubated at 30°C for 12-15 hours. Plasmid loss on the original plasmid marker plate (observed by the presence of colonies footprint) was looked for by comparison with either the 'master' plate or the introduced plasmid marker replica plate, on which there would be no loss of colonies. Single colonies were then picked from the plate on
which no loss had occurred by it being matched with the lost colonies on its
replica plate. The single colonies were then appropriately purified by growing
them in the Sc-media of their marker genes. Plasmid dependence was then
confirmed by no growth when about 4μl of the cells were diluted with 100μl of the
culture media and plated on the original plasmid marker Sc-media and grown on the
replacing plasmid marker Sc-media. The newly created strains were then used for
a mock library transformation with the appropriate plasmid to test for the
usefulness or otherwise of the strain in library screening. (See the flow charts
on creation of yeast strains for the creation of the specific yeast strains in
Figures 6 and 7).
Transformation of GU1 and GU2 Strains with plasmids, U5C, U9C, U12C, UGC, and UC.

Plate on Sc-URA3 and grow for 2.5 days.

Pick single colonies and grow in Sc-URA3 for 2.5 days.

Plate 200ul of 1/10th dilution of the culture onto Sc-URA3 for 2.5 days.

Replica plate onto Sc-URA3 and Sc-LEU2.

Sc-LEU2 (look for plasmid loss from here).

Sc-URA3 (pick leu2 colonies from here).

Recheck for growth and no growth on Sc-URA3 and Sc-LEU2 respectively (Plasmid dependence).

Mock library transformation with YEpl3 and YEp9.

Figure 6: Flow Chart showing the creation of haploid strains GL1(GL2) of clones 5, 9, 12, UGC and UC.
**Creation of LG1(LG2)-TG Strains**

Transformation of GL1-9, GL1-12, GL2-9 and GL2-12 Strains with pTGC

Plate on Sc-TRP and grow at 30°C for 2.5 days

Replica plate onto Sc-TRP and Sc-URA3

Sc-URA3 look for ura3 lost colonies here)

Sc-TRP (Pick ura3 and TRP colonies here)

Confirm plasmid dependence

Mock transformation

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**Figure 7: Creation of LG1(LG2)-TG Strains**

The strains GL1(GL2)-9 and 12 were transformed with plasmid TGC and plated on SC-TRP. Selection of the strains were based on growth on SC-TRP and no growth on SC-URA media since the new strains have TRP1 as their marker gene.
Plasmid Constructs

The plasmids were constructed as illustrated in the figures (diagrams) (8, 9, 10, 11). Refer to legends in each case for description of the constructs.

2.2.8 Construction of TRP/GPA/CAN plasmid (pTGC)

Restriction endonuclease XhoI oligonucleotide linker ligation was used to change a Smal to a XhoI site in pTGS to give pTGX. This was performed by cutting pTGS with Smal enzyme. This was followed by phosphatasing the cut ends with calf intestine phosphatase (CIP). The cleaved and phosphatased pTGS was then purified and pelleted with chloroform/phenol and cold 100% ethanol respectively. Meanwhile, the XhoI oligonucleotide linker was kinased using T4 DNA kinase. The phosphatasing was necessary to prevent the religation of the compatible Smal ends. The phosphatased pTGS and the kinased XhoI oligonucleotide linker were then ligated using T4 DNA ligase.
Figure 8: Construction of pTGC

This illustrates the various steps involved in the construction of plasmid TGC. Restriction enzyme digests and ligation reactions were mainly used in the construction. The Smal site was changed to a Xhol site using a Xhol oligonucleotide linker to form plasmid TGX. The autonomous replicating sequence (ARS) was then cut from plasmid KSars. The ARS fragment was then ligated to TGX which had been cut with PstI and Xhol to form plasmid TGSXars. pTGSXars was then ligated to the SalI/Xhol fragment of CANI gene to form plasmid TGC. pTGC has TRP1 marker, ARS, CAN1 and GPA1 genes as its relevant genotype.
The PstI to XhoI restriction fragment of autonomous replicating sequence (ARS) was cut from plasmid ksARS and ligated to pTGX which had been cut with XhoI and PstI and gel purified to give pTGSXars. The plasmid TGSXars was then cut with XhoI, phosphatased and ligated to SalI/XhoI fragment of canavanine gene (CAN1) which was cut from the plasmid pYeCAN to give pTGSXarsCAN which was shortened to pTGC (Figure 8). SalI and XhoI cut to leave compatible sticky ends, but when ligated, neither site is retained.

All the constructs on the pathway leading to the final plasmid were confirmed using appropriate restriction digests and run on agarose gels alongside DNA markers and vector digests. The pTGC was used to create the LG1(LG2)-TG strains (Figure 7).

2.2.9 Construction of YEp9

YEp9 was constructed using YEp13M4 which has LEU2 as its marker gene, as the vector. YEp13M4 was cut with Sacl and SmaI restriction enzymes. The digested YEp13M4 was then agarose gel-purified and ligated to a purified Sacl/SmaI fragment of Clone9 (Figure 9).

2.2.10 Construction of plasmid RS416 "9"

Plasmid RS416, a centromere (CEN) plasmid and a URA3 based vector was used. The vector RS416 was KpnI and ClaI double digested and gel purified. Similarly, clone9 was KpnI and ClaI double digested using their compatible buffer, and gel purified.
Figure 9: Construction of YEp9

This shows steps involved in construction of YEp9. First, the vector YEp13M4, which has LEU2 as its marker was cut with Sacl and Smal double digest. This was then purified and ligated to the Sacl/Smal fragment of clone9 to form YEp9. YEp9 has MATa2, LEU2 marker and a 2μm origin of replication genes.
The gel purified RS416 was then ligated to the Kpn1/Cla1 fragment of MATa2 using T4 DNA ligase to give pRS416"9", a CEN based plasmid (Figure 10).

All other plasmids were similarly constructed by ligating cohesive restriction ends of purified fragments and vectors. Restriction enzyme digests were mainly used to confirm the plasmids. However, the yeast plasmids were further confirmed by marker gene prototrophy.

2.2.11 Disruption of MATa2

In order to test the importance of MATa2 in recovering the gpal strains, the MATa2 transcript was disrupted using URA3 marker gene.

A Hind III restriction endonuclease site was inserted into the XbaI recognition site on the gene subcloned into pBluescript (pks) vector forming the plasmid KSEK using HindIII oligonucleotide linker (Figure 31). The plasmid KSEK was cut with XbaI, phosphatased using calf intestine phosphatase and the DNA pelleted with ethanol. 1.0μg of the HindIII linker was phosphorylated using T4 polynucleotide kinase reaction and ligated to the pKESK phosphatased plasmid. This was followed by insertion of the 1.17Kb Hind III fragment of the URA3 marker gene into the newly created Hind III site based on HindIII cohesive end compatibility and T4 DNA ligase reaction (Fig 26).
Figure 10: Construction of Centromere plasmid.

This illustrates the steps involved in construction of plasmid RS416"9". The vector pRS416 was cut with Clal and Kpn1 and ligated to the Clal/Kpn1 fragment of clone 9 to form pRS416"9". Plasmid RS416"9" has CENS origin of replication, URA3 marker and MATa2 genes.
The various restriction fragments of CAN1 were transferred into pUV2, pUG, pYGC5, pYGC9, pYGC12 from pKCC to form the CAN1 versions of the respective plasmids. First, Xhol/SalI fragment of CAN1 was subcloned into the multiple cloning site of Bluescript Ks(−/+) to form pKCC. This increased and varied the restriction sites flanking the CAN1 gene. The plasmids UV2, UG, YGC5, YGC9 and YGC12 were then cut with Kpn1/SalI, SalI, Kpn1/BamI, Xhol/SalI and Kpn1/ClaI respectively and ligated to the various fragments of CAN1 which were cut with the corresponding enzymes to enhance sticky ends compatibility ligations.
The *SacI/KpnI* restriction fragment of the *URA3* disrupted gene (*mata2::URA3*) was then subcloned into pUV2 vector to give the plasmid UVEK-URA. The pVEK-URA was then transformed with GU1 and GU2 haploid yeast strains and plated on SC-URA (fig.27). Single colonies were picked after 2 days and inoculated into SC-URA media for another 2 days and plated on SC-URA-ARG+CAN. The disruption of the *MATa2* gene was confirmed with restriction endonuclease digests of both the vectors and the vectors plus the inserts.

