G-PROTEIN MEDIATED SIGNAL TRANSDUCTION IN SACCHAROMYCES CEREVISIAE

BY

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A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF A MASTER OF PHILOSOPHY DEGREE IN BIOCHEMISTRY AT THE UNIVERSITY OF GHANA, LEGON

DEPARTMENT OF BIOCHEMISTRY
UNIVERSITY OF GHANA
LEGON, GHANA

SEPTEMBER, 1995

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 .

Signed: .....

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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.

ABSTRACT

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 MATa and MATα *gpa1::HIS3* haploid cell types whereas *MCM1* complements only MATa *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 *matα2::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 *MATα2*, *YGC1* and *MCM1* are components or modulate component(s) of the signaling pathway. It also showed that *MATα2* is even a more potent negative regulator of the signaling pathway than *GPA1*, since overexpression is not a prerequisite for negatively regulating the pathway. *MATα2* does not belong to the G protein family since it has no GTP/GDP binding and/or exchange domains.

TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABBREVIATIONS

G protein	Guanine nucleotide binding protein
FUS	Genes whose activation leads to the Fusion of the Cell
GPA1	Yeast G protein alpha subunit
MATα2	Mating type locus alpha 2 gene
YGC	Yeast G protein complementing clones
MCM1	Minichromosome maintenance gene
STE	Genes whose inactivation leads to sterility of the cell
CDC	Cell division cycle genes
βME	β-mecarptoethanol
SDS	Sodium dodecyl sulfate
LB	Luria-Bertani medium
MES	2(-N-morpholino) ethanesulfonic acid
YPD	Yeast peptone dextrose media
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, (2µm) 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 α cells of the budding yeast, *Saccharomyces cerevisiae*, are able to grow vegetatively or can mate to form a diploid a/α 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 a-factor and respond to α-factor, and cells of α-mating type produce α-factor and respond to a-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-ß (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 presumed serine/threonine protein kinases. For yeast, we now have the outline of 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 α cells produce different receptors and different mating factors. a/α 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, *MATα2* 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 (a-factor and α-factor) binding to integral membrane protein receptors, and cells of α-mating type produce an a-factor receptor. Both receptors are coupled to the same heterotrimeric G protein, Gαβγ. 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 Gα, which leads to release of the βγ subunit of the G protein. Gβγ 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 a 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 α 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 norphology 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.

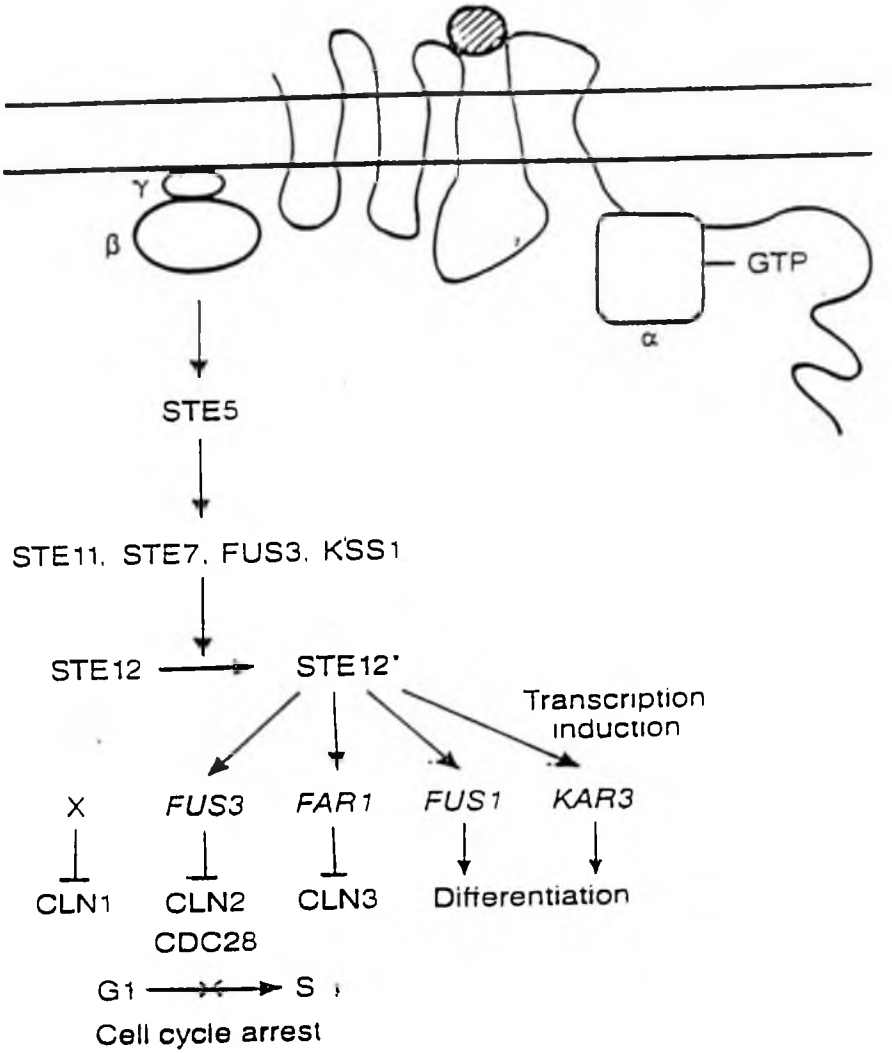

Figure 1: Differentiation and cell cycle arrest in response to pheromone.

Binding of pheromone (hatched circle) alters the conformation of the receptor. As a result of an attendant conformational change Gα.GTP replaces GDP, and Gβγ is released. Activated Gβγ 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 proteins(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.

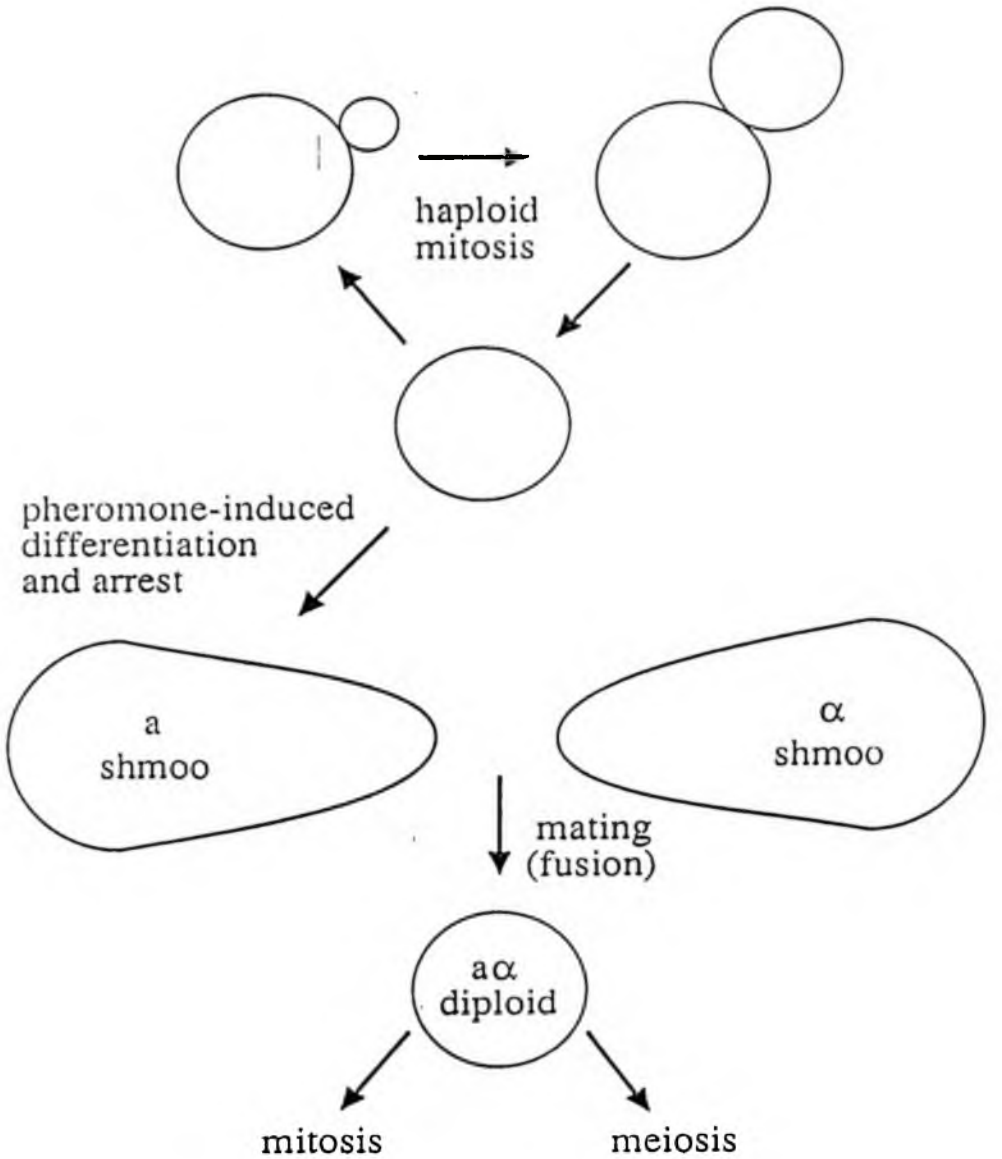

Figure 2: Yeast Cell Types

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.

In particular, mutations in *STE2*, *STE4*, *STE5*, *STE7*, *STE11*, *STE12*, *STE18*, *STE20*, and *FUS3* all confer resistance to α-factor by disrupting the signaling pathway (Mackay & Manney,1974; Hartwell, 1980; Whiteway *et al.*, 1989; Leberer *et al.*, 1992; Elion *et al.*, 1990). Many of the genes involved in phenomone production were identified as mutants defective in mating (Wilson & Herskowitz , 1987).

1.2.3 The α-Factor Receptor

α-Factor is an unmodified peptide of thirteen amino acid residues (Duntze *et al.*, 1970) and is necessary for mating by α cells (Kurjan, 1985). It activates **a** cells via the α-factor receptor encoded by the STE2 gene (Nakayama *et al.*, 1985) and is degraded by a specific extracellular peptidase encoded by the *BAR1* gene (Sprague & Herskowitz, 1981). Both of these genes are expressed only in **a** cells (Kronstad *et al.*, 1987). Mutations in *STE2* affect pheromone response only of **a** 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 *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 *et al.*, 1991). Supporting the notion that these hydrophobic domains are important for α-factor receptor function,their size and position is conserved in the *Saccharomyces kluyveri* α-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 $6x10^{-9}$ (Jenness *et al.*, 1986) to $2x10^{-8}$ (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 a-Factor Receptor

a-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 **a** cells (Michaelis & Herskowitz, 1988). Its synthesis and secretion follow a strikingly different route from α-factor (Kuchler *et al.*, 1989). The a-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 a-factor since it is hydrophobic and exhibits a high level of nonspecific binding. Synthetic a-factor is active in inducing cellular responses at nanomolar concentrations, thus suggesting that affinity of the **a**-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 a-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 a-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* a-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 α-factor receptor responds to a-factor- instead of α-factor (Nakayama *et al.*, 1987; Bender & Sprague , 1989). An α cell engineered to produce the α-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 better to its own α-factor than to the heterologous α-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* α-factor(Marsh & Henskowitz , 1988), thereby demonstrating that the *STE2* protein is the specificity determinant for α-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 ß-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 G_qS. The yeast system may prove useful for genetic analysis of mammalian receptors, since mutant receptors with altered signaling properties are easily identifiabde by plate assay.

1.2.7 Yeast G Protein

The a-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 G_q subunit from the $G_{\beta\gamma}$ 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 Gßγ subunit rather than the Gα subunit that is responsible for activating signaling targets (Dietzel & Kurjan , 1987; Whiteway *et al.*, 1987). Activation of phospholipase A_2 by Gßγ has been reported to occur in bovine rod outer segments (Jelsema & Axelrol, 1987). Also Gßγ derived from G_s G_i and G_o 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 G_{β} or G_{γ} show reduced α-factor binding (Jenness *et al.*, 1987; Blumer & Thorner , 1990) which suggest that $G_{\beta\gamma}$ is necessary for proper association of G_{α} with the receptor. Yeast membranes exposed to the non-hydrolyzable GTP analogue, $GTP_{\gamma}S$, 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 Gα Subunit of Yeast G protein

Yeast G_{α} genes were identified by cross-hybridization, to a rat $G_{\alpha}i$ 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 G_{α} 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 G_{α} 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 Gα in this study.

The yeast Gα subunit contains 472 amino acid residues and is 45% identical to rat Gαi (Miyajima *et al.*, 1987). Like the mammalian Gα, 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 Gα 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 G_q 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 Gα causes the pathway to become activated, Gα is obviously not required to propagate signal for activation. Rather, Gα is formally a negative regulator of signaling necessary to maintain the pathway in a quiescent state, apparently by binding to Gβγ.

Studies of mammalian Gα and receptors in yeast demonstrate specific interactions between components involved in signaling. Yeast cells lacking yeast Gα but expressing mammalian Gα 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 ß-adrenergic receptor expressed in yeast requires coexpression of Gαs (King *et al.*, 1990). Hybrid studies with yeast and mammalian Gα suggest that the C-terminus of Gα may be required for interaction with the receptor (Kang *et al.*, 1990). Some point mutations in the C-terminus of Gα 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 a cells than in α cells, which suggest that it interacts less well with the a-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 Gα can interact with two receptors that lack obvious sequence homology. It may also be possible to identify determinants on the receptor that interact with Gα by exchanging regions of *STE2* and *STE3* and determining their ability to function with these Gα mutants. Also, again using genetic approaches, several kinds of "activated" mutations of the *GPA1* gene have been characterized. A $GPA1^{val-50}$ 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, $GPA1^{lys-355}$ and $GPA1^{lys-364}$, were selected from a pool of mutants based on phenotypic changes (Stone and Reed , 1990). The alignment of Gα primary structures shows that the mutations correspond to Val-49 mutation of Gαs proteins. According to the model described above, constitutive activation of GP1α 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 βγ-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 ß- and γ- subunits respectively cannot interact with *GPA2*. This implies that an additional set of genes that code for ß-and γ-subunits interacting with *GPA2* must be present in yeast cells.

1.2.10 Gßγ Subunits of Yeast G protein

The ß 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 ß 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 Gß (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*^{Hpl}), cause lethality only in a and α haploid cells but not in a/α diploids in

which several essential components of the signaling pathway (such as *STE5*, *STE12*, and *FUS3*) are turned off.

The *STE4*^{Hpl} mutations may cause synthesis of a Gß subunit that is insensitive to inhibition by the Gα subunit, but which preserves its ability to interact with downstream components of the pathway.

The importance of the balance between Gα and Gßγ has also been demonstrated by studies in which different subunits are overproduced. Overexpression of Gß(*STE4*) alone, or with Gγ (*STE18*), leads to constitutive mating-factor responses (Whiteway *et al.*, 1990; Cole *et al.*, 1990). This induction is overcome by overproduction of Gα, (Whiteway *et al.*, 1990: Cole *et al.*, 1990), presumably by converting free Gßγ subunits back to G_{α}ßγ heterotrimers and restoring the normal ratio of the subunits. Over expression of G_{α}(*SCG1*) has also been observed to counter the growth inhibition of certain **a** strains (mutants defective in the *SST2* gene) exposed to α-factor (Kang *et al.*, 1990; Cole *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 Gγ-subunits is found. In Rasp21, this motif signals postranslational modification of the C-terminus, which is required for membrane association and biological activity (Willumsen *et al.*, 1984). Studies have demonstrated that the conserved cysteine in the motif is the site of polyisoprenylation of both Ras (Casey *et al.*, 1989) and Gγ proteins (Mumby *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

(Finegold *et al.*, 1990).