2.2.12 Mating Assays

Patch mating tests were performed by replica-plating patches of cells to a lawn of the tester strains on permissive plates. Patches of the haploid strains carrying these respective plasmids; SPI(pUV2), SPI(pYGC9) GU1(pUV2), GU1(pYGC9), FY250(pUV2), FY250(pYGC9) GU2(pUV2) and GU2(pYGC9) were streaked on YPD plate to give the "master" plate. The master plate was then incubated at 30°C for 60 hours for the cells to grow. The plate was then replicated onto a velvet, then from the velvet onto SC-URA plate and SC-HIS-TRP plate which had been spread with DC14 cells. The master plate was re- replica plated onto a new velvet followed by replication from the velvet onto SC-HIS-TRP spread with DC17 cells. Diploids were selected on the SC-HIS-TRP plates whilst the SC-URA plate served as the control. The plates were incubated at 30°C overnight.
2.2.13 Quantitative Mating Assay

Single colonies of the strains with their plasmids were picked from the YPD plate and grown in YPD media for 60 hrs. $10^5$ cells of each strain were counted and mixed with $10^5$ cells of the tester strains DC14 and DC17 in TE buffer and incubated at 30°C for 3 hours to allow the cells to mate. 1.5 mls of SC-HIS-TRP was added to each tube to select for the diploids. The diploid cells were counted from drops of the cell culture fixed on Hematocrit under a Nikon phase-contrast microscope.

2.2.14 Sequencing

Both DNA strands of MATa2 were sequenced by the dideoxy chain termination method (Sanger et. al., 1977). The sequenase version 2.0, a genetic variant of bacteriophage T7 DNA polymerase and [a-32P]dATP were used. The MATa2 gene was subcloned into pBluescript 11KS(+/−) using cohesive restriction ends. MATa2 was cleaved into almost two equal parts and subcloned into multicloning sites of pks forming the plasmids KSEX and KSSX (Figure 29 and 30). Unlike Sanger’s method which stressed on single stranded template sequencing vectors such as bacteriophages, the use of plasmids called for denaturation of the plasmids prior to the annealing reaction to create single stranded templates.

The purified RNA-free plasmid DNAs were prepared using the Qiagen method. 7µg of plasmid DNA in 200µl total volumes were alkaline-denatured by adding, 20µl of 0.2M NaOH containing 0.2mM EDTA and incubated at 37°C for 30 minutes. The denatured DNAs were neutralized by adding 20µl of 3M sodium acetate (pH5.2) and the DNA precipitated with 600µl of ethanol ($-70^\circ$C, 15 min). After washing the pelleted DNA with 70% ethanol, it was redissolved in 6µl of distilled water. 2µl of sequenase reaction buffer and 2µl of T7 and T3 DNA polymerase primers were
then added. Annealing of primers to the template was done by warming the capped tubes containing the DNA templates and primers to 65°C for 2 min and leaving to slowly cool from 65°C to 30°C in a temperature block. The rest of the reactions were as described by Sanger et. al., (1977).

2.2.15 Transformation of Bacteria with Plasmids

Plasmid DNAs and their DNA inserts were routinely propagated by transforming *E. Coli* strain DH5α with the plasmids. The DH5α cells were cultured in 250ml flasks (OD₆₀₀ = 250), usually in 100ml LB+ ampicillin media. The cells were then spun down at 3,500 rpm in a Sorvall GS3 rotor set at 4°C. The supernatants were then decanted and the pellets combined in 20ml transformation buffer (TFB) (see appendix) kept on ice for 10 min and then centrifuged at 4°C for 10 min at 3,500 rpm in a Sorvall GS3 rotor. The supernatant was well decanted and 2ml TFB added to the pellets. 70μl of DnD (See appendix) and water to 10ml total volume was added gently, swirled and kept on ice for 15 min. Another 70μl DnD aliquot was added, swirled and again kept on ice for 15 min. 200μl of cells were then aliquoted into eppendorf tubes and either 4μl of ligation reaction added or a lesser concentration of purified plasmid DNA added and kept on ice for 30 min. These were followed by 90 seconds heat shocking at 42°C after which the cells were kept on ice for 1 min and quickly spun at 6,000xg for 3 seconds in a microfuge. The supernatant was then removed and the cells resuspended in 200μl of SOC (see appendix) and incubated for 50 min at 37°C thus allowing the cells to recover. After the incubation period, the cells were spread on LB+ Amp plates and incubated at 37°C overnight. However, at certain times part of these competent cells were frozen at -80°C and readily used when needed except that the efficiency of transformation slightly fell below that of the freshly prepared competent cells.
2.2.16 **Transformation of Yeast with Plasmids**

Most often, yeast transformations were used to determine the function of genes cloned into plasmids and also for marker genes prototrophy. In this work, most of the yeast strains used for the transformations were haploid strains.

Cells were cultured to $0.5 \times 10^7$ to $3.0 \times 10^7$ cells/ml usually in 100ml YPD culture media in 250ml flasks. The cells were spun for 5 min at 2,000rpm using Beckman bench-top centrifuge at room temperature. After decanting the supernatant, the cells were resuspended in 20ml 0.1M LiOAC, containing 1M sorbitol per 50 ml culture and kept at room temperature for 10 min. The cells were then spun at 2,000rpm for 5 min at room temperature in a Beckman bench-top centrifuge and resuspended as before in 0.1M LiOAC, 1M sorbitol. The cells were then pelleted again by spinning at 2,000rpm for 5 min at room temperature and $6.5 \times 10^7$ cells were resuspended per ml in 0.1M LiOAC+1M sorbitol followed by addition of 20μl denatured carrier DNA per ml (Salmon sperm DNA 5mg/ml stock). 0.3ml cells were aliquoted into 1.5 ml eppendorf tubes and about 10μg (20μl) DNA added, mixed and kept at 30°C for 15 min without shaking. 0.7ml of 50% PEG in TE (Polyethylene glycol in Tris EDTA) was added to each tube, mixed by inversions and incubated at 30°C for 30 min with inversions every 10 min. This was followed by the addition of 0.1M DMSO (Dimethyl sulfoxide) with immediate mixing and heat shocking at 42°C for 5 min. The cells were then spun down at 7,500xg for 5 sec in a microfuge and resuspended in 200μl TE plus penicillin/streptomycin after the supernatants were removed. The cells were then plated on the appropriate plate and grown at 30°C for 60 hours.
2.2.17 Mini Plasmid Preparation Procedure

Two-and-half (2½) mls of LB+AMP bacteria culture was grown at 37°C overnight with shaking. 1.5mls of the culture was then spun in microcentrifuge tubes for 10 seconds and the supernatant decanted such that about 60μl of it was left in the tube into which the cells were resuspended completely (using 200μl pipet). 300μl of TENS solution (10mM Tris-HCl, pH 7.5, 1mM EDTA, 0.1N NaOH, 0.5% SDS) was added and vortexed for 4 sec until the cells lysed and the mixture became viscous (up and down pipetting also lysed the cells). 150μl of 3.0M sodium acetate, (pH5.2) was then added and vortexed for 5 seconds to mix completely. Cell debris and chromosomal DNA were pelleted by spinning for 2 min at 12,000xg in a microfuge, the supernatant transferred to a fresh tube and 900μl of cold 100% ethanol added and mixed well by inverting the eppendorf tubes. Plasmid DNAs and RNAs were pelleted by spinning for 5 min at 12,000xg in a microfuge. The supernatants were then discarded and the pellet, having a white appearance rinsed twice with 1ml 70% cold ethanol. Residual ethanol was removed after another quick spin and the DNA pellets were then resuspended in 30-40μl of TE buffer or sterile deionised water.