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 $G_{\alpha}\beta\gamma$ 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 G_α 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 G_α 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 G_α 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 G_α. Physiological analysis of a cdc 39-ts mutant indicates that *CDC39* does not control synthesis of a functional Gα subunit, but rather raises the intriguing possibility that it might play a role in communication between the activated receptor and Gα, or be involved in stabilizing the GDP-bound form of Gα (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/α strains defective in *GPA1*(*SCG1*) are viable whereas a/α 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 1:

Genes involved in Pheromone Signal Transduction in Budding Yeast

Gene	Function	Null mutation phenotype	Cell-type expression
STE2	α–facor receptor	Unresponsive	a
STE3	a-factor receptor	Unresponsive	α
GPA1(SCG1)	Gα subunit	Constitutive (lethal)	a,α
STE4	Gβ subunit	Unresponsive	a,α
STE18	Gγ subunit	Unresponsive	a,α
STE20	Protein Kinase	Unresponsive	All
STE5	Unknown	Unresponsive	a,α
FUS3	Protein Kinase	Unresponsive	a,α
STE7	Protein Kinase	Unresponsive	All
STE11	Protein Kinase	Unresponsive	All
KSS1	Protein Kinase	See text	Not reported
STE12	Transcriptional activator	Unresponsive	a,α
SGV1	Protein Kinase	Hypersensitive	Not reported
SST2	Unknown	Hypersensitve	a,α
CDC36	Unknown	Constitutive(lethal)	All
CDC39	Unknown	Constitutive(lethal)	All
CDC72(NMT1)	Myristoyl transferase	Constitutive(lethal)	All
CDC73	Unknown	Constitutive(lethal)	All
SRM1	Unknown	Constitutive(lethal)	All

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, a cells produce an-extracellar 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 a-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, G_{α}, G_{β} 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 hyperphos-phorylated in response to α-factor (Marsh *et al.*, 1991).

Mutants of G_{α} with a Gly to val substitution at position 50 (analogous to the ras^{val12} mutation that reduces the intrisic GTPase activity of ras) exhibit a complex phenotype that appears to indicate that Gα 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 Gα subunit (Gα-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 Gα-stimulated adaptive response.

Cole & Reed (, 1991) reported that the *STE4* protein (Gß) 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 Gα subunit is required for this phosphorylation. These observations lead to the hypothesis that the G_q- and *SGV1*- dependent recovery process proposed by Miyajima *et al.*, (1989) may function by stimulating phosphorylation of Gß (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 Gα or some other G protein subunit since certain Gα mutant alleles are epistatic to *sst2* mutations (Kurjan *et al.*, 1991). Also, overexpression of Gα partially overcomes an *sst2* mutation (Dietzel & Kurjan , 1987). The *SST2* product may stimulate the intrinsic GTPase activity of Gα 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*, Gß, 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 other wise 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 G_{α} and Gß and *STE5* or other STE genes. As noted earlier, null mutations in *GPA1*(*SCG1*) cause constitutive behaviour of the

pathway, whereas null multations 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 *gpaI ste11* strain exhibits the properties of the *ste11* mutant. These observations are interpreted to indicate that the STE11 protein acts after Gα 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 Gß subunit, encoded by the $STE4^{Hpl}$ 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 Gß (or functioning at the same step in the pathway as Gß). 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 Gßγ subunit is presumed to interact with one or more proteins to propagate the signal. The gene product that lie immediately downstream of Gßγ - *STE*20, *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 Gßγ subunits. This supposed interaction between the Gßγ and *STE*20 product is however not clear

and must be substantiated by further genetic and biochemical studies. Thus, identification of the target for Gßγ 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 IP_3 is controlled by activation of the G protein (Iyengar & Birnbauner , 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 *FUS3* is to inactivate the *CLN3* protein perhaps by phosphorylating it (Elion *et al.*, 1990). It is not possible to know from these observations if *CLN3* is a direct substrate of *FUS3*. These studies of *FUS3* and *KSS1* reveal some of the complexities likely to be encountered in studying protein kinases with multiple substrates and overlapping specificities; it appears that *FUS3* has at least two substrates, only one of which is a substrate for *KSS1* (Marsh *et al.*, 1991).

KSS1 was identified because its expression, when carried on a high copy number plasmid, allows cells defective in the *SST2* gene to become partially resistant to α-factor (Courchesne *et al.*, 1989). Given that *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 *KSS1* should inhibit the functioning of the pathway.

The *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 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 *STE4* (47 kd), *STE7* (55kd), *STE12* (112 kd) or *FUS3* (40kd) Rhodes *et al.*, 1990), and neither does it correspond to *STE20* (102kd) Leberer *et al.*, 1992). Whether p78 has a role or not in signal transduction is unknown. There are numerous possible substrates for *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*, *STE*20 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 (pheronome 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 STE*20 genes are expressed in all cell types, even in a/α cells which do not respond to mating factors. Their role in a/α 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/α 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 (Appletauer & Achstetter , 1989). These include genes involved in cell fussion 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 *AGα1* (coding for α-agglutinin), which in induced more than 20-fold by a-factor

(Lipke *et al.*, 1989), *AGA1* (coding for an a-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 (MFα1) 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 a-specific and α-specific genes analysed *(MFa1, MFa2, 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 Gßγ) 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 *STE12* could regulate its entry into the nucleus (Baeuerle & Baltimore, 1988).

1.2.14 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 transcient 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 *CLN1, CLN2,* and *CLN3*, which are required for progression from G1 to S and are thought to be required for activity of *CDC28*, the budding yeast homologue of $p34^{cdc2}$ (Wittenberg *et al.*, 1990). Arrest in the cell cycle requires that all three G1 cyclins be inactivated (Richardson *et al.*, 1989), which may occur through the action of three separate inhibitors (Chang & Herskowitz , 1990). Two of the inhibitors (*FUS3* and *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 *et al.*, 1990; Chang & Herskowitz , 1990). *FUS3* appears to be responsible for inactivating *CLN3* (Elion *et al.*, 1990, , 1991), and *FAR1* for inactivating *CLN2* (Chang & Herskowitz , 1990). The compound responsible for inhibiting *CLN1* is unknown. The response pathway apparently triggers cell-cyle arrest by enhancing activity of these inhibitors of the G1 cyclins. Transcription of both *FUS3* and *FAR1* is induced several fold by mating factors Elion *et al.*, 1990; Chang & Herskowitz , 1990). In addition, *FAR1* is rapidly phosphorylated

after treatment of **a** 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, **a** 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. MAT**a** differs from MATα in the substitution of a 650-bp **a**-specific sequence for a non-homologous 750-bp α-specific sequence (Nasmyth and Tatchell , 1980). The two genes at MATα, α1 and α2, 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 **a** mating functions, which are otherwise antagonistic to α mating functions (Strathern *et al.*, 1980). In diploid cells, the same inhibition of **a** mating functions is exerted, but in addition, it acts in conjunction with the a1 gene to regulate MAT transcription, principally the repression of α1 transcription and to allow sporulation (Nasmyth *et al.*, 1981). The *MATα2* gene product is therefore necessary for preventing the expression of both **a** and α mating types in **a**/α diploids (figure 3), (also Nasmyth et al.,

1981). *In vivo*, α2 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 α2 to a different set of operators (Herskowitz , 1989). The yeast α2 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 α-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 cells 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.

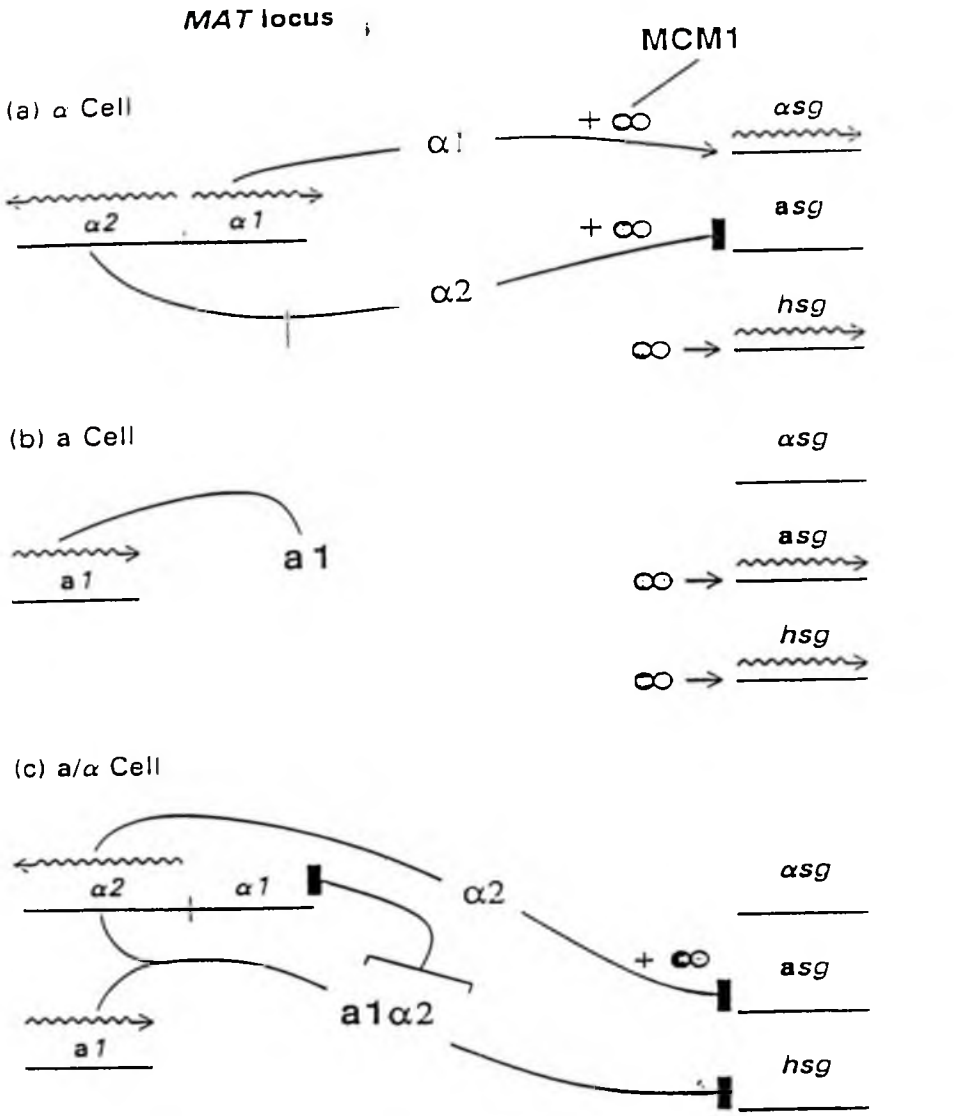

Figure 3: Regulation of cell types by MAT locus genes and MCM1

Regulation of a-specific genes (asg), α-specific genes (αsg), and haploid specific genes (hsg) in α, a and a/α yeast cells by regulatory proteins encoded at the MAT locus together with *MCM1*, a constitutive DNA-binding protein. 3a shows transcription of αsg 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 a1-α2 proteins and α1 transcription repression by a1-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

Strain	Relevant Genotype	Source
SP1	*MATQ leu2, ura3, his 3 ade8 trp1, TYR1, gal2, can1*	Colicelli
FY250	*MATa ura3, his3, leu2, trp1*	Colicelli
DC14	*MATa his1*	Colicelli
DC17	*MATa his1*	Colicelli
DC124	*MATa leu2, ura3, his4, trp1 ade8*	Colicelli
SP1/DC124	*MATa/MATα, leu2/leu2, his3/HIS3, HIS4/his4, ura3/ura3, trp1/trp1, ade8/ade8*	Colicelli
GU1	*MATa gpa1:HIS3 leu2, ura3, can1 (pTLCG)*	Colicelli
GU2	*MATα gpa1::HIS3, leu2, ura3, can1 (pTLCG)*	Colicelli
LG1-TG	GU1 with plasmid replacement for *TRP1/GPA1/CAN1*	This work
LG2-TG	GU2 with plasmid replacement for *TRP1/GPA1/CAN1*	This work
LG1-UG	GU1 with plasmid replacement for *URA3/GPA1*	This work
LG2-UG	GU2 with plasmid replacement for *URA3/GPA1*	This work
GL1-5	GU1 with plasmid replacement for *URA3/MCM1/CAN1*	This work
GL1-9	GU1 with plasmid replacement for *URA3/MATα3/CAN1*	This work
GL1-12	GU1 with plasmid replacement for *URA3/YGC1/CAN1*	This work
GL1-UGC	GU1 with plasmid replacement for *URA3/GPA1/CAN1*	This work
GL1-UC	GU1 with plasmid replacement for *URA3/CAN1*	This work
GL2-5,9 12, UGC and UC	All created by GU2 replacements for the respective plasmids as in GL1-strains	This work

Table 3

Plasmids Used in this Study

Plasmid	Relevant Genotype	Source
pTLGC	*LEU2/GPA1/CAN1*	Colicelli
pUV2	pUC118/*URA3*	Colicelli
YEP13M4	*LEU2* vector	Colicelli
pRS416	*URA3/CEN* plasmid	Colicelli
pYeCAN	*CAN1* Source	Colicelli
pKS	pBluescript vector	Colicelli
pTGC	*TRP1/GPA1/CAN1*	This work
pUGC	*URA3/GPA1/CAN*	This work
pU5C	*URA3/MCM1/CAN1*	This work
pU9C	*URA3/MATa2/CAN*	This work
pU12C	*URA3/YGC1/CAN*	This work
pUC	*URA3/CAN1*	This work
YEp9	*LEU2/MATa2*	This work
pRS416"9"	*CEN/URA3/MATa2*	This work
pUVEK	pUV2/*Eag1-kpn1* fragment of *MATa2*	This work
pUVEK-URA3	pUV2/mata2::URA3	This work
pKSEK	pKS/*MATa2*	This work
pKSSX	pKS/*Sma1-Xba1* fragment of *MATa2*	This work

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:

Adenine	0.75g
Arginine	1.5g
Asparagine	1.5g
Aspartic Acid	1.5g
Histidine	1.5g
Isoleucine	1.5g
Leucine	3.0g
Lysine	1.5g
Methionine	1.5g
Phenylalanine	1.5g
Proline	1.5g
Serine	1.5g
Threonine	1.5g
Tyrosine	1.5g
Tryptophan	1.5g
Uracil	1.5g
Valine	1.5g

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 50 ml 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^{0}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-^{32}P)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 30^0C with shaking at 250rpm on a rotary platform. This was to enable the GU cells lose the *GPA1/LEU2/CAN1* maintenance plasmid.

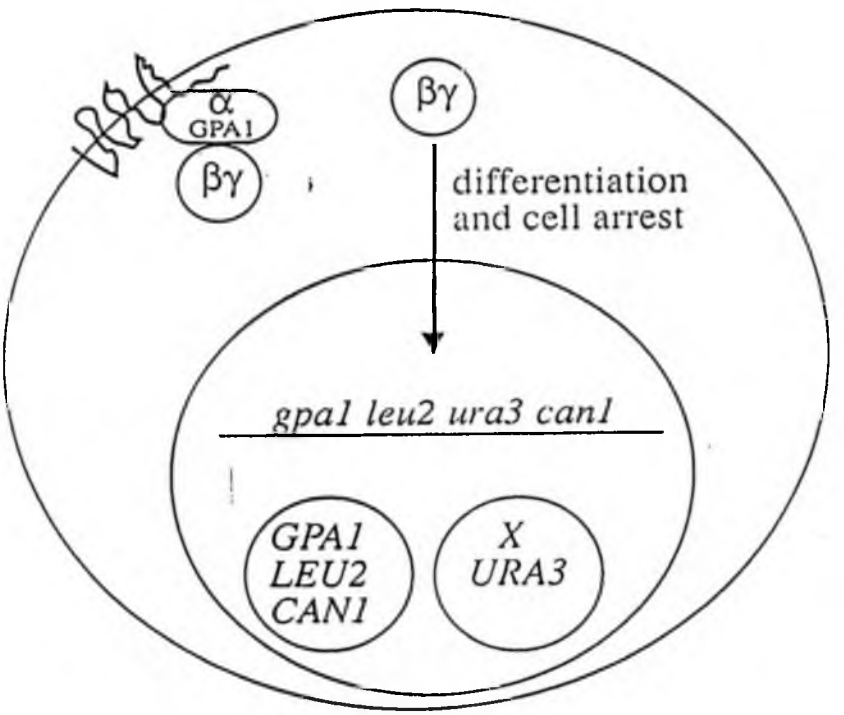

Figure 4: Complementation of GPA1

The diagram shows the selection procedure based on the complementation of gpa1 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 ($\alpha\beta\gamma$) is also shown. Where the $\beta\gamma$ 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 a-factor.

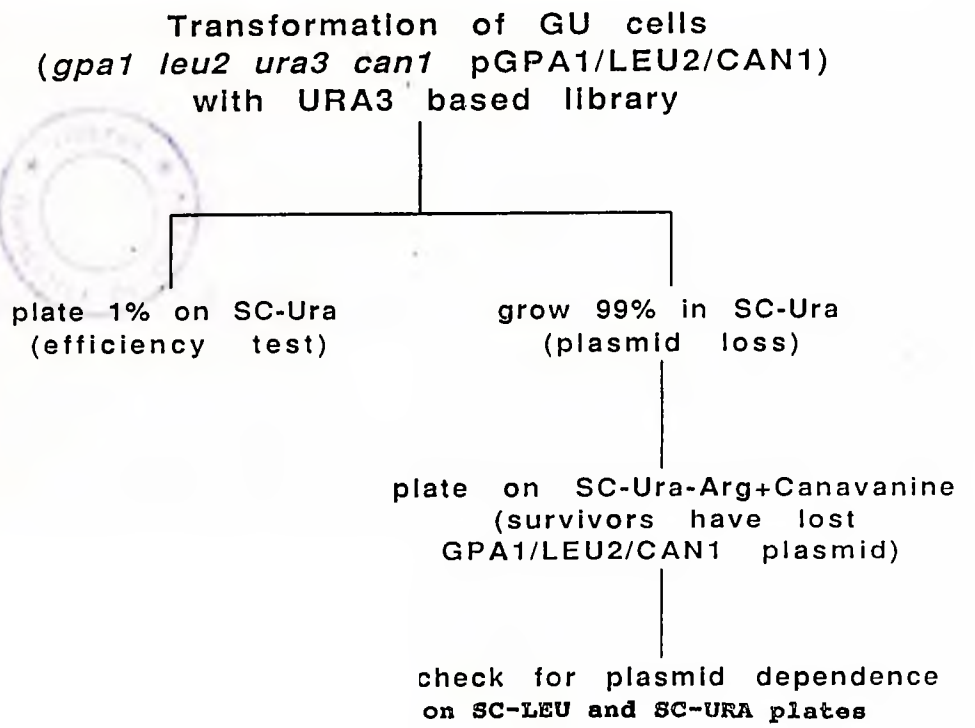

Figure 5: Flow Chart describing the selection of high copy suppressors of *gpa1*

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 *Eco*R1 and *Hind*III 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 mls 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 *Eco*R1 fragment of *GPA1* was labelled with ^{32}P using the modifying enzyme T4 polynucleotide kinase reaction. The *GPA1*-labelled probe was then denatured by heating at 100^{0}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^{0}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 ^{32}P-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^{0}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 0.1XSCC containing 0.5% SDS, at room temperature. The filter was incubated for 45 min at 37^{0}C with gentle agitation and then transferred into a 68^{0}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 *Eco*R1 and *Hind*III, 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 *Hind*III digest and ϕX174 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^{0}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 MATα) 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 30^0C for $2\frac{1}{2}$ to 3 days. Then 5µl of the culture media was plated on the SC-plate where the replacing plasmid could grow better for $2\frac{1}{2}$ to 3 days at 30^0C. 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 30^0C 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 occured by it being matched with the lost colonies on it's 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).

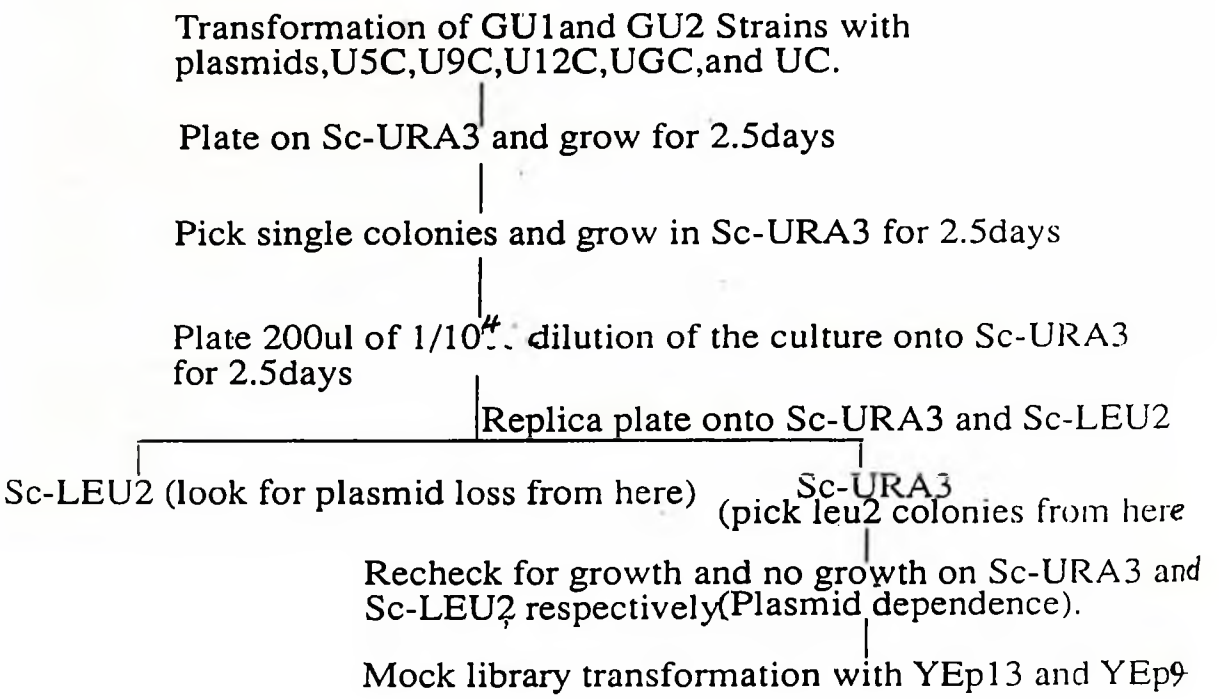

Figure 6: Flow Chart showing the creation of haploid strains GL1(GL2) of clones 5,9 12, UGC and UC.

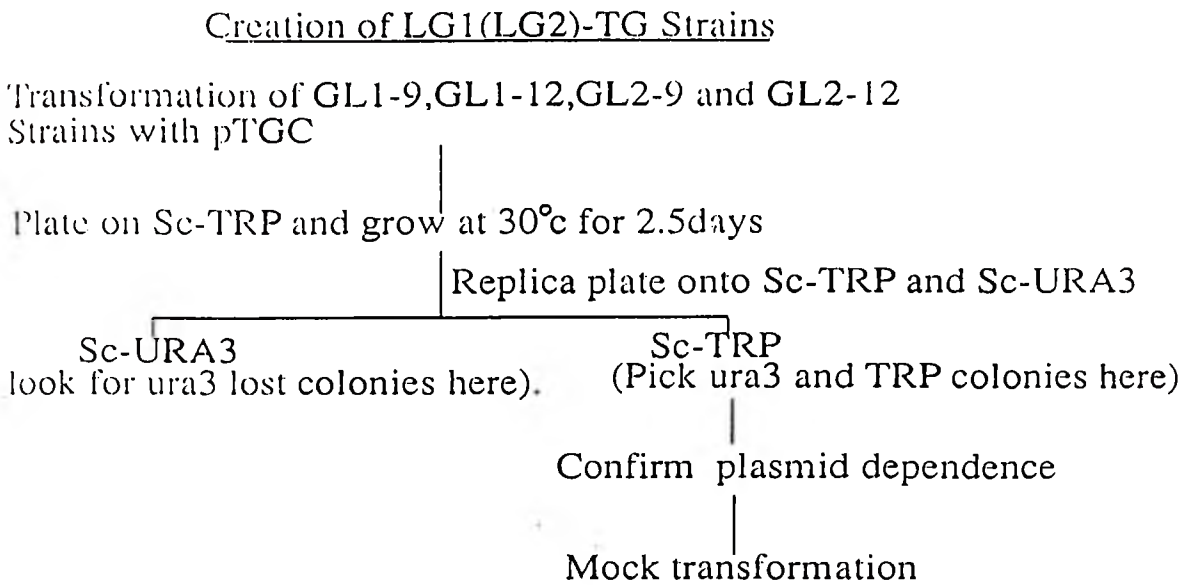

Figure 7: Creation of LG1(LG2)-TG Strains

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 theirs 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 *Xho*1 oligonucleotide linker ligation was used to change a *Sma*1 to a *Xho*1 site in pTGS to give pTGX. This was performed by cutting pTGS with *Sma*1 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 *Xho*1 oligonucleotide linker was kinased using T4 DNA kinase. The phosphatasing was necessary to prevent the religation of the compatible *Sma*1 ends. The phosphatased pTGS and the kinased *Xho*1 oligonucleotide linker were then ligated using T4 DNA ligase.

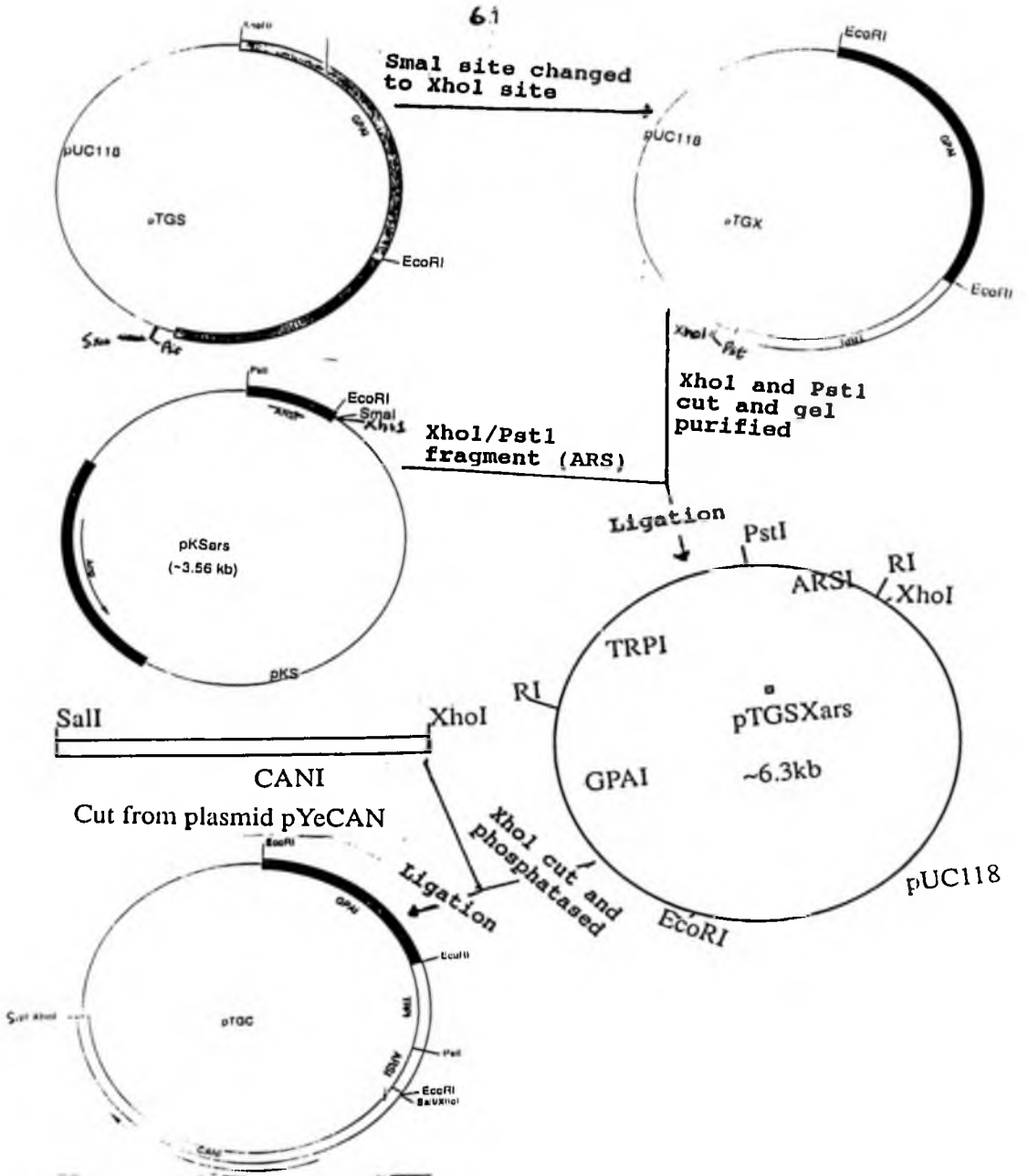

Figure 8: Construction of pTGC

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 *SmaI* site was changed to a *XhoI* site using a XhoI oligonucleotide linker to form plasmid TGX. The autonomous replicating sequence (ARS) was then cut from plasmid KSars. pKSars was formed by ligating the *PstI/XhoI* fragment of ARS to Bluescript KS+. The ARS fragment was then ligated to TGX which had been cut with *PstI* and *XhoI* to form plasmid TGSXars. pTGSXars was cut with XhoI phosphatased and then ligated to the *SalI/XhoI* fragment of *CAN1* gene to form plasmid TGC. pTGC has *TRP1* marker, *ARS*, *CAN1* and *GPA1* genes as its relevant genotype.

The Pst1 to *Xho*1 restriction fragment of autonomous replicating sequence (ARS) was cut from plasmid ksARs and ligated to pTGX which had been cut with *Xho*1 and *Pst*1 and gel purified to give pTGSXars. The plasmid TGSXars was then cut with *Xho*1, phosphatased and ligated to *Sal*1/*Xho*1 fragment of canavanine gene (*CAN1*) which was cut from the plasmid pYeCAN to give pTGSXarsCAN which was shortened to pTGC (Figure 8). *Sal*1 and *Xho*1 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 *Sac*1 and *Sma*1 restriction enzymes. The digested YEp13M4 was then agarose gel-purified and ligated to a purified *Sac*1/*Sma*1 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 *Kpn*1 and *Cla*1 double digested and gel purified. Similarly, clone9 was *Kpn*1 and *Cla*1 double digested using their compatible buffer, and gel purified.