2.2.18 Preparation of Yeast Genomic DNA

The cells were cultured in a rich medium such as YPD overnight at 30°C in an incubator with shaking at 250rpm. 10ml portions of the cultured cells were spun down at 2,000rpm in Beckman bench-top centrifuge for 5 min and resuspended in 1 ml TE, and transferred into eppendorf tubes. The cells were then spun down for 30 min in a microfuge at 7,500xg. Cells were then resuspended in 1 ml buffer containing 1M sorbitol, 0.1M Tris (pH 7.5), 50mM EDTA, 50mM GME (3.5ml/ml) plus 0.5mg zymolyase/ml, and incubated for 40 min at 37°C, pelleted for 30 sec in
microfuge at 6,000xg and the spheroplasts resuspended in 0.5ml buffer containing 0.1M Tris(pH7.5) and 50mM EDTA. 25μl of 10% SDS was added, mixed and heated at 50°C for 10 min, followed by the addition of 200μl of 5M KOAC and kept on ice for 30 min. This was followed by a 10 min spinning in the cold at 12,000xg in a microfuge and the supernatant transferred into a new tube, 1ml ethanol added, mixed and kept at room temperature for 5 min. It was then spun at 12,000xg in a microfuge for 5 min, the pellet washed with 70% ethanol and resuspended in 300μl TE. 3ml of 10% SDS was added, mixed and also 10μl of 5mg/ml Proteinase K was added, mixed and incubated at 37°C for 1 hr. The mixture was then phenol and chloroform extracted and 30μl of 3M NaOAC or 75μl of 6M NH₄OAC added. 600μl of ethanol was then added and the mixture kept at -20°C for 5 min. The genomic DNA was then pelleted by spinning at 12,000xg in a microfuge for 5 min. The pelleted DNA was then washed with 70% ethanol, dried in a speed vacuum and dissolved in 50μl TE.

2.2.19 Qiagen Plasmid Midi Preparations

The protocol described below is as given in the QIAGEN plasmid Midi and Maxi preparations. This protocol involves elution of DNA through columns making the plasmid DNA free from chromosomal DNA and RNA. The cultured DH5α cells usually in 200ml LB plus ampicillin medium were spun at 3,500 rpm in a Sorvall GS3 rotor for 10 mins and the pellets suspended in 4ml P1 buffer. Four ml's of buffer P2 was added, mixed gently and incubated at room temperature for 5min. Then 4ml of buffer P3 was added, mixed immediately but gently and centrifuged at 4°C for 30 min at 12,000xg. The supernatant was promptly removed and applied to QIAGEN-tip 100 which has been already equilibrated with 5ml of buffer QBT and allowed to enter the resin by gravity flow. The QIAGEN-tip 100 was then washed
with 10 ml of buffer QC and the DNA eluted with 5ml of buffer QF into a new tube. The DNA was then pelleted with 3.5ml of isopropanol which had been previously equilibrated to room temperature by centrifuging at 4°C using the Sorvall ultracentrifuge set at 12,000xg. The DNA was washed with 70% ethanol, vacuum dried for 10min and redissolved in 100μl TE. This was mainly the protocol used for purifying large quantities of plasmid DNA.

2.2.20 Revertants

Clone 9 revertants were selected to compare their morphology with those of normal cells. GL1 (GL2)-9 or GU1(GU2)-U9C strains were grown in 3ml YPD or SC - complete media at 30°C for 3 days. About 10μl of the culture was then plated on SC-URA-ARG+CAN and incubated at 30°C for 2 days. The morphology of the revertants was then observed under a Nikon phase-contrast microscope after fixing the cells on microslides. The fixing was done by aliquoting 1.0μl of the cells onto a microslide and 1.5 ml of a buffer containing 1M sorbitol and 0.1MTris EDTA (pH7.5). These were then covered with a microslide cover and viewed under the microscope.

2.2.21 Mock Transformations

Mock transformations were done as described by the flow charts to check the background of strains which were to be used for screening libraries. The strains were transformed with the appropriate vector plasmid. 5% of the transformation was then plated on the appropriate media and the rest grown in 2.5mls of the same media. 5μl of the cells in the media were then plated on the selective plate that destroyed the strain maintenance plasmids. The 5% of the transformed cells on the plate were just to determine the efficiency of the transformation. A clean background as indicated by no colony or 1-3 colonies confirmed the usefulness of the strains; colonies of more than 10 had a poor background and were discarded.
3.1 Isolation of the \textit{MATa2, YGC1} and \textit{MCM1} Genes

High copy number plasmids that were able to complement the \textit{gpa1} defect in \textit{gpa1::HIS3} cells were isolated from a yeast library in the plasmid UV (a URA3 marker based vector). Several plasmids containing nonhomologous inserts (Fig.4 and 12) were able to complement the \textit{gpa1} mutation (in some cases only partially). Two nonhomologous plasmids that allowed complementation in both \textit{MATa} and \textit{MATa} \textit{gpa1} strains were analyzed in detail. The first plasmid, pYGC9 contains the \textit{MATa2} gene which has previously been characterized but its function of complementing \textit{gpa1} yeast strains has not been unraveled. The second plasmid, pYGC12 contains what is known as the yeast G – protein complementation gene1 (YGC1) which has hitherto not been characterized. Another plasmid pYGC5 contains the \textit{MCM1} gene which was known to be a general transcription activator. However, because the \textit{MCM1} product complements only \textit{MATa} \textit{gpa1} cells but not \textit{MATa} \textit{gpa1} strains unlike \textit{MATa2} and \textit{YGC1}, there was little characterisation of it here. The ability of the YGC9 plasmid to suppress the \textit{gpa1} mutation suggested that it might encode a component of the pheromone response and/or recovery pathway or have a function similar to a component of this pathway; therefore, this gene was characterized further. From the Southern hybridization results (figures 12, 13 and 14) it was clear that none of the inserts in plasmids YGC5, YGC9 and YGC12 had a sequence homology to G-protein alpha subunit (\textit{GPA1}) gene of \textit{Saccharomyces cerevisiae}. Another reason why much attention was paid to the pYGC9 insert was that its \textit{gpa1}-complemented cells looked more viable. Additionally, the size of the insert was comparatively smaller. The restriction map of the pYGC9 insert is shown in
figures 16, 19 and 20, whilst those of pYGC5 and pYGC12 are shown in figures 17 and 18, respectively. Comparing the three restriction maps of the inserts to that of GPA2 (another G-protein alpha subunit in yeast) also showed no sequence or restriction mapping similarity. It was important to compare the sequences and/or restriction maps of the inserts to that of GPA1 since GPA1 is known to be the inhibitor of the pheromone signal transduction pathway in Saccharomyces cerevisiae.

Plasmids derived by deletions or subcloning of fragments of the pYGC9 inserts were constructed and tested for their ability to suppress the gpal mutation, as described in figure 20. Multicopy plasmids containing a 4.8 kb Hind III/EcoRl fragment of pYGC9 were able to complement the gpal mutation (figure 22). When the size was narrowed down to 1.8kb Eagl/Kpn1 (Figure 19) or Cla1/Kpn1 (Figure 20) fragments of pYGC9, it was still able to complement the gpal lethality both in MATa and MATa cell types. Thus the active part of pYGC9 involved in the complementation is located between Eagl and Kpn1.

3.2 The Southern Analysis

The Southern blot showed that GPA1 does not hybridize to the high copy suppressor plasmids (Figure 12). In Figure 12, the faint signals seen in lanes 2-10 correspond to plasmid sequences because the probe fragment contained a small amount of contaminating vector fragment that was also radioactively labeled. Figure 13 indicated that clone9 hybridized to all the other clones except clones 5 and 12. the band in lane 10 (Figure 13) was due to a residual signal from the hybridization on (Figure 12) which was not completely removed by the stripping procedure. Figure 14 showed that clones 5 and 12 are each unique since they do not hybridize to each other.
3.3 Phenotype of \textit{MATa2} Disruption

To test for the importance of \textit{MATa2} to the pheromone signal transduction pathway, the gene was disrupted with \textit{URA3} marker gene (Figure 26). Insertion of a HindIII fragment of the \textit{URA3} marker gene into the XbaI site changed to HindIII site within the 1.8kb EagI/KpnI fragment eliminated the ability of the plasmid to complement \textit{gpal} (Figures 26 and 27). This result indicated that the gene required for complementation is contained within the EagI-KpnI fragment, since its disruption led to the cells lethality (Figure 27).