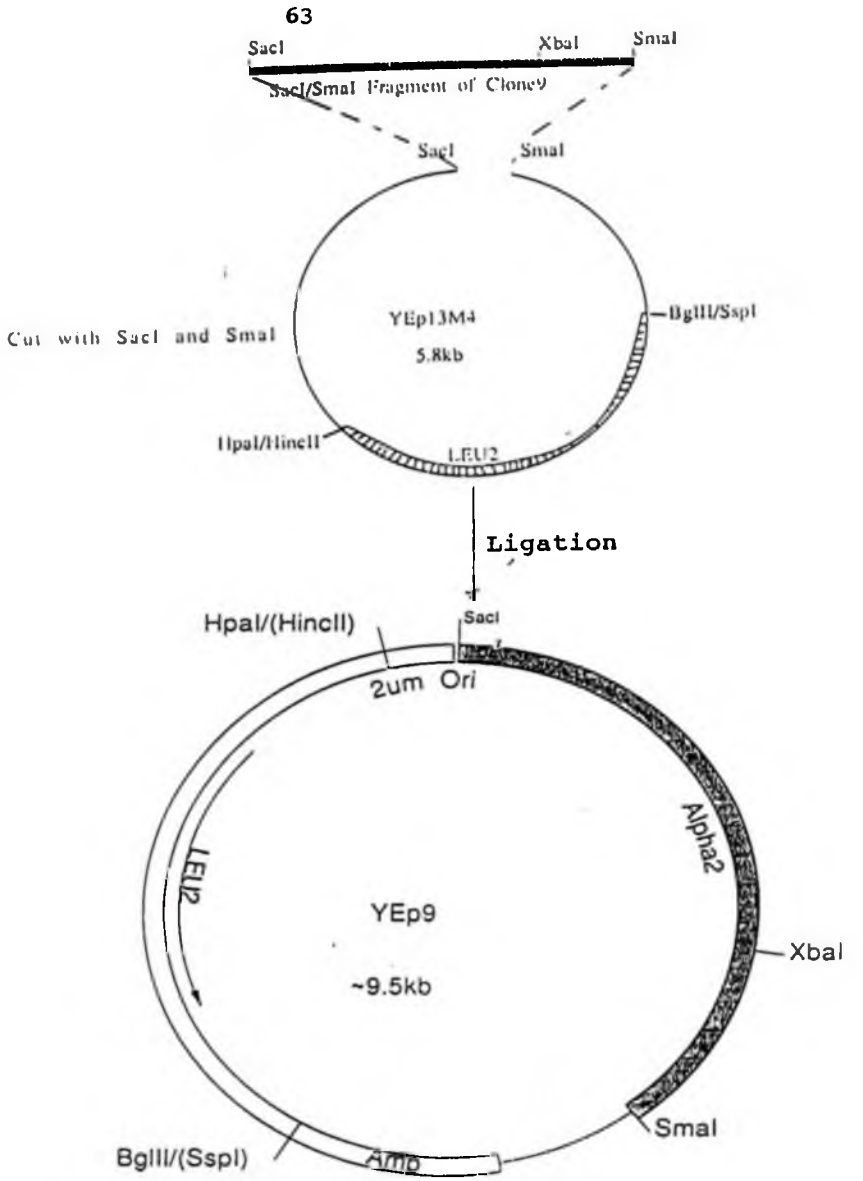

Figure 9: Construction of YEp9

This shows steps involved in construction of YEp9. First, the vector YEp13M4, which has LEU2 as its marker was cut with *SacI* and *SmaI* double digest. This was then purified and ligated to the *SacI/SmaI* fragment of clone9 to form YEp9. YEp9 has *MATα2*, LEU2 marker and a 2μm origin of replication genes.

The gel purified RS416 was then ligated to the *Kpn*1/*Cla*1 fragment of MATα2 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 MATα2

In order to test the importance of MATα2 in recovering the *gpa1* strains, the MATα2 transcript was disrupted using *URA3* marker gene.

A *Hind* III restriction endonuclease site was inserted into the Xba1 recognition site on the gene subcloned into pBluescript (pks) vector forming the plasmid KSEK using *Hind*III oligonucleotide linker (Figure 31). The plasmid KSEK was cut with *Xba1*, phosphatased using calf intestine phosphatase and the DNA pelleted with ethanol. 1.0µg of the *Hind*III 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 *Hind*III cohesive end compatibility and T4 DNA ligase reaction (Fig 26).

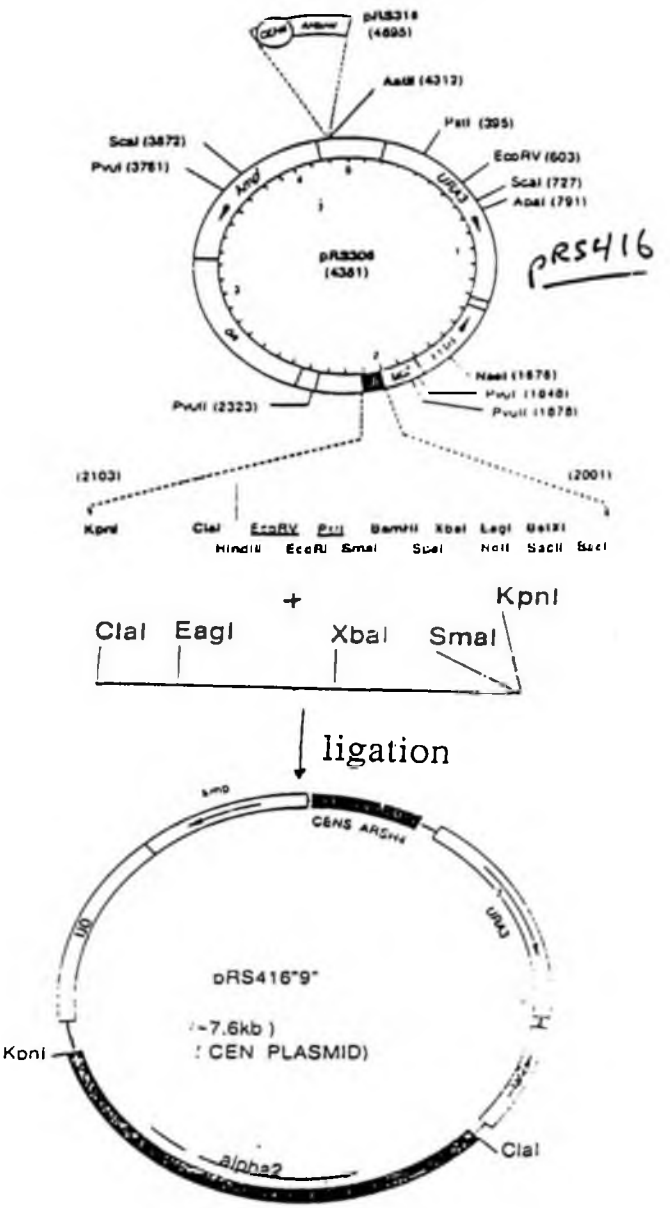

Figure 10: Construction of Centromere plasmid.

This illustrates the Steps involved in construction of plasmid RS416"9". The vector pRS416 was cut with *ClaI* and *KpnI* and ligated to the *ClaI/KpnI* fragment of clone 9 to form pRS416"9".
Plasmid RS416"9" has CENS origin of replication, *URA3* marker and *MATα2* genes.

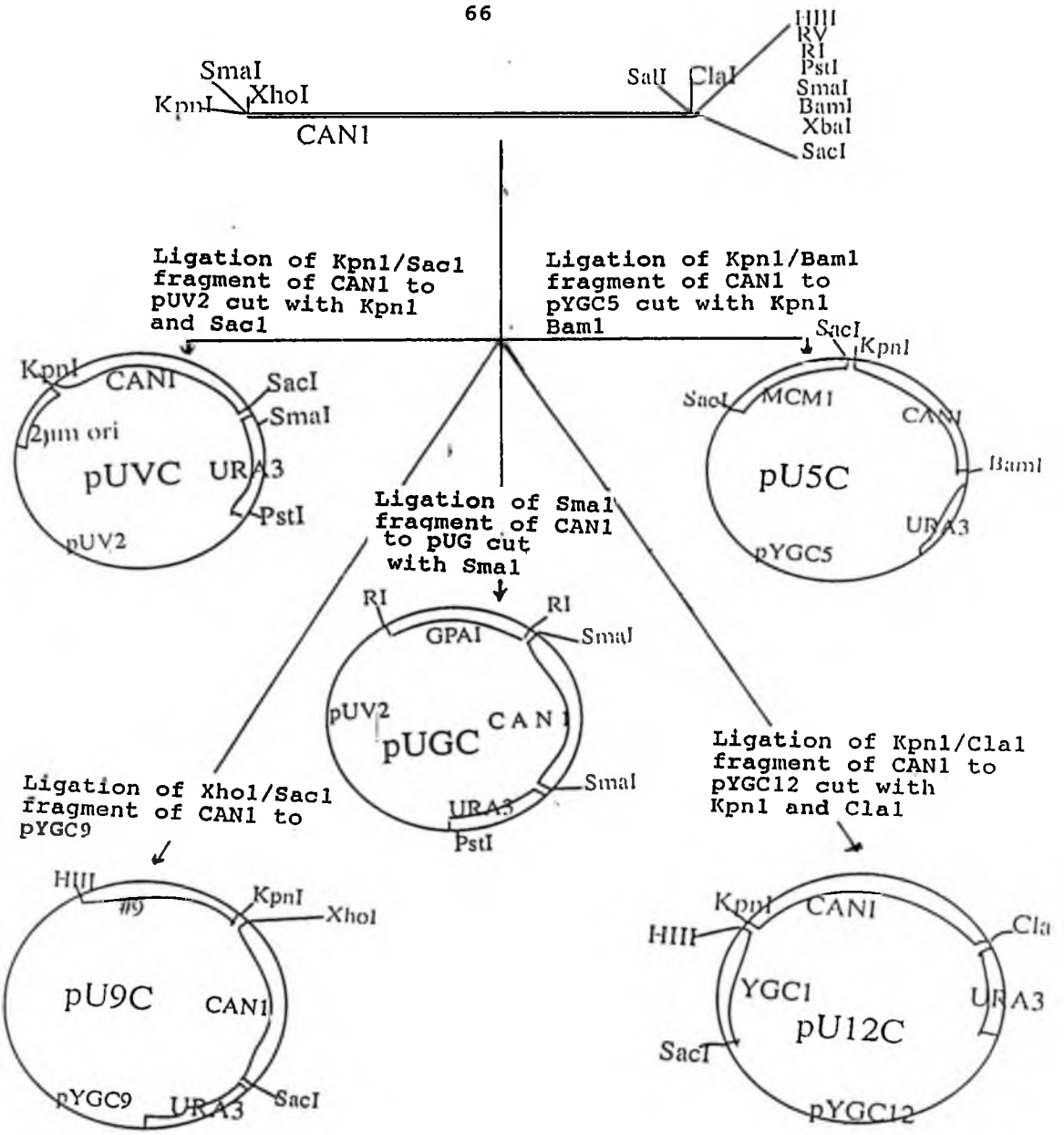

Figure 11: Construction of UGC, U5C, UVC, U9C and U12C plasmids

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, *Xho1/Sal1* 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/Sac1, Sma1, Kpn1/Bam1, *Xho1/Sac1* and *Kpn1/Cla1* respectively and ligated to the various fragments of *CAN1* which were cut with the corresponding enzymes to enhance sticky ends compatibility ligations.

The *Sac1/Kpn1* 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), SP1(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 30^0C for 60hours 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 30^0C 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. 10^6 cells of each strain were counted and mixed with 10^6 cells of the tester strains DC14 and DC17 in TE buffer and incubated at 30^0C 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 MATα2 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 [α-^{32}P]dATP were used. The *MATα2* gene was subcloned into pBluescript 11KS(+/-) using cohesive restriction ends. *MATα2* 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 37^0C 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 (-70^0C, 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^{0}C for 2 min and leaving to slowly cool from 65^{0}C to 30^{0}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 (OD_{600} = 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^{0}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^{0}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^{0}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^{0}C thus allowing the cells to recover. After the incubation period, the cells were spread on LB+ Amp plates and incubated at 37^{0}C overnight. However, at certain times part of these competent cells were frozen at -80^{0}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 $0.5x10^7$ to $3.0x10^7$ 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 $6.5x10^8$ 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 30^0C 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 30^0C 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 42^0C 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 30^0C for 60 hours.

2.2.17 Mini Plasmid Preparation Procedure

Two-and-half ($2\frac{1}{2}$) mls of LB+AMP bacteria culture was grown at 37^0C 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, O.IN 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 lyses 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 30^0C 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 ßME (3.5ml/ml) plus 0.5mg zymolyase/ml, and incubated for 40 min at 37^0C, pelleted for 30 sec in

microfuge at 6,000xg and the sphereoplasts 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^{0}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^{0}C for 1 hr. The mixture was then phenol and chloroform extracted and 30µl of 3M NaOAC or 75µl of 6M NH_4OAC added. 600µl of ethanol was then added and the mixture kept at -20^{0}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 mls 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^{0}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 4^0C 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 30^0C for 3 days. About 10µl of the culture was then plated on SC-URA-ARG+CAN and incubated at 30^0C 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.

CHAPTER THREE

RESULTS

3.1 Isolation of the *MATα2*, *YGC1* and *MCM1* Genes

High copy number plasmids that were able to complement the *gpa1* defect in *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 *gpa1* mutation (in some cases only partially). Two nonhomologous plasmids that allowed complemen-tation in both MATa and MATα *gpa1* strains were analyzed in detail. The first plasmid, pYGC9 contains the *MATα2* gene which has previously been characterized but its function of complementing *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 *MCM1* gene which was known to be a general transcription activator. However, because the *MCM1* product complements only MATa *gpa1* cells but not MATα *gpa1* strains unlike *MATα2* and *YGC1*, there was little characterisation of it here. The ability of the *YGC9* plasmid to suppress the *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 (*GPA1*) gene of *Saccharomyces cerevisiae*. Another reason why much attention was paid to the pYGC9 insert was that its *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 p*YGC12* 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 *gpa1* mutation, as described in figure 20. Multicopy plasmids containing a 4.8 kb Hind III/EcoR1 fragment of pYGC9 were able to complement the *gpa1* mutation (figure 22). When the size was narrowed down to 1.8kb Eag1/Kpn1 (Figure , 19) or Cla1/Kpn1 (Figure 20) fragments of pYGC9, it was still able to complement the *gpa1* lethality both in MATa and MATα cell types. Thus the active part of pYGC9 involved in the complementation is located between *Eag1* 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 *MATα2 Disruption*

To test for the importance of MATα2 to the pheromone signal transduction pathway, the gene was disrupted with URA3 marker gene (Figure 26). Insertion of a HindIII fragment of the URA3 marker gene into the Xba1 site changed to HindIII site within the 1.8kb Eag1/Kpn1 fragment eliminated the ability of the plasmid to complement *gpa1* (Figures 26 and 27). This result indicated that the gene required for complementation is contained within the Eag1-Kpn1 fragment, since its disruption led to the cells lethality (Figure 27).

3.4 Overexpression of *MATα2 is Not a Prerequisite for GPA1* Complementation

In order to test whether overexpression of the MATα2 product is necessary for the suppression of *gpa1* strains, the 1.8kb Eag1/Kpn1 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 30^0C (Figure 25). The ability of the centromere plasmid to suppress the *gpa1* mutation suggests that overexpression was not required for complementation by *MATα2 (Figure 25).*

No *GPA1*-containing plasmid was isolated in the original screen for suppression of *gpa1* mutation (Figure 12). To be certain that suppressing the *gpa1* 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 *gpa1* 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.