3.4 Overexpression of \textit{MATa2} is Not a Prerequisite for \textit{GPA1} Complementation

In order to test whether overexpression of the \textit{MATa2} product is necessary for the suppression of \textit{gpal} strains, the 1.8kb EagI/KpnI fragment of pYGC9 was cloned into a centromere plasmid vector RS416 to obtain pRS416"9" (Figures 10 and 24). Centromere plasmids maintain the plasmid copy number at one per cell. A selection was imposed for uracil prototrophy and growth at 30°C (Figure 25). The ability of the centromere plasmid to suppress the \textit{gpal} mutation suggests that overexpression was not required for complementation by \textit{MATa2} (Figure 25).

No \textit{GPA1}-containing plasmid was isolated in the original screen for suppression of \textit{gpal} mutation (Figure 12). To be certain that suppressing the \textit{gpal} did not require a mutation that had arisen during cloning, overlapping clones were isolated by hybridization to the pYGC9 insert (Figure 13) and shown to be capable of suppression of the \textit{gpal} mutation. Figure 15 shows one of the pictures taken during restriction mapping of clone 9. In the picture, double digests of clone9 using some restriction endonucleases are shown.
Figure 12: High Copy Suppressors are not GPA1

High copy suppressors plasmids were digested with EcoRI and HindIII, run on a 0.8% agarose gel, blotted onto a nitrocellulose filter and probed with the $^{32}$P labeled 1.9kb EcoRI fragment of GPAI. Lane 1: Lambda HindIII digest marker: Lanes 2-9: suppressors 5,6,7,9,11,12,14 and 20, respectively. Lane 10: GPAI EcoRI fragment.
Figure 13: Suppressor 9 Hybridizes to Most Other Suppressors

The same nitrocellulose blot (used in fig.12) was stripped in a boiling water bath and rehybridized to a $^{32}$P-labeled probe made from suppressor 9. The vector fragment hybridizes in each lane except lanes 1 and 10. The insert fragments of suppressors 6, 7, 9, 11, 14 and 20 also hybridize but not suppressors 5 and 12. The band in lane 10 is a residual signal from the previous hybridization that was not completely removed by the stripping procedure.
Figure 14:  **Suppressors 5 and 12 are each unique**

High copy suppressor plasmids 5 and 12 were digested with EcoR1 and HindIII, run on an agarose gel and transferred to a nitrocellulose filter. The filter was then probed with ^32P labeled clone 12. Lane 1, Lambda DNA-HindIII digest marker; lane 2, clone 5; lane 3, clone 12. These results show that clones 5 and 12 have 2 different inserts.
Figure 15: Restriction Mapping of Clone 9

This shows an example of several double restriction digests during the mapping of clone 9. Lane 1 Lambda DNA-HindIII digest marker [sizes of fragments from origin are 9.42, 6.56, 4.36, 2.32, and 2.03, respectively. Lane 2, Clal EcoRl; Lane 3, Clal+Xhol; Lane 4, Clal+Pstl; Lane 5, Clal+Sacl; Lane 6, Clal+Apal Lane 7, Pst+ Hind III; Lane 8, Xhol+Hind III; Lane 9, Xhol+Sacl; Lane 10, Xhol+EcoRl; Lane 11, Xhol+Apal; Lane 12, Apal; Lane 13, Apal+KpnI; Lane 14, Apal+EcoRl; Lane 15, Apal+Sacl; Lane 16, Sacl; Lane 17, Sacl+Hind III; Lane 18, EcoRl+Hind III; Lane 19, EcoR+Sacl. Digests were run on 0.8%agarose gel.
Figure 16: Restriction Map of Clone 9

Clone 9 was mapped using restriction endonuclease digests run on agarose and polyacrylamide gels alongside marker DNAs. Restriction sites highlighted are unique on the insert. The EcoRI site highlighted is almost at the centre of the insert. The size of the insert is 4.8kb.

Figure 19: Restriction Map of MAT\alpha2

This Eag/KpnI fragment of clone9 Complements the gap1 lethality both in MAT\alpha and MAT\alpha Yeast strains.
The Smal/SphI fragment and the Smal/XhoI segments are both able to complement the gpal in MATα cells. The restriction sites are: EcoRI (R1), KpnI, Smal, Clal, SphI, SacI, XhoI, XbaI, Clal, PvuII and HindIII.

ORF stands for the open reading frame, and its direction is shown by the arrow.

Figure 17: Restriction Map of Clone 5
Figure 17: Restriction Map of Clone 5

The Smal/SphI fragment and the Smal/Xhol segments are both able to complement the gpal in MATa cells. The restriction sites are: EcoRI (RI), KpnI, Smal, Clal, SphI, SacI, Xhol, Xbal, Clal, PvuII and HindIII.

ORF stands for the open reading frame, and its direction is shown by the arrow.
Figure 10: Restriction Map of Clone 12

Figure A shows the ORF of YGC1 from the N-terminal end. This fragment of YGC1 is fully capable of complementing the gpal lethality: Fig. B shows restriction map of the original clone 12 (YGC1). Restriction sites are indicated by the names of the enzymes.
Figure indicates that the active fragment of clone9 which is complementing the gpal is between ClaI and Kpnl. Complementation is based on the rescuing of gpal cells. Regions tested for their ability to complement the gpal mutation in pUV plasmids are indicated in rectangular bars. Restriction sites Rl(EcoRI), HindIII, SacI, ClaI, Xbal and Kpnl. Ability to complement the gpal is indicated with positive signs (+) and inability to do so with negative signs (-).
3.5 MATa2 has no Sequence Homology to G-proteins

The pYGC9 insert could not be identified from its restriction map. However, when about 500 bp of the Eag1/Kpn1 fragment was sequenced using the Sanger dideoxy method and the sequence fed into the BLAST Database search for homology, the MATa2 gene was identified (Figure 21). The MATa2 sequence had already been reported by Nasmyth and Tatchell (, 1980). The MATa2 gene had no homology to any known G-protein and/or its subunit(s). It had no classified region implicated in guanine-nucleotide binding and GTPase activity. The MCM1 product is also not known to have either GDP/GTP-binding site or GTPase activity. However, in the case of the YGC1, the sequence did not have any GDP or GTP binding domain, but further characterization is going on currently to classify this gene.

3.6 MATa2 Suppresses Mating in both MATa and MATa gpa1 yeast cells

Table 6 shows the average of two qualitative mating assay results of pYGC9 in both MATa and MATa cell types. Judging from the number of diploid cells selected on the SC-His-Trp prototroph, there seemed to be suppression of mating by pYGC9 in both GUI and GU2 cells as compared to SP1 and FY250 haploid cells. GUI and GU2 are gpa1 strains whereas SP1 and FY250 are GPA1 haploid strains. Suppression of the mating seemed to be more pronounced in MATa gpa1 cells than MATa gpa1 cell types.

3.7 The Loss of MATa2 Function Results in late G1 Arrest

Microscopic examination of the spores containing a disrupted MATa2 showed that they germinated and went through several cell divisions.
Figure 21: Sequence of MATa2.

The regions underlined are those that were sequenced before using the BLAST Database Search to look for sequence homology.
Figure 22: Clone 9(a2) rescues both MATα and MATα cells

Plates show growth on Sc-URA-ARG+CAN1. Survivors lost the pLEU2/GPA1/CAN1 plasmid as indicated by no growth on the pUV2 plates. (A) GI transformed with clone9 and pUV2 at left and right respectively. (B) GI2 transformed with clone9 and pUV2 at left and right respectively.
Confirmation of Plasmid RS416'9'.

This shows restriction enzyme digests of the plasmid RS416''9'', a CEN plasmid. The various plasmid constructs were similarly confirmed. Here, pRS416'9' was cut with Clal and Kpn1. Lane1 is the Lambda DNA-HindIII digest marker (sizes from origin are 9.42, 6.56, 4.36, 2.32, 2.03). The smaller (lower) band corresponds to the MATa2 gene, whilst the larger (upper) band is the CEN plasmid vector RS416'9' (lanes 2 to 13).
**Table 5:**

Transformation Results

<table>
<thead>
<tr>
<th>Yeast Strain</th>
<th>Mating Type</th>
<th>Plasmid</th>
<th>Complementation of gpal</th>
</tr>
</thead>
<tbody>
<tr>
<td>GU1</td>
<td>MATa</td>
<td>clone 9(α2)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>clone 5(MCM1)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>clone12(YGC1)</td>
<td>+</td>
</tr>
<tr>
<td>GU2</td>
<td>MATα</td>
<td>clone9(α2)</td>
<td>+</td>
</tr>
<tr>
<td></td>
<td></td>
<td>clone5(MCM1)</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>clone12(YGC1)</td>
<td>+</td>
</tr>
</tbody>
</table>

The transformation results indicate that clones 9 and 12 complement gpal in both MATa and MATα cell types whereas clone5 only complement gpal in MATa cells. The positive signs indicate the ability of the plasmids to keep the gpal cells alive whereas the negative sign shows the inability to do so.
Table 6

MATING ASSAY RESULTS

<table>
<thead>
<tr>
<th>Mating plasmid</th>
<th>Mating type of yeast strain</th>
<th>DC17(α-cells), Number of colonies</th>
<th>DC14(α-cells), Number of diploid colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>GU2 with pUV2</td>
<td>MATα</td>
<td>0</td>
<td>75</td>
</tr>
<tr>
<td>GU2 with pYGC9</td>
<td>MATα</td>
<td>0</td>
<td>5</td>
</tr>
<tr>
<td>FY250 with pUV2</td>
<td>MATα</td>
<td>2</td>
<td>2,540</td>
</tr>
<tr>
<td>FY250 with pYGC9</td>
<td>MATα</td>
<td>1</td>
<td>996</td>
</tr>
<tr>
<td>GUI with pUV2</td>
<td>MATα</td>
<td>612</td>
<td>0</td>
</tr>
<tr>
<td>GUI with pYGC9</td>
<td>MATα</td>
<td>32</td>
<td>0</td>
</tr>
<tr>
<td>SPI with pUV2</td>
<td>MATα</td>
<td>1210</td>
<td>0</td>
</tr>
<tr>
<td>SPI with pYGC9</td>
<td>MATα</td>
<td>1500</td>
<td>1</td>
</tr>
</tbody>
</table>

The results show that clone9 complemented strains mated with both MATα and MATα mating type cells. The assay results also indicate, however, that MATα cells containing pYGC9 mate better than MATα cells. Clone9 suppresses mating in wild type MATα cells (FY250) but not in wild type MATα cells (SPI) as shown. The tester or mating type strains are DC17(α-cells) and DC14(α-cells).
Map of plasmid RS416"9"

This shows a centromere plasmid RS416"9" map which was constructed and transformed with the GU1 and GU2 yeast strains to find out whether complementation of gpal by MATa2 depended on the copy number or not. The URA3 marker, the CEN centre and the alpha2 genes are shown with the arrows indicating their direction of transcription.
Figure 25: Rescue of Cells In Single Copies by Clone 9

pRS416"9" is a CEN plasmid containing clone9, which is capable of complementing the gpal both in MATa and MATa cells. Thus, complementation is independent of the copy number. A; GU1 transformed with the plasmids RS416"9" and RS414 at left and right respectively. B; GU2 transformed with the plasmids RS416"9" and RS414 at left and right respectively. Cells were plated on Sc-URA-ARG+CAN.
Figure 26: Disruption of Clone 9

Figure 26 shows the disruption of alpha2 with the URA3 Marker. The HindIII site was inserted at the XbaI site and the HindIII fragment of URA3 was used to frame-shift the alpha2 gene. The disrupted gene was confirmed by restriction enzyme digests. The XbaI site was cut with XbaI endonuclease, phosphatase using calf intestine phosphatase (CIP) and the HindIII oligonucleotide linker which had been phosphorylated using T4 polynucleotide kinase, inserted at the original XbaI site using the T4 DNA ligase reaction. The introduced HindIII site was then cut with HindIII endonuclease and the HindIII fragment of URA3 inserted there to frame-shift the open reading frame (ORF) of the alpha2 gene. Figure 27 shows that this disruption is lethal to the cells.
Disruption of MATα2 Leads to Constitutive Cell-Cycle Arrest.

(A): GU1 transformed with clone9 and pUVEK-URA showing at the left and right respectively. (B): GU2 transformed with clone9 and pUVEK-URA at left and right respectively. Transformed colonies were shown on SC-URA-ARG+CAN plates. This figure indicates that the disruption is lethal to both MATα and MATα cell types. Plasmid UVEK-URA contains MATα2 which has been disrupted with URA3 marker. Growth of transformed GU1 and GU2 cells with MATα2 and no growth with UVEK-URA show that disruption of MATα2 is lethal to the cells indicating that the signaling pathway is constitutively arrested.
Micromanipulation of these cells revealed that most of the cells were unbudded and some of them showed an aberrant cell morphology, similar to the "shmoo" of cells arrested by mating factors (table 7 and plate 1). This phenotype is characteristic of cells arrested in late G1 phase. Also, figure 27 shows that disruption of the MATa2 gene product is lethal to both MATa and MATα cell types.

### 3.8 Plasmid Constructs and Creation of New Yeast Strains

In an attempt to screen mammalian cDNA library for possible pYGC9, pYGC5 and pYGC12 analogs, plasmids U5C, U9C, U12C, UGC and UC were constructed (figure 11). These were used to create new hosts for GU1 and GU2, forming the GL1(GL2)-5,9,12, UGC and UV strains all of which had the URA3 gene as their selective marker and canavanine 1 gene for the inhibition of these plasmids in the strains (Figure 6, 11). It, however, turned out that the background for these strains obtained from the mock transformations were too high to be used for screening purposes.

Another plasmid TGC (TRP1/GPA1/CAN1) was constructed which had the autonomous replicating sequence (ARS) giving it an advantage to be lost easily (Figure 7, 8). The TRP1 is the marker gene since the cDNA library had the LEU2 gene as its selective marker. Construction of pTGC initially involved a lot of techniques including PCRings out the CAN1 gene from the wild type yeast genomic DNA.
Figure 28: Map of plasmid TGC

Plasmid TGC was constructed and its strain created. The idea was to use this strain for screening the mammalian library. *EcoRI* fragment of *GPA1* is shown as shaded part. *TRP1* marker, autonomous replicating sequence (*ARS1*), and the *CANI* marker genes are also shown.
The arrows indicate the direction of sequencing of the Eagl/Xbal fragment of MATα2. The Eagl/Xbal fragment of MATα2 was subcloned into Bluescript (pKS+1/-) for sequencing purposes. The T7 and T3 promoters of pKS were useful since T7 and T3 sequencing primers are readily available in the market.
Figure 30: Plasmid KSXS

Direction of the arrows indicates direction of sequencing by the sequence version 2.0 enzyme. In plasmid KSXS, the Xbal to Smal fragments of MATα2 were subcloned into vector pks. The T7 and T3 promoters were helpful since T7 and T3 sequencing primers are commercially available.
Outline of plasmid KSEK.

This plasmid was constructed and used for the disruption of MATa2 with URA3 Marker gene. The marker gene was inserted at the Xbal site after the HindIII restriction site was introduced. First, Xbal endonuclease was used to digest KSEK. Since Xbal is unique on KSEK, a HindIII restriction endonuclease oligonucleotide that fitted into the sticky ends of the Xbal restriction site was introduced and ligated to it using T4 DNA ligase.
**Table 7**