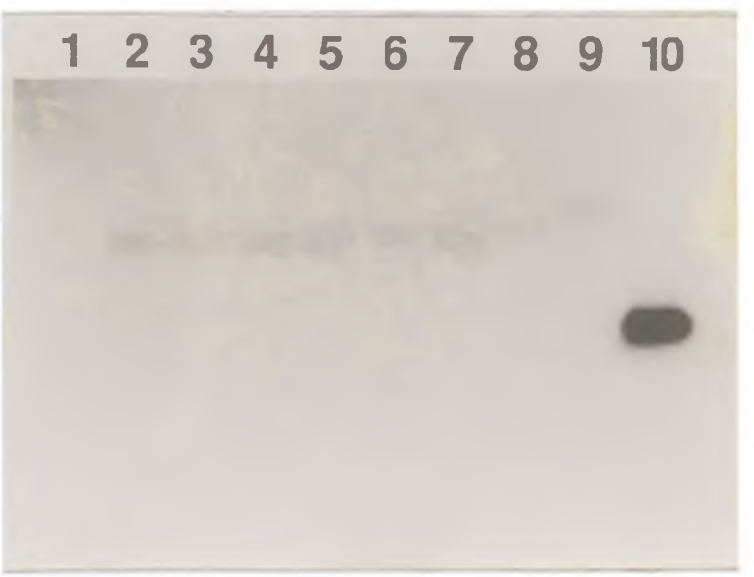

Figure 12: High Copy Suppressors are not GPA1

High copy suppressors plasmids were digested with *Eco*R1 and *Hind*III, run on a 0.8% agarose gel, blotted onto a nitrocellulose filter and probed with the ^{32}P labeled 1.9kb *Eco*R1 fragment of *GPA1*. Lane 1: Lambda *Hind*III digest marker: Lanes 2-9: suppressors 5,6,7,9,11,12,14 and 20, respectively. Lane 10: *GPA1 Eco*R1 fragment.

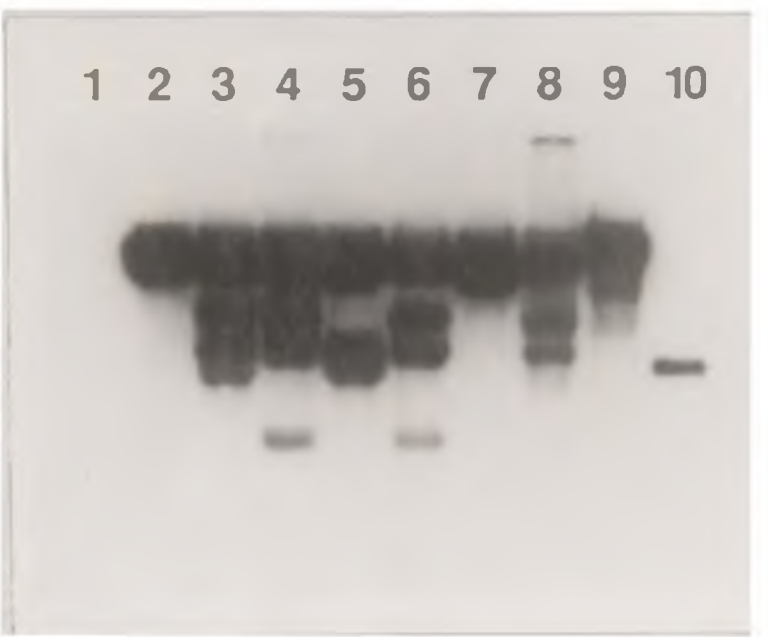

Figure 13: Suppressor 9 Hybridizes to Most Other Suppressors

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.

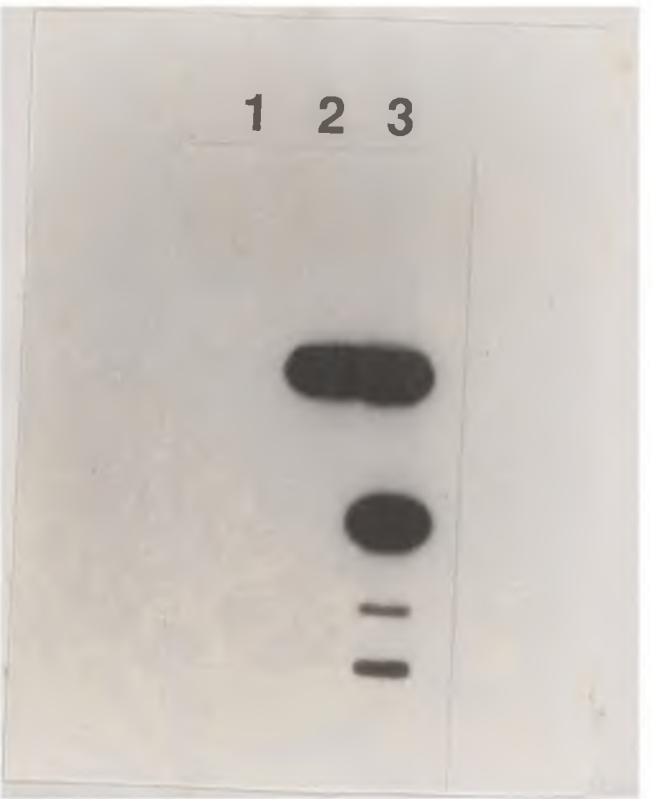

Figure 14: **Suppressors 5 and 12 are each unique**

High copy suppressor plasmids 5 and 12 were digested with *Eco*R1 and *Hind*III, run on an agarose gel and transferred to a nitrocellulose filter. The filter was then probed with ^{32}P 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, Cla1 EcoR1; Lane 3, Cla1+Xho1; Lane 4, Cla1+Pst1; Lane 5, Cla1+Sac1; Lane 6, Cla1+Apa1 Lane 7, Pst+ Hind III; Lane 8, Xho1+Hind III; Lane 9, Xho1+Sac1; Lane 10, Xho1+EcoR1; Lane 11, Xho1+Apa1; Lane 12, Apa1; Lane 13, Apa1+Kpn1; Lane 14, Apa1+EcoR1; Lane 15, Apa1+Sac1; Lane 16, Sac1; Lane 17, Sac1+Hind III; Lane 18, EcoR1+Hind III; Lane 19, EcoR+Sac1. Digests were run on 0.8%agarose gel.

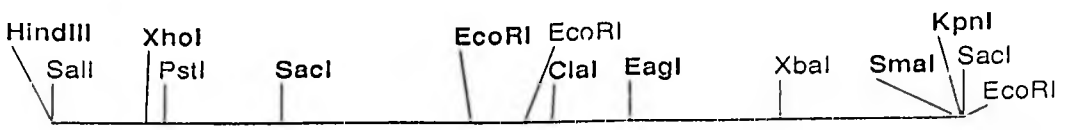

Figure 16: Restriction Map of Clone 9

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 *Eco*R1 site highlighted is almost at the centre of the insert. The size of the insert is 4.8kb.

Figure 19: Restriction Map of MATα2

This *Eag/Kpn1* fragment of clone9 Complements the gpa1 lethality both in MATa and MATα Yeast strains.

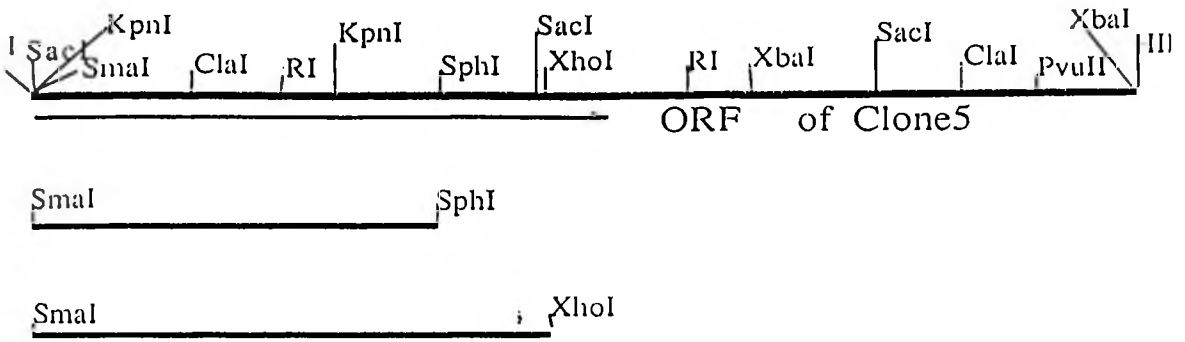

Figure 17: Restriction Map of Clone 5

The SmaI/SphI fragment and the *SmaI/XhoI* segments are both able to complement the *gpa1* in MATa cells. The restriction sites are: *EcoRI* (RI), KpnI, *SmaI*, *ClaI*, *SphI*, *SacI*, *XhoI*, *XbaI*, *ClaI*, *PvuII* and *HindIII*.
ORF stands for the open reading frame, and its direction is shown by the arrow.

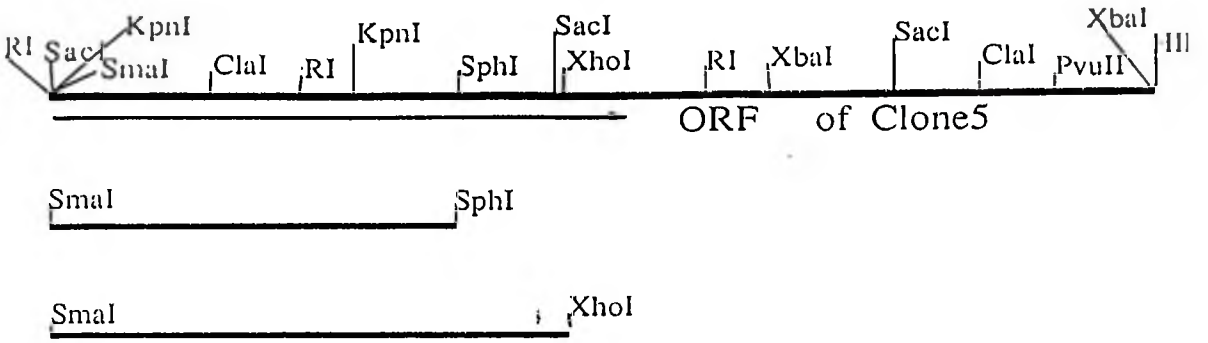

Figure 17: **Restriction Map of Clone 5**

The SmaI/SphI fragment and the *Sma*I/*Xho*I segments are both able to complement the *gpaI* in MATa cells. The restriction sites are: *EcoRI* (RI), KpnI, *Sma*I, *Cla*I, *Sph*I, *Sac*I, *Xho*I, *Xba*I, *Cla*I, *Pvu*II and *Hind*III.
ORF stands for the open reading frame, and its direction is shown by the arrow.

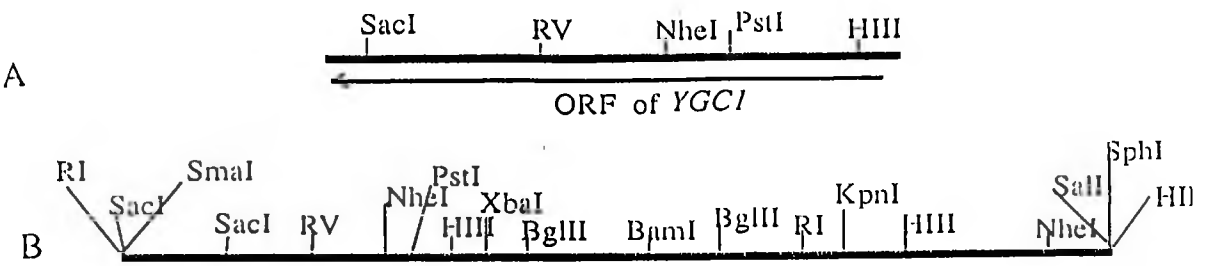

Figure 18: Restriction Map of Clone 12

Figure A shows the ORF of YGC1 from the N-terminal end. This fragment of *YGC1* is fully capable of complementing the *gpa1* lethality: Fig. B shows restriction map of the original clone 12 (*YGC1*). Restriction sites are indicated by the names of the enzymes.

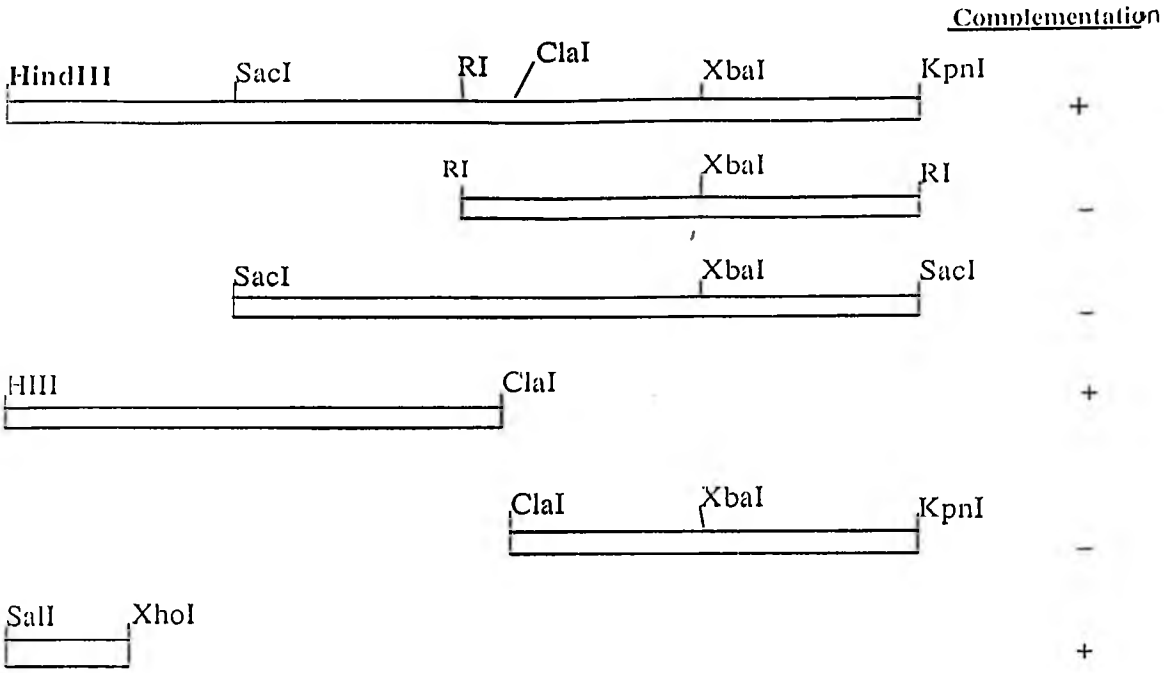

Figure 20: Clone 9 Deletions and suppression of *gpa1*

Figure indicates that the active fragment of clone9 which is complementing the *gpa1* is between *Cla1* and *Kpn1*. Complementation is based on the rescuing of *gpa1* cells. Regions tested for their ability to complement the *gpa1* mutation in pUV plasmids are indicated in rectangular bars. Restriction sites R1(*Eco*R1) *Hind*III, *Sac*1, *Cla*1, *Xba*1 and Kpn1. Ability to complement the *gpa1* is indicated with positive signs (+) and inability to do so with negative signs (-).

3.5 *MATα2* 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 *MATα2* gene was identified (Figure 21). The *MATα2* sequence had already been reported by Nasmyth and Tatchell (, 1980). The MATα2 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 MATα2 Suppresses Mating in both MATa and MATα *gpa1* yeast cells

Table 6 shows the average of two qualitative mating assay results of pYGC9 in both MATa and MATα 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 GU1 and GU2 cells as compared to SP1 and FY250 haploid cells. GU1 and GU2 are *gpa1* strains whereas SP1 and FY250 are *GPA1* haploid strains. Suppression of the mating seemed to be more pronounced in MATα *gpa1* cells than MATa *gpa1* cell types.