**MORPHOLOGY OF REVERTANTS OF gpa1 CELLS**

<table>
<thead>
<tr>
<th>Strain</th>
<th>Cells</th>
<th>Colonies</th>
</tr>
</thead>
<tbody>
<tr>
<td>9Rev1</td>
<td>some shmoos, small</td>
<td>large</td>
</tr>
<tr>
<td>9Rev2</td>
<td>high percent, large shmoos</td>
<td>small</td>
</tr>
<tr>
<td>9Rev3</td>
<td>some shmoos, small</td>
<td>medium</td>
</tr>
<tr>
<td>9Rev4</td>
<td>high percent, large, long shmoos</td>
<td>small</td>
</tr>
<tr>
<td>9Rev5</td>
<td>some shmoos, small</td>
<td>big</td>
</tr>
<tr>
<td>9Rev6</td>
<td>very high percent</td>
<td>small</td>
</tr>
<tr>
<td>9Rev7</td>
<td>high percent, large shmoos</td>
<td>small-med.</td>
</tr>
<tr>
<td>9Rev8</td>
<td>some shmoos, small</td>
<td>big</td>
</tr>
<tr>
<td>9Rev9</td>
<td>high percent, small, some long shmoos</td>
<td>big</td>
</tr>
<tr>
<td>9Rev10</td>
<td>high percent, small</td>
<td>big</td>
</tr>
<tr>
<td>9Rev11</td>
<td>high percent, large shmoos</td>
<td>small-med.</td>
</tr>
<tr>
<td>9Rev12</td>
<td>moderate to high percent, small shmoos</td>
<td>big</td>
</tr>
<tr>
<td>9Rev13</td>
<td>some shmoos, small</td>
<td>medium</td>
</tr>
<tr>
<td>9Rev14</td>
<td>moderate to high percent</td>
<td>medium</td>
</tr>
<tr>
<td>9Rev15</td>
<td>high percent, large shmoos</td>
<td>small-med.</td>
</tr>
<tr>
<td>9Rev16</td>
<td>high percent, small shmoos</td>
<td>big</td>
</tr>
<tr>
<td>9Rev17</td>
<td>high percent, small semi-shmoos</td>
<td>big</td>
</tr>
<tr>
<td>9Rev18</td>
<td>high percent, small shmoos</td>
<td>small</td>
</tr>
<tr>
<td>9Rev19</td>
<td>high percent, large, round shmoos</td>
<td>small-med.</td>
</tr>
<tr>
<td>9Rev20</td>
<td>high percent, large semi-shmoos</td>
<td>small</td>
</tr>
<tr>
<td>9Rev21</td>
<td>high percent, very large, round shmoos</td>
<td>small-med.</td>
</tr>
<tr>
<td>9Rev22</td>
<td>high percent, round shmoos, 25% cell death</td>
<td>small</td>
</tr>
<tr>
<td>9Rev23</td>
<td>high percent, shmoos, some cell chains</td>
<td>big</td>
</tr>
</tbody>
</table>

Reversion of the strains may occur as a result of a switch of the uraj of the chromosome with the URA3 of the U9C plasmid. Reversion also occurs as a result of mutations in the genes involved in the pathway. The cells were observed under the Nikon phase-contrast microscope. The revertants were created from clone 9 by growing a number of colonies picked from SC-URA-ARG-CAN plate in SC-URA media. The strain example 9rev1 means revertant number one from clone 9 etc. Cells are described by their sizes, percentage of the cells shmoosed per colony and the shape of the shmoos as viewed under the microscope. The colonies grown are also described by their sizes, whether big, small or medium.
Plate 1 shows the morphology of Wild Type and clone 9 revertant cells. The "shmoo" shaped cells can be observed on the Wild Ty pe revertant.
The pathway for the differentiation of the pyC1 cells shows differentiation of the pyC1 cells as compared with the wild type cells. This might be due to mutations or alterations in genes such as STRA18 or new gene A.
However, the PCR CAN was truncated and not active because the oligonucleotide primers used did not flank the CAN1 gene completely. Plasmid YepCAN was however obtained and the entire CAN gene cut out for the construction of pTGC (figure 8). The LG1 (LG2)-TG strains created from the TGC gave a very good mock transformation background.

These LG1 (LG2)-TG strains were used to isolate two genes from a mammalian cDNA library (Colicelli, personal communication) by virtue of their ability to complement gpal yeast cells. Both genes were not previously characterized. These mammalian genes have no sequence homology to GPA1.

Reversion of strain occured as a result of switch of the ura3 of the chromosome with the URA3 of the U9C plasmid as shown in table 7. Reversion may also have occured as a result of mutations in the genes involved in the pathway (table 7 and plate 1).

Plate 1 shows the shmoo morphology of revertants derived from clone 9. Plate 2 shows differentiation of YGC1 cells compared with wild type cells. Plate 2 also showed that cell cycle arrest can be distinguished from cell differentiation.
CHAPTER FOUR

DISCUSSION AND CONCLUSIONS

4.1 General Strategy for Selecting the High Copy Suppressor Clones

The yeast strain GU1 having the genotype \textit{MATa/gpal::HIS3/ leu2/ura3/can1} was kept alive with the plasmid TLCG whose relevant genotype is \textit{GPA1/LEU2/CAN1}, since the \textit{gpal} strain would have remained in the constitutively cell arrested state (death). Transforming the GU1 strains with the clones \((X/URA3)\) caused the GU1 to harbor two plasmids at the same time, that is plasmids TLCG and the clones (figure 4). When the transformed cells were plated on Sc-URA-ARG+CAN media, the plasmid TLCG was destroyed because \textit{CAN1} is a toxic arginine (ARG) analog which allows the arginine permease to accumulate canavanine drug (Figure 5). \textit{can1} is a recessive mutation which eliminates the arginine permease, thus preventing canavanine drug from entering the cell. The GU1 cells and the clones are therefore resistant to canavanine. Because the CAN1 gene is tagged to the \textit{GPA1} maintenance plasmid, survival of cells after the destruction of the \textit{GPA1} means that the high copy suppressor plasmids were responsible for keeping the GU1 cells alive or complementing the \textit{gpal::HIS3} lethality (figures 4, 5, 22 and table 5).

4.2 Identification of \textit{MATa2, YGC1} and \textit{MCM1} Genes Involved in the Pheromone Response Pathway

\textit{Saccharomyces cerevisiae} genes \textit{MATa2, YGC1} and \textit{MCM1} have been identified by isolation of plasmids that were able to complement or suppress a \textit{gpal::HIS3} mutation.
MATa2 gene is known to be required for the determination of both haploid and diploid cell types. In haploid cells, it is necessary for inhibiting the expression of a mating functions which are otherwise antagonistic to a mating functions (Strathern et al., 1980). In diploid cells, the same inhibition of a mating functions is exerted by MATa2, but in addition, it acts in conjunction with the a1 gene to regulate MAT transcription, principally the repression of a1 transcription and to allow sporulation (Figure 3). In vivo, a2 protein represses transcription of two sets of cell type-specific genes by binding to operator sites together with either the a1 or MCM1 proteins (Figure 3). The a2 protein is also a member of the homeodomain superfamily of DNA-binding proteins that regulate development in eukaryotic cells (Wolberger et al., 1991).

MCM1 was first identified as a gene involved in maintenance of artificial minichromosomes in yeast. It has also been shown to serve as a general transcriptional regulator of several genes including mating-type specific genes. Biochemical data suggest that the Mcm1 protein coactivates a-specific genes and corepresses a-specific genes by binding to a 10 base pair dyad symmetry element in their upstream regions (Elble and Tye, 1991).

Yeast G-protein complementing gene (YGC1) has not been characterised, hence its function or mode of action is not known. However, it has been established here that this novel gene complements or suppresses a gpal::HIS3 mutation in high copy plasmids (table 5).

In this study, another important function of MATa2 gene has been established, that is complementation of gpal yeast strains (figure 20, 22 and 25; table 5). The a2 protein can suppress gpal mutations, including null mutations not only when overexpressed in multicopy plasmids but also in single copy centromere plasmids (figures 22 and 25). This result is however contrary to the
earlier findings that SCG1 (also known as GPA1) can only complement supersensitive strains (sst2-1) mutations when overexpressed (Dietzel and Kurjan, 1987). The ability of \textit{MATa2} to suppress the pathway even in single copy (figure 25) not only show its negative regulatory influence on the pathway but also that it is an even more potent inhibitor of the signal pathway than GPA1. The \textit{gpa1} mutation results in hypersensitivity to pheromone and a defect in recovery from the cell-cycle arrest caused by exposure to pheromone. The ability of \textit{MATa2} and \textit{YGC1} to suppress \textit{gpa1::HIS3} mutation in both \textit{MATa} and \textit{MATa} cells (table 5, figure 22) and \textit{MCM1} in \textit{MATa} cells (table 5) suggests that \textit{MATa2}, \textit{YGC1} and \textit{MCM1} might be components of the pathway involved in pheromone response and/or recovery or that they might have a biochemical function similar to GPA1 or some other components of this pheromone signal transduction pathway.

4.3 Implications for the Involvement of MATa2 and YGC1 in the Pheromone - Induced Signaling Pathway

It is very surprising that MATa2 (figure 21) and YGC1 which does not show any level of sequence homology to GPA1 or to the a subunits of G-proteins which are involved in a number of different signal transduction systems should suppress GPA1 mutants in haploid cells (Figure 22). The fact that some of the suppressor mutations may occur in the effector and/or other molecules in the pathway has not been ignored. In this case, the mutant effector and/or molecule(s) may be altered not to generate the arrest signal. Therefore this type of mutation may be able to suppress the lethality of \textit{gpa1::HIS3} mutations. This possibility has however been ruled out based on the control transformation results and the consistency of the complementation results obtained (Figure 22).
To investigate further the possibility of α2 protein involvement in the pathway, mata2 disruption mutant was created (figure 26). This shows a constitutive arrest of the cell-cycle at the late G1 phase (figure 27). The α2 revertants also showed the peculiar "shmoo" shaped cells which are very familiar with the arrested cells (plate 1). The cellular morphology of mata2:: URA3 suggests that the MATα2 is involved in the pheromone response pathway. The ability of α2 to suppress gpal mutations further supports the hypothesis of being involved in the signaling pathway. Although the possibility of being involved in the pathway is favoured, one has not eliminated the probability that MATα2 is required for growth per se, and that suppression of the gpal phenotype is indirect. The results and observations are consistent with a simple model (Figure 1) for the role of the yeast Ga (Gpal), Gβ (Ste4) and Gγ (Ste18) subunits in the activation of the pheromone response pathway. Genetic results indicate that Gβγ functions downstream of Ga to activate the pathway, presumably by activating a downstream effector which is currently unidentified. In the absence of pheromone, Ga is presumed to bind GDP tightly and interacts with Gβγ to inhibit the pathway. In the presence of pheromone, the pheromone - receptor interaction relieves this negative control by promoting GDP and GTP exchange on Ga, resulting in dissociation of Ga from Gβγ and the free Gβγ then activates the pathway. In a gpal null mutant, free Gβγ is present and constitutively activates the pathway leading to G1 arrest and morphological alterations. In analogous manner, the MATα2 and YGC1 gene products seem to be playing the role of GPA1 in gpal mutant cells either directly or indirectly on the pathway. It is however amazing and unexpected that MATα2 and YGC1 should play the role of GPA1 in the pathway because apart from not having sequence homologies, MATα2 is not known to bind GDP/GTP and neither can it respond to conformational changes after guanine
nucleotide treatment since it has no GTP binding, exchange and hydrolysis domains. It is however pertinent to note that since the two receptor-pheromone interactions are interchangeable (Nakayamua et al., 1987) and the phenotypes associated with a2 are the same in both a and a cells, the mechanism of $\text{MATa2}$ action is likely to be the same in both mating types.

The partial sterility of $gpa1$ cells expressing a2 proteins (table 6) suggested that the protein was able to interact with a downstream component of the pheromone response pathway to keep the pathway inactivated but was unable to interact effectively with pheromone receptors to elicit activation of the pathway in response to pheromone. Another possible explanation is that the protein is able to interact with yeast $\text{GBP}$ as a conformational analog of Gpal (GDP), thus preventing activation of the pathway. Since the resulting cells are sterile and unable to respond to pheromone, this suggests that the a2 protein cannot interact functionally with the pheromone receptors. From table 6, it is obvious that there is a very strong reduction in mating efficiency in both GU1 and GU2 cells kept alive with $\text{MATa2}$. GU1 cells that are kept alive with $\text{MCM1}$ and $\text{YGC1}$ also showed similar pattern of suppression of mating efficiency. It could also be inferred from table 6 that wild type MATa cells (FY250) show a large reduction in mating. This observation could further be explained on the basis of non-specific and noneffective, interaction with the pheromone receptors to elicit activation of the pathway in response to pheromone.

Lethality of the cell with disrupted $\text{MATa2}$ (figure 27) can be explained by the uncoupling of the effector molecule from a2 protein. The effector, which may be unlocked from the mating factor receptor complex, may elicit a constitutive signal from cell-cycle arrest regardless of the presence of mating factors (figures 26 and 27). It was also observed, through microscopic examination of the
MATa2 disrupted spores and micromanipulation of the mata2::URA3 tetrapods, that most of the cells were unbudded and some of them showed an aberrant cell morphology similar to the "shmoo" of cells arrested by mating factors (table 7 and plate 1). This observation supports the idea that MATa2 disruption results in continuous production of a cell-cycle arrest signal (figure 27) and promotion of conjugation in the absence of a mating factor signal.

The clone9 revertants (table 7 and plate 1) observed are of interest because some are clearly in known pathway genes. These were the ones that yielded sterile, morphologically normal cells which were not examined further, but are probably genes such as STE4, STE18 and the Effector molecule. The revertants may also contain new genes which give cells that are still differentiated but are going through mitosis (table 7 and plate 2). The differences in these growing shmoo revertants may reflect different phenotypes from various mutations in the same gene.

4.4 Possible Models of MATa2, YGC1 and MCM1 Actions

The results of this study that support the role of a2 and Ygc1 proteins in the mating factor signal transduction pathway include; (a) the arrest phenotype of haploids when a2 and Ygc1 expressions were turned off, is characteristic of cells arrested in late G1 phase and some of these arrested cells exhibit shmoo morphology (figure 2, table 7 and plate 1) and (b) disruption of a2 is lethal in haploid cells (figure 27) which indicates a2 is a haploid essential gene for cellular growth.

To explain the mechanism of action of a2, Ygc1 and Mcm1, one could say that they are either involved directly in the signaling pathway and/or that these proteins act to modulate a component of the pathway, most probably Gβγ or the
effector molecule. Indeed, some other products are known to be involved in vivo in modulating the signaling response. Inactivation of these genes leads to activation of the signaling pathway; hence, these products, just like a2, can be considered as negative components of the pathway. The genes with this behaviour are *CDC36* and *CDC39* (Neiman et al., 1990; de Barros Lopes et al., 1990), *CDC72* and *CDC73* (Dietzel & Kurjan, 1987). The abbreviation CDC stands for cell division cycle genes. The a2 protein might play a role in communication between the activated receptor and Gβγ or be involved in stabilizing the putative effector and/or Gβγ proteins. In this way, inactivating a2 and Ygc1 would definitely have an effect on the pathway (figure 27).

There are a group of genes whose transcripts are expressed in haploids but repressed in a/a diploids. Repression requires both the a1 protein, encoded at MATa, and the a2 protein encoded at MATa, which make up a1-a2 activity (figure 3, Jensen et al., 1983). It is proposed that the transcription of *GPA1* would be under negative control by a1-a2 activity (figure 3 and Miyajima et al., 1987). *GPA1* would be expressed in haploid a cells because of the absence of a2 products and in a cells because of the absence of a1 product. However, a 20 base pair consensus sequence common to the 5' ends of haploid specific genes that are negatively regulated by a1-a2 has not yet been found upstream of *GPA1* except for some sequence homology 50 base pairs upstream of the translation start (Miller et al., 1984).

If a1-a2 can regulate *GPA1* negatively, and if the regulation involves a direct contact between a1-a2 and *GPA1*, then one could argue that probably the region of contact between the a1-a2 complex and *GPA1* might be structurally similar to that of Gβγ subunits of yeast G-protein. If this is possible, then in the absence of *GPA1*, that is in *gpa1::HIS3* yeast strains, a1-a2 or probably Mcm1-
a2 could bind the Gβγ nonspecifically because of the assumed similarity in the region of contact between GPA1 and Gβγ. Thus in MATα cells, a1 and/or Mcm1 might repress the Gβγ activity whereas in MATα cells, the a2 component of the complex is expected to functionally repress the Gβγ activity. One could however be tempted to say that this model could be inadequate because only the a2 is able to suppress the pathway both in MATα and MATα cell types. The argument could have been more valid if gpal::HIS3 yeast strains are complemented by a2 only in MATα cells (table 5). However, one did not overlook the fact that probably, high copy expression of the a2 proteins activated the transcription of its putative complexing counterpart in MATα cells to suppress the signaling pathway. Here, it is pertinent to note that a2 protein does not repress the pathway only when expressed in a high copy plasmid but also in a single copy centromere plasmid (figure 25). MCM1 complements gpal only in MATα cells probably because it forms a complex with a2 (figure 3) to modulate the pathway by repressing a-specific genes involved in the transduction of the pathway whilst depressing a-specific genes.