3.7 The Loss of *MATα2* Function Results in late G1 Arrest

Microscopic examination of the spores containing a disrupted *MATα2* showed that they germinated and went through several cell divisions.

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                10          20          30          40          50          60
   1 CAGTTACAAA CATCTTAGTA GTGTCTGAGG AGAGGGTTGA TTGTTTATGT ATTTTTGCGA
  61 AATATATATA TATATATTCT ACACAGATAT ATACATATTT GTTTTTCGGG CTCATTCTTT
 121 CTTCTTTGCC AGAGGCTCAC CGCTCAAGAG GTCCGCTAAT TCTGGAGCGA TTGTTATTGT
 181 TTTTTCTTTT CTTCTTCTAT TCGAAACCCA GTTTTTGATT TGAATGCGAG ATAAACTGGT
 241 ATTCTTCATT AGATTCTCTA GGCCCTTGGT ATCTAGATAT GGGTTCTCGA TGTTCTTCTT

               310         320         330         340         350         360
 301 TGCAAACCAA CTTTCTAGTA TTCGGACATT TTCTTTTGTA AACCGGTGTC CTCTGTAAGG
 361 TTTAGTACTT TTGTTTATCA TATCTTGAGT TACCACATTA AATACCAACC CATCCGCCGA
 421 TTTATTTTTC TGTGTAAGTT GATAATTACT TCTATCGTTT TCTATGCTGC GCATTTCTTT
 481 GAGTAATACA GTAATGGTAG TAGTGAGTTG AGATGTTGTT TGCAACAACT TCTTCTCCTC
 541 ATCACTAATC TTACGGTTTT TGTTGGCCCT AGATAAGAAT AGTAATATAT CCCTTAATTC

               610         620         630         640         650         660
 601 AACTTCTTCT TCTGTTGTTA CACTCTCTGG TAACTTAGGT AAATTACAGC AAATAGAAAA
 661 GAGCTTTTTA TTTATGTCTA GTATGCTGGA TTTAAACTCA TCTGTGATTT GTGGATTTAA
 721 AAGGTCTTTA ATGGGTATTT TATTCATTTT TTCTTGCTTA TCTTCCTTTT TTTCTTGCCC
 781 ACTTCTAAGC TGATTTCAAT CTCTCCTTTA TATATATTTT TAAGTTCCAA CATTTTATGT
 841 TTCAAAACAT TAATGATGTC TGGGTTTTGT TTGGGATGCA ATTTATTGCT TCCCAATGTA

               910         920         930         940         950         960
 901 GAAAAGTACA TCATATGAAA CAACTTAAAC TCTTAACTAC TTCTTTTAAC CTTCACTTTT
 961 TATGAAATGT ATCAACCATA TATAATAACT TAATAGACGA CATTCACAAT ATGTTTACTT
1021 CGAAGCCTGC TTTCAAAATT AAGAACAAAG CATCCAAATC ATACAGAAAC ACAGCGGTTT
1081 CAAAAAAGCT GAAAGAAAAA CGTCTAGCTG AGCATGTGAG GCCAAGCTGC TTCAATATTA
1141 TTCGACCACT CAAGAAAGAT ATCCAGATTC CTGTTCCTTC CTCTCGATTT TTAAATAAAA