Another plausible mechanism through which MATα2 can complement gpal lethality would be to bind to the putative biological effector of Gβγ which might act at the same level as Gβγ thereby preventing the transduction of the signal from Gβγ to the effector molecule. In this way, the signaling pathway would be suppressed due to the competition between a2 and Gβγ for binding to the effector. Logically if the a2 is transcribed in higher copies, it would advantageously out compete the Gβγ thereby suppressing the pathway. This model can only be validated after the effector molecule is isolated, cloned and shown both genetically and biochemically to bind to a2 protein.
One could also envisage that the possibility of α2 and Ygcl suppressing the pathway could be that the pathway is not simply linear, at least upstream, that is at the level of the G proteins. The possibility of the pathway being branched at the G proteins level is one of the main objectives of this study. Hybridization analysis by Dietzel and Kurjan (, 1987) indicates that there are at least two additional GPA1 homologs in S. cerevisiae. Isolation of these homologs could have thrown more light in understanding the pathway if they could be shown to be involved. Ascertaining the possible involvement of these homologs may confirm the branching of the pathway upstream and also show whether there is a common effector in the pathway, similar to the cAMP pathway as described by Stryer and Gilman (, 1986). If it is confirmed that the pathway is branched, then α2 and Ygcl proteins could be components or modulators of the branched pathway. This stem from the fact that disruption of these proteins leads to similar phenotypes as in GPA1 disruption (figure 27 and table 7). It is rather unfortunate that the screening of the GPA1 homologs does not yield any of the supposed Ga subunits present in S. cerevisiae. Surprisingly, the screening does not even produce GPA1 (figure 12).

The emerging view of gene regulation is one of combinatorial control. The specific level of expression of a given gene may result from the interplay of a multiplicity of factors, each contributing differently to the final level of transcription. Thus, from a limited pool of DNA-binding factors, a virtually unlimited range of binding specificities and levels of expression may be generated. The same factor may act positively or negatively, depending on the context of its binding sites and/or on the other factors with which it interacts (Berk and Schmidt , 1990). For example, Serum response factor (SRF), the vertebrate homolog of the yeast MCM1 gene binds together with the ternary complex...
factor to activate transcription of c-fos (Norman et al., 1988). Repression of
c-fos is mediated by SRF acting at the same site (Rivera et al., 1990) —
presumably in conjunction with other transcription factors that interact with
other transcription factors, MCM1 may interact with some other factor in MATa
cells to suppress the pathway and may bind in the same site or interact with
other factors in MATa cells to enhance the transduction of the pheromone signal.
Also an increase in the abundance of these transcription factors may shift
equilibrium in the cell and reduce the expression of proteins required for cell
arrest and differentiation. This could result in the ability of a2 and Mcml to
suppress gpa1 lethality. This may be unraveled by doing Northern blots. The fact
that the a2 protein has no particular motif to bind GTP/GDP unlike Gpa1 and has
no sequence homology to any of the known Ste proteins indicates that the pathway
might be branched whereby a2 would be involved in the branched pathway or if the
pathway is simply linear as envisaged, then a2 might modulate some component(s)
on this linear or the branched pathway either directly or indirectly. The fact
however remains that a2 protein is strongly involved in the signaling pathway
that leads to cell-cycle arrest at the G1 phase since disruption of the MATa2
gene results in constitutive arrest of the pathway (figure 26 and 27). Thus, this
study shows that the signaling pathway seems to be more complex and one of
combinatorial control by structural and functional genes than simply by G-
proteins and the Ste proteins most of which are known to be protein kinases with
homology to protein kinase C. Hence the involvement of a2, Ygc1 and Mcml has
given more impetus to understanding GPA1 signal transduction in S. cerevisiae.
Generation of several point mutants of MATa2 and the study of these mutants in
terms of arresting the signaling pathway would throw more light into the
mechanism of suppression of a2 protein.
4.5 New Yeast Strains

The plasmid TGC was constructed (figure 8) and subsequently used to create a new host for GU1 and GU2 haploid cells forming LG1- and LG2-TG respectively (Figure 7). These yeast strains were very useful in screening a mammalian cDNA library leading to the isolation of two mammalian analogs of GPA1 which were able to complement gpa1 haploid cells (Colicelli, personal communication). The yeast strains have ARS origin of replication enabling the plasmid to be easily lost when desired. It also has canavanine gene (an arginine analog) for selection purposes and TRP1 as its selective marker since the cDNA library available was LEU2 based.

Analysis of the signal transduction pathway whereby MATa2 and YGC1 genes elicit physiological changes in the responding cells is likely to provide important insights into the mode of action of other hormonal factors in higher eukaryotes. It is also not unexpected that the identification and involvement of MATa2, YGC1 and MCM1 would throw more light on the intricacies of the mechanism of signal transduction in yeast and subsequently in multicellular eukaryotes such as mammals. Understanding the mechanism of the signal pathway would further reveal how growth is stringently controlled and how cancer cells occur due to the defect in this cellular growth control mechanism.
APPENDIX

**Tris-borate (TBE) Working Solution**

0.5x: 0.045M Tris-borate

0.001M EDTA

**10x Sodium dodecyl sulfate (SDS)**

Dissolve 100g of electrophoresis-grade SDS in 900ml of H\(_2\)O. Heat to 68°C to assist dissolution. Adjust the pH to 7.2 by adding a few drops of Conc.HCL. Adjust the volume to 1 liter with H\(_2\)O.

**20x sodium citrate (SSC)**

Dissolve 175.3g of NaCl and 88.2g of sodium citrate in 800ml of H\(_2\)O. Adjust the pH to 7.0 with a few drops of a 10N NaOH. Adjust the volume to 1 liter with H\(_2\)O.

**20 x SSPE**

Dissolve 175.3 of NaCl, 27.6g of Na\(_2\)HPO\(_4\).H\(_2\)O and 7.4 g of EDTA in 800ml of H\(_2\)O. Adjust the pH to 7.4 with NaOH (---6.5ml of a 10N solution). Adjust the volume to 1 liter with H\(_2\)O.

**DnD Solution**

To prepare 10ml of DnD

Dithiothreitol 1.53

DMSO 9ml

1M potassium Acetate (pH7.4) 100ml

H\(_2\)O to 10ml
Sterilise the DnD solution
by filtration through millex
SR membrane unit (Millipore)
and store at -20°C
in sterile 0.5ml microfuge
tubes.

Preparation of 1 liter TFR

<table>
<thead>
<tr>
<th>Reagent</th>
<th>Amount required/liter</th>
<th>Final Concentration</th>
</tr>
</thead>
<tbody>
<tr>
<td>1M MES (pH63)</td>
<td>10ml</td>
<td>10mM</td>
</tr>
<tr>
<td>MnCl$_2$.4H$_2$O</td>
<td>8.91g</td>
<td>45mM</td>
</tr>
<tr>
<td>CaCl$_2$.2H$_2$O</td>
<td>1.47g</td>
<td>10mM</td>
</tr>
<tr>
<td>KCL</td>
<td>7.46</td>
<td>100mM</td>
</tr>
<tr>
<td>Hexamminecobalt chloride</td>
<td>0.80g</td>
<td>3mM</td>
</tr>
</tbody>
</table>

**SOC Medium**

Per liter:

To 950ml of deionized H$_2$O, add:

bacto-tryptone     20g
bacto-yeast extract 5g
NaCl              0.5g
Shake until the solutes dissolve. Add 10 ml of a 25 mM solution of KCl. Adjust the pH to 7.0 with 5 N NaOH (.2 ml). Adjust the volume of the solution to 1 liter with deionized H₂O. Sterilize by autoclaving for 20 minutes at 121°C on liquid cycle. Allow it to cool to 60°C or less and then add 20 ml of a sterile 1 M solution of glucose. Just before use, add 5 ml of sterile solution of 2 M MgCl₂.

**Prehybridization solution**

50% formamide

6xSSC (or SSPE)

0.05xBLOTTO (Bovine Lacto Transfer Technique Optimizer 5% nonfat dried milk dissolved in water containing 0.02% sodium azide).
REFERENCES