              1210        1220        1230        1240        1250        1260
1201 TCCAAATTCA CAGGATAGCG TCTGGAAGTC AAAATACTCA GTTTCGACAG TTCAATAAGA
```

Figure 21: Sequence of MATα2.

The regions underlined are those that were sequenced before using the BLAST Database Search to look for sequence homology.

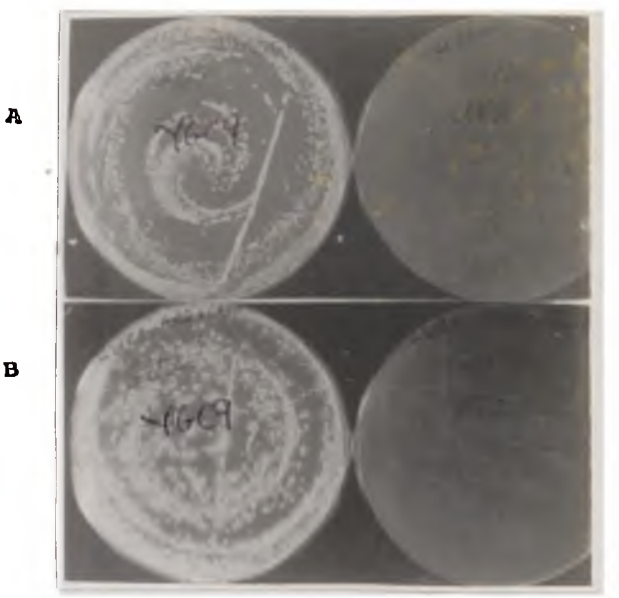
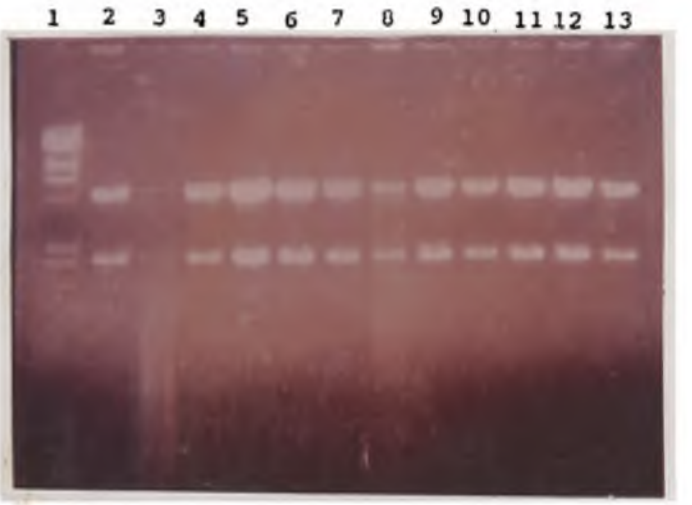


Figure 22: **Clone 9(α2) rescues both MATa and MATα cells**

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) GU1 transformed with clone9 and pUV2 at left and right respectively. (B) GU2 transformed with clone9 and pUV2 at left and right respectively.



Figuro 23: **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 ClaI and KpnI. Lanel is the Lambda DNA-*Hind*III digest marker (sizes from origin are 9.42, 6.56, 4.36, 2.32 and 2.03). The smaller (lower) band corresponds to the MATα2 gene, whilst the larger (upper) band is the CEN plasmid vector RS416 (lanes 2 to 12).

Table 5:

Transformation Results

Yeast Strain	Mating Type	Plasmid	Complementation of *gpa1*
GU1	MATa	clone 9(*α2*)	+
		clone 5(*MCM1*)	+
		clone12(*YGC1*)	+
GU2	MATα	clone9(*α2*)	+
		clone5(*MCM1*)	–
		clone12(*YGC1*)	+

The transformation results indicate that clones 9 and 12 complement gpa1 in both MATa and MATα cell types whereas clone5 only complement gpa1 in MATa cells. The positive signs indicate the ability of the plasmids to keep the gpa1 cells alive whereas the negative sign shows the inability to do so.

Table 6

MATING ASSAY RESULTS

Mating plasmid	Mating type of yeast strain	DC17(α-cells), Number of colonies	DC14(a-cells, Number of diploid colonies
GU2 with pUV2	MATα	0	75
GU2 with pYGC9	MATα	0	5
FY250 with pUV2	MATα	2	2,540
FY250 with pYGC9	MATα	1	996
GUI with pUV2	MATa	612	0
GUI with pYGC9	MATa	32	0
SPI with pUV2	MATa	1210	0
SPI with pYGC9	MATa	1500	1

The results show that clone9 complemented strains mated with both MATa and MATα mating type cells. The assay results also indicate, however, that MATa cells containing pYGC9 mate better than MATα cells. Clone9 suppresses mating in wild type MATα cells (FY250) but not in wild type MATa cells (SP1) as shown. The tester or mating type strains are DC17(α-cells) and DC14(a-cells)

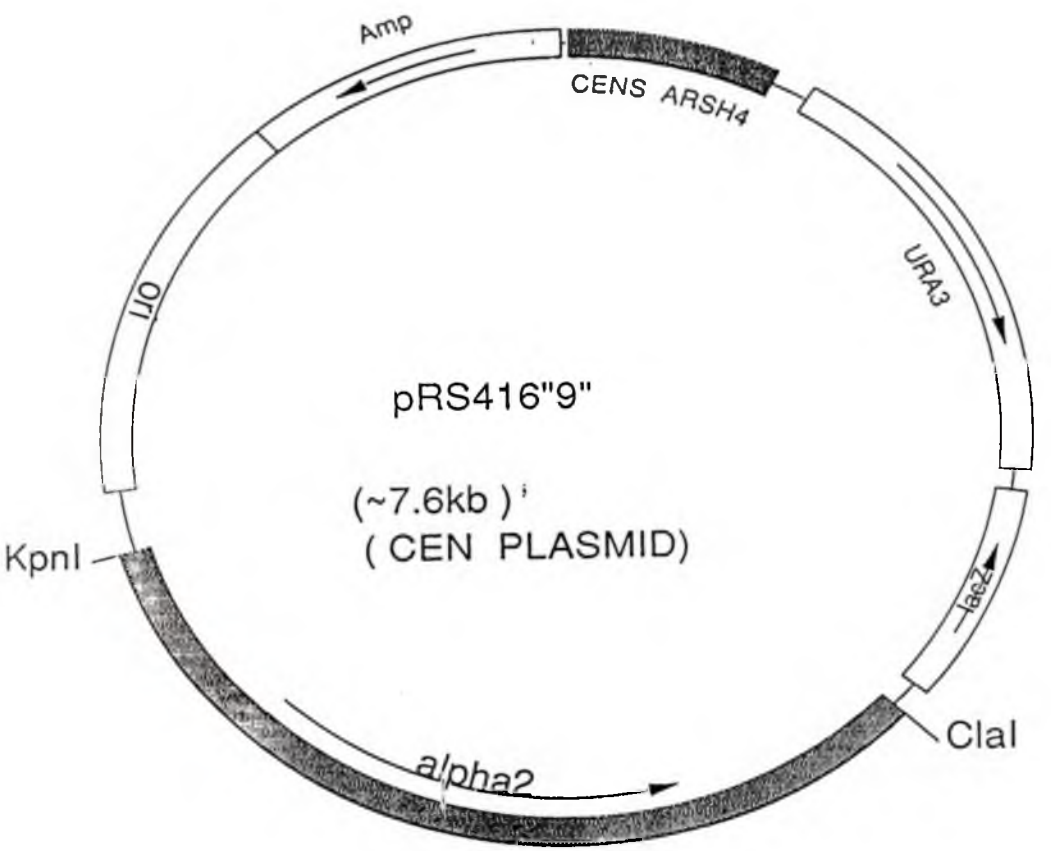


Figure 2 **Map of plasmid RS416"9"**

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 *gpa1* by *MATα2* 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.

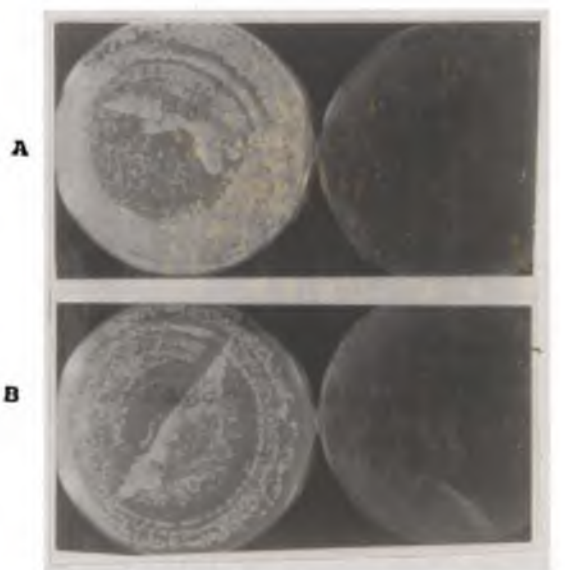


Figure 25: Rescue of Cells In Single Copies by Clone 9

pRS416"9" is a CEN plasmid containing clone9 which is capable of complementing the gpa1 both in MATa and MATα cells. Thus, complementation is independent of the copy number. A; GU1 transformed with the plasmids RS416"9" and RS416 at left and right respectively. B; GU2 transformed with the plasmids RS4169 and RS416 at left and right respectively. Cells were plated on Sc-URA-ARG+CAN.

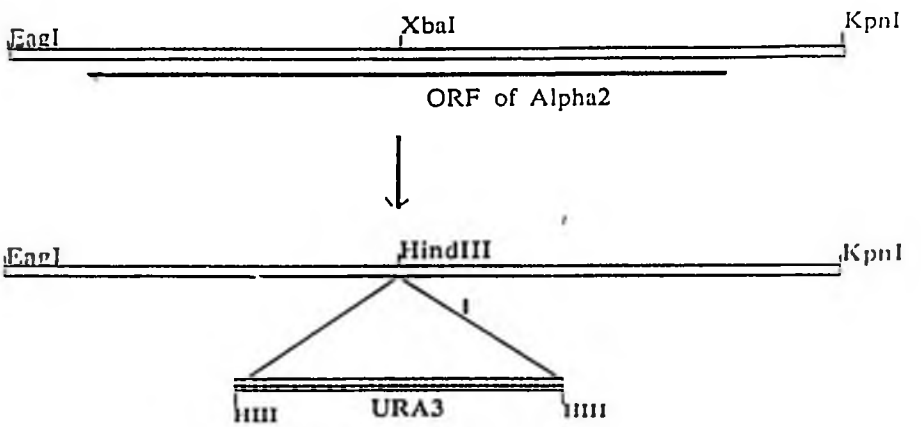


Figure 26: Disruption of Clone9

Figure 26 shows the disruption of alpha2 with the URA3 Marker. The *Hind*III site was inserted at the *Xba*1 site and the *Hind*III fragment of *URA*3 was used to frame-shift the alpha2 gene. The disrupted gene was confirmed by restriction enzyme digests. The *Xba*1 site was cut with *Xba*1 endonuclease, phosphatased using calf intestine phosphatase (CIP) and the *Hind*III oligonucleotide linker which had been phosphorylated using T4 polynucleotide kinase, inserted at the original *Xba*1 site using the T4 DNA ligase reaction. The introduced *Hind*III site was then cut with *Hind*III endonuclease and the *Hind*III 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.

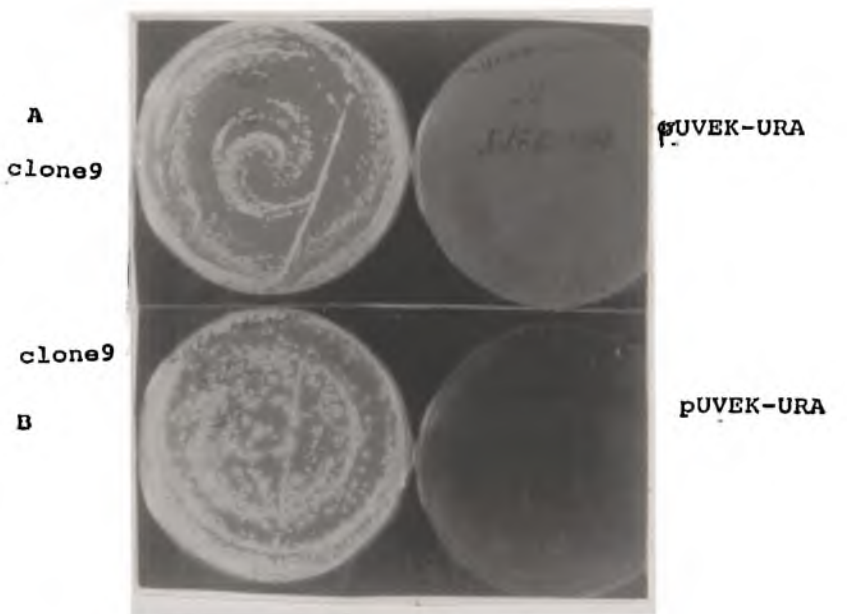


Figure 27: Disruption of *MATα2* Leads to Constitutive Cell-Cycle Arrest.

(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 MATa 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.

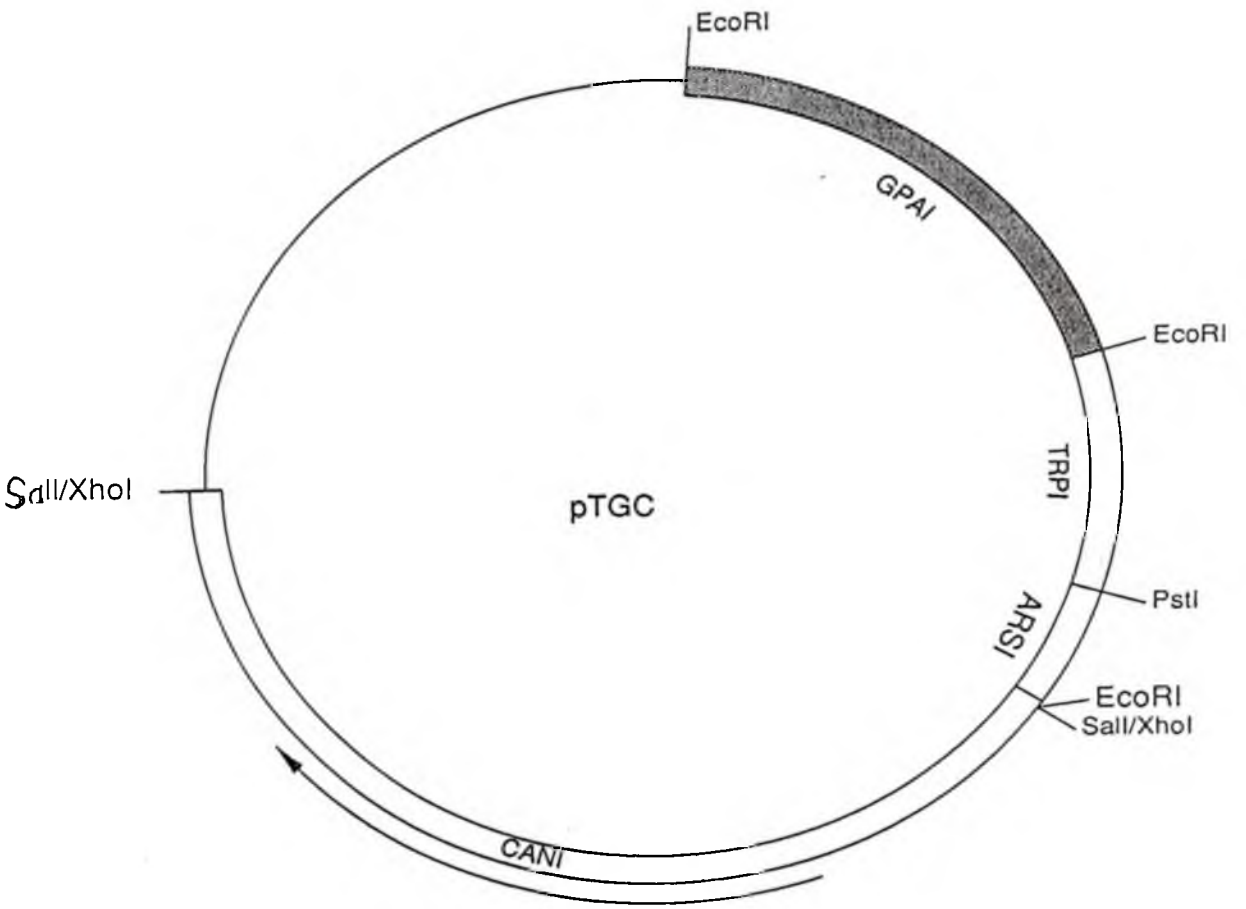


Figure 28: Map of plasmid TGC

Plasmid TGC was constructed and its strain created. The idea was to use this strain for screening the mammalian library. *EcoR1* fragment of *GPA1* is shown as shaded part. *TRP1* marker, autonomous replicating sequence (*ARS1*), and the *CAN1* marker genes are also shown.

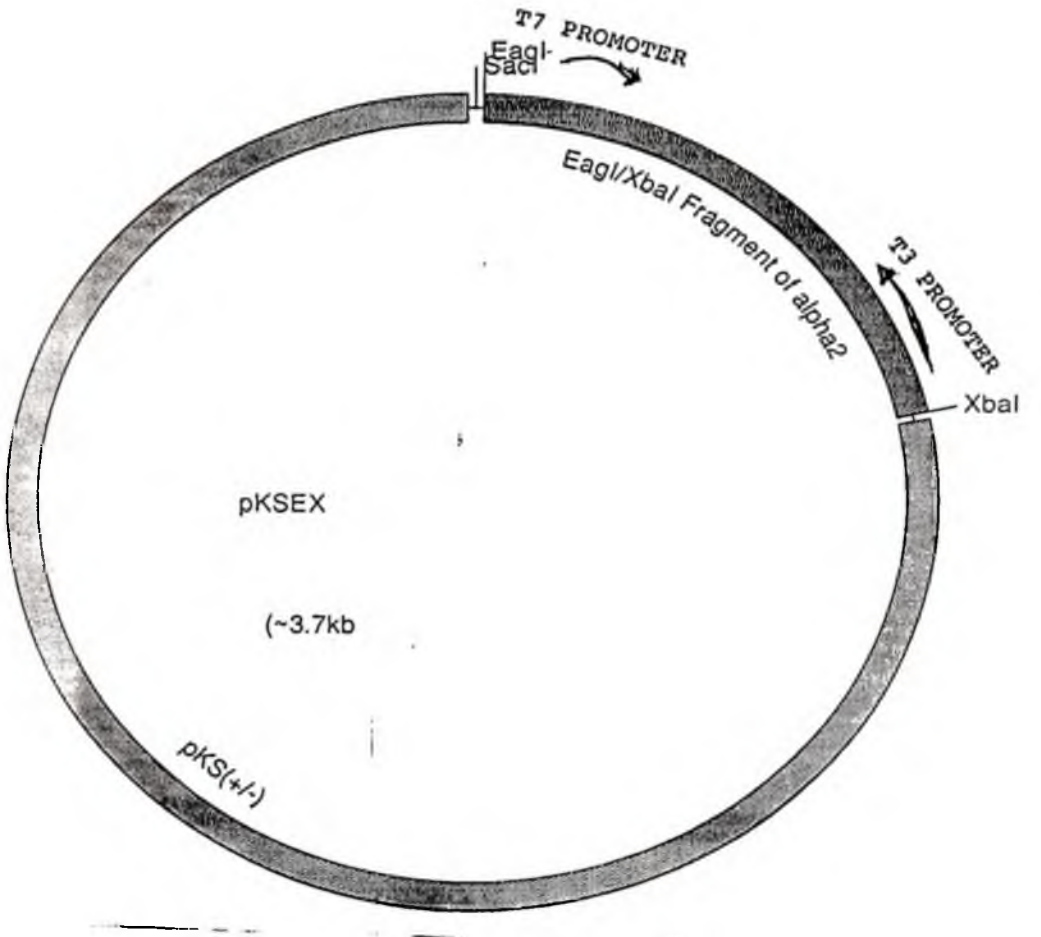


Figure 29: Plasmid KSEX.

The arrows indicate the direction of sequencing of the Eag1/Xba1 fragment of MATα2. The Eag1/Xba1 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.

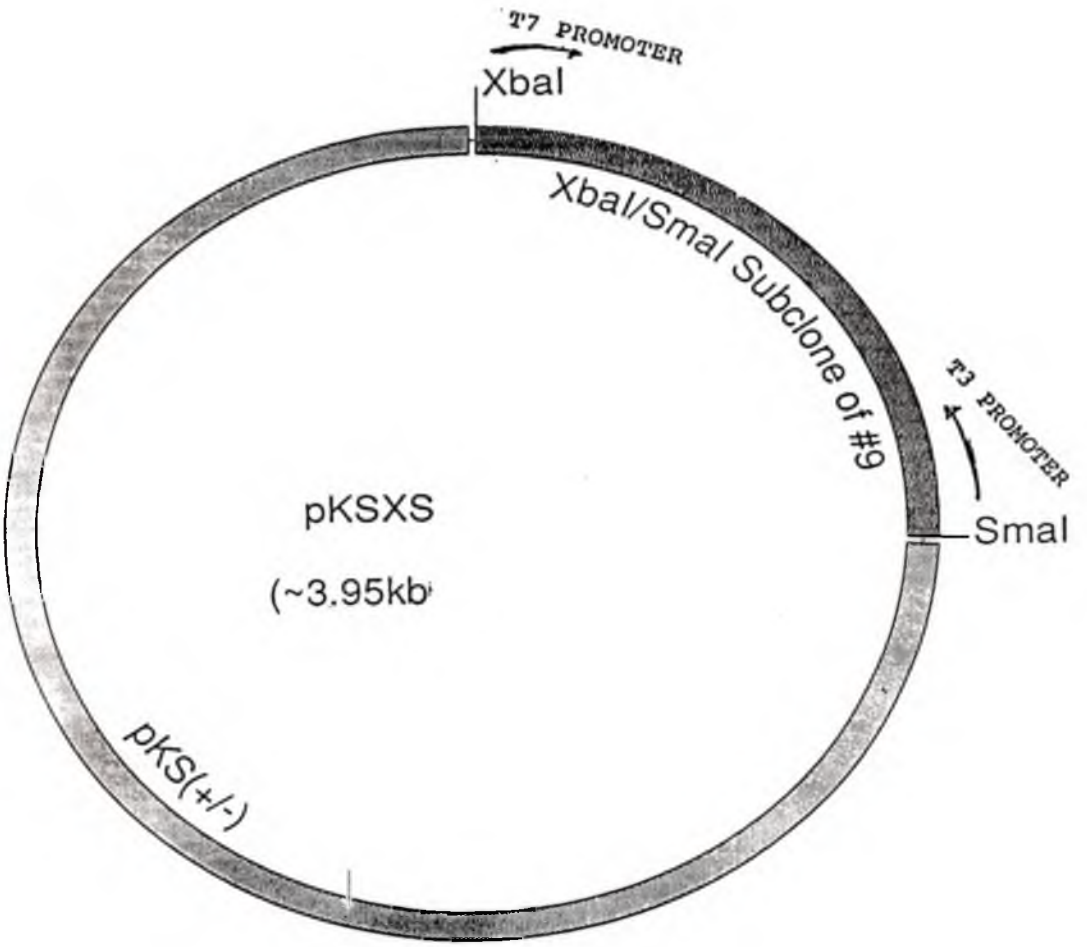


Figure 30: Plasmid KSXS

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

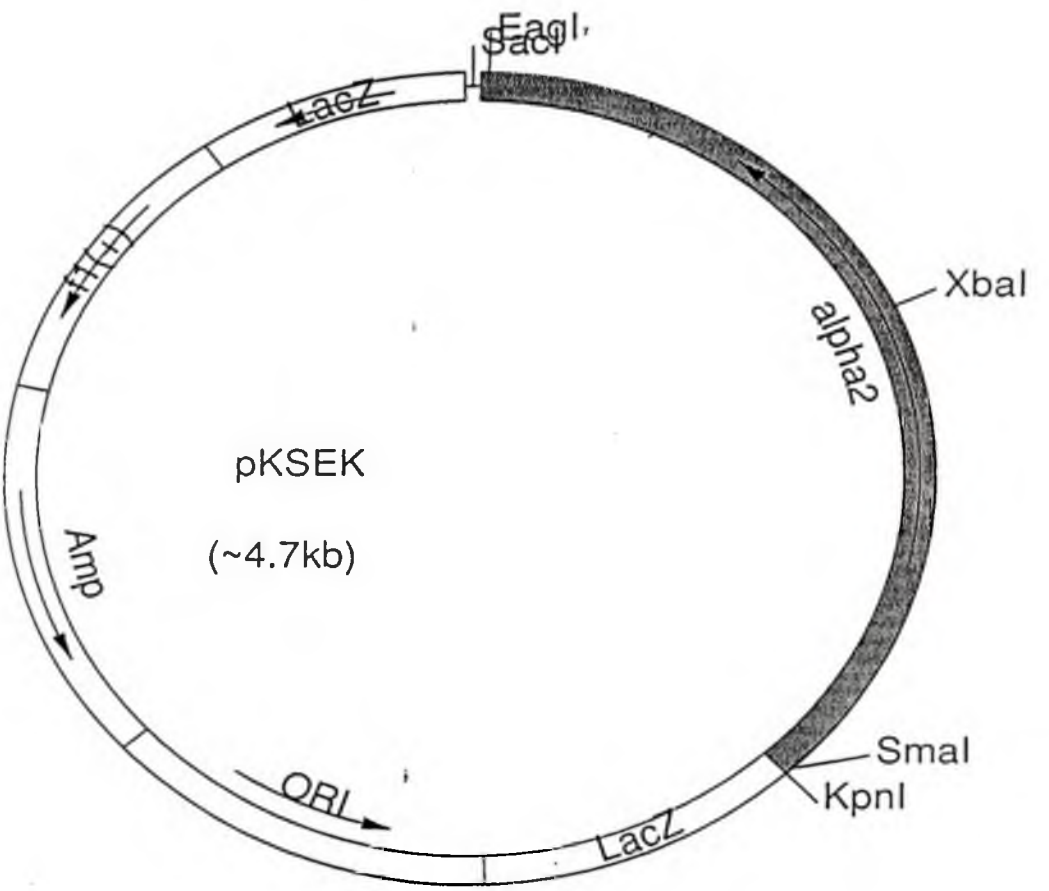


Figure 31: **Outline of plasmid KSEK.**

This plasmid was constructed and used for the disruption of *MATα2* with *URA3* Marker gene. The marker gene was inserted at the *Xba1* site after the *Hind*111 restriction site was introduced. First, *Xba1* endonuclease was used to digest KSEK. Since *Xba1* is unique on KSEK, a *Hind*III restriction endonuclease oligonucleotide that fitted into the sticky ends of the *Xba1* restriction site was introduced and ligated to it using T4 DNA ligase.

Table 7

MORPHOLOGY OF REVERTANTS OF gpa1 CELLS

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

Reversion of the strains may occur as a result of a switch of the *ura3* 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 micros-cope. The revertants were created from clone9 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 shmooed 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.

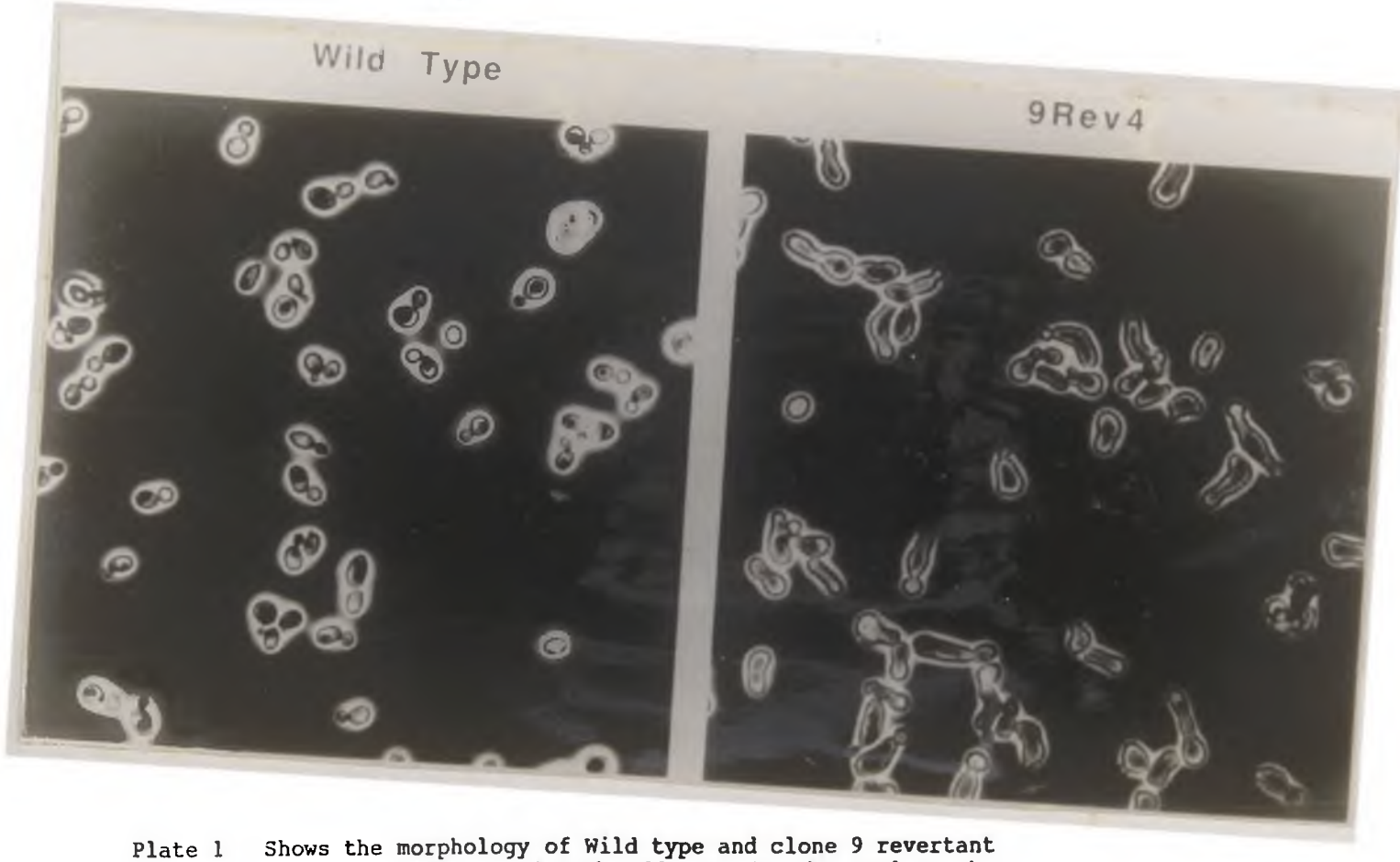


Plate 1 Shows the morphology of Wild type and clone 9 revertant cells the "Shmoo" shaped cells can be observed on the 9Rev4

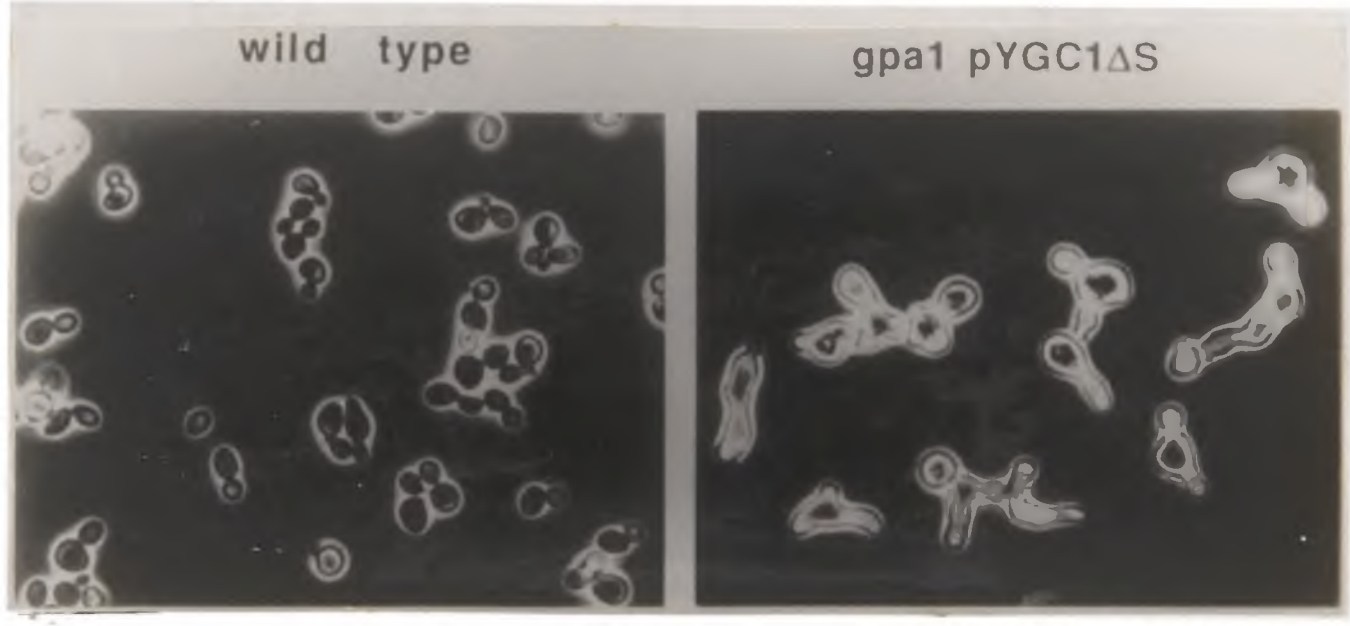


Plate 2 Shows differentiation of the pYGC1 cells as compared with the wild type cells. This might be due to mutations or alterations in genes such as STE4, STE18 or new gene in the pathway.

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 *gpa1* 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 *MATa/gpa1::HIS3/ leu2/ura3/can1* was kept alive with the plasmid TLCG whose relevant genotype is *GPA1/LEU2/CAN1*, since the *gpa1* 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 *CAN1* is a toxic arginine (ARG) analog which allows the arginine permease to accumulate canavanine drug (Figure 5). *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 *GPA1* maintenance plasmid, survival of cells after the destruction of the *GPA1* means that the high copy suppressor plasmids were responsible for keeping the GU1 cells alive or complementing the *gpa1::HIS3* lethality (figures 4, 5, 22 and table 5).

4.2 Identification of *MATa2*, *YGC1* and *MCM1* Genes Involved in the Pheromone Response Pathway

Saccharomyces cerevisiae genes *MATa2*, *YGC1* and *MCM1* have been identified by isolation of plasmids that were able to complement or suppress a *gpa1::HIS3* mutation.

MATα2 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 α mating functions (Strathern et al., 1980). In diploid cells, the same inhibition of **a** mating functions is exerted by *MATα2*, but in addition, it acts in conjunction with the **a**1 gene to regulate MAT transcription, principally the repression of α1 transcription and to allow sporulation (Figure 3). *In vivo,* α2 protein represses transcription of two sets of cell type-specific genes by binding to operator sites together with either the **a**1 or MCM1 proteins (Figure 3). The α2 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 α-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 *gpa1::HIS3* mutation in high copy plasmids (table 5).

In this study, another important function of *MATα2* gene has been established, that is complementation of *gpa1* yeast strains (figure 20,22 and 25; table 5). The α2 protein can suppress *gpa1* 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 *MATα2* 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 *gpa1* mutation results in hypersen-sitivity to pheromone and a defect in recovery from the cell-cycle arrest caused by exposure to pheromone. The ability of *MATα2 and YGC1* to suppress *gpa1::HIS3* mutation in both MATa and MATα cells (table 5, figure 22) and *MCM1* in MATa cells (table 5) suggests that *MATα2*, *YGC1* and *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 MATα2 and YGC1 in the Pheromone - Induced Signaling Pathway

It is very surprising that MATα2 (figure 21) and *YGC1* which does not show any level of sequence homology to *GPA1* or to the α 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 *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 matα2:: URA3 suggests that the *MATa2* is involved in the pheromone response pathway. The ability of α2 to suppress gpa1 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 *MATa2* is required for growth *per se*, and that suppression of the *gpa1* phenotype is indirect. The results and observations are consistent with a simple model (Figure 1) for the role of the yeast Gα (Gpa1), Gß (Ste4) and Gγ(Ste18) subunits in the activation of the pheromone response pathway. Genetic results indicate that Gßγ functions downstream of Gα to activate the pathway, presumably by activating a downstream effector which is currently unidentified. In the absence of pheromone, Gα is presumed to bind GDP tightly and interacts with Gßγ to inhibit the pathway. In the presence of pheromone, the pheromone - receptor interaction relieves this negative control by promoting GDP and GTP exchange on Gα, resulting in dissociation of Gα from Gßγ and the free Gßγ then activates the pathway. In a *gpa1* null mutant, free Gßγ is present and constitutively activates the pathway leading to G1 arrest and morphological alterations. In analogous manner, the *MATa2* and *YGC1* gene products seem to be playing the role of *GPA1* in *gpa1* mutant cells either directly or indirectly on the pathway. It is however amazing and unexpected that *MATa2 and YGC1* should play the role of *GPA1* in the pathway because apart from not having sequence homologies, *MATa2* is not known to bind GDP/GTP and neither can it respond to conforma-tional 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 (Nakayanua et al., 1987) and the phenotypes associated with α2 are the same in both a and α cells, the mechanism of *MATα2* action is likely to be the same in both mating types.

The partial sterility of *gpa1* cells expressing α2 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 Gβγ as a conformational analog of Gpa1 (GDP), thus preventing activation of the pathway. Since the resulting cells are sterile and unable to respond to pheromone, this suggests that the α2 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 *MATα2*. *GU1* cells that are kept alive with *MCM1* and *YGC1* also showed similar pattern of suppression of mating efficiency. It could also be inferred from table 6 that wild type MATα 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 *MATα2* (figure 27) can be explained by the uncoupling of the effector molecule from α2 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

MATα2 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 α2 and Ygc1 proteins in the mating factor signal transduction pathway include; (a) the arrest phenotype of haploids when α2 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 α2 is lethal in haploid cells (figure 27) which indicates α2 is a haploid essential gene for cellular growth.

To explain the mechanism of action of α2, 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 Gßγ or the

effector molecule. Infact 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 α2, 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 α2 protein might play a role in communication between the activated receptor and Gßγ or be involved in stabilizing the putative effector and/or Gßγ proteins. In this way, inactivating α2 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/α diploids. Repression requires both the a1 protein, encoded at MATa, and the α2 protein encoded at MATα, which make up a1-α2 activity (figure 3, Jensen *et al.*, 1983). It is proposed that the transcription of *GPA1* would be under negative control by a1-α2 activity (figure 3 and Miyajima *et al.*, 1987). *GPA1* would be expressed in haploid a cells because of the absence of α2 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-α2 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-α2 can regulate *GPA1* negatively, and if the regulation involves a direct contact between a1-α2 and *GPA1*, then one could argue that probably the region of contact between the a1-α2 complex and *GPA1* might be structurally similar to that of Gßγ subunits of yeast G-protein. If this is possible, then in the absence of *GPA1*, that is in *gpa1::HIS3* yeast strains, a1-α2 or probably Mcm1-

α2 could bind the Gßγ nonspecifically because of the assumed similarity in the region of contact between *GPA1* and Gßγ. Thus in MATα cells, a1 and/or Mcm1 might repress the Gßγ activity whereas in MATa cells, the α2 component of the complex is expected to functionally repress the Gßγ activity. One could however be tempted to say that this model could be inadequate because only the α2 is able to suppress the pathway both in MATa and MATα cell types. The argument could have been more valid if *gpa1::HIS3* yeast strains are complemented by α2 only in MATa cells (table 5). However, one did not overlook the fact that probably, high copy expression of the α2 proteins activated the transcription of its putative complexing counterpart in MATα cells to suppress the signaling pathway. Here, it is pertinent to note that α2 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 *gpa1* only in MATa cells probably because it forms a complex with α2 (figure 3) to modulate the pathway by repressing a-specific genes involved in the transduction of the pathway whilst depressing α-specific genes.

Another plausible mechanism through which *MATα2* can complement *gpa1* lethality would be to bind to the putative biological effector of Gßγ which might act at the same level as Gßγ thereby preventing the transduction of the signal from Gßγ to the effector molecule. In this way, the signaling pathway would be suppressed due to the competition between α2 and Gßγ for binding to the effector. Logically if the α2 is transcribed in higher copies, it would advantageously out compete the Gßγ 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 α2 protein.

One could also envisage that the possibility of α2 and Ygc1 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 Ygc1 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 Gα 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 MATα 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 α2 and Mcm1 to suppress *gpa1* lethality. This may be unraveled by doing Northern blots. The fact that the α2 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 α2 would be involved in the branched pathway or if the pathway is simply linear as envisaged, then α2 might modulate some component(s) on this linear or the branched pathway either directly or indirectly. The fact however remains that α2 protein is strongly involved in the signaling pathway that leads to cell-cycle arrest at the G1 phase since disruption of the *MATα2* 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 α2, Ygc1 and Mcm1 has given more impetus to understanding *GPA1* signal transduction in *S. cerevisiae*. Generation of several point mutants of *MATα2* and the study of these mutants in terms of arresting the signaling pathway would throw more light into the mechanism of suppression of α2 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

10% Sodium dodecyl sulfate (SDS)

Dissolve 100g of electrophoresis-grade SDS in 900ml of H_2o Heat to 68^0C 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_2o.

20x sodium citrate (SSC)

Dissolve 175.3g of Nacl and 88.2g of sodium citrate in 800ml of H_2o. Adjust the pH +7.0 with a few drops of a 10N NaoH, Adjust the volume to 1 liter with H_2o.

20 x SSPE

Dissolve 175.3 of Nacl, 27.6g of $NaH_2PO_4.H_2o$ and 7.4 g of EDTA in 800ml of H_2o. Adjust the pH to 7.4 with NaoH (--6.5ml of a 10N solution). Adjust the volume to 1 liter with H_2o.

DnD Solution

To prepare 10ml of DnD

dithiothreitol	1.53
DMSO	9ml
1M potassium Accetate (pH7.4)	100ml
H_2o to 10ml	

Sterilise the DnD solution
by filtration through millex
SR membrane unit (Millipore)
and store at -20^{0}C
in sterile 0.5ml microfuge
tubes.

Preparation of 1 liter TFB

Reagemt	Amount required/liter	Final Concentration
1M MES (pH63)	10ml	10mM
$MnCl_2.4H_2o$	8.91g	45mM
$CaCl_2.2H_2o$	1.47g	10mM
KCL	7.46	100mM
Hexamminecobalt chloride	0.80g	3mM

SOC Medium

Per liter:

To 950ml of deionized H_2o, add:

bacto-tryptone	20g
bacto-yeast extract	5g
Nacl	0.5g

Shake until the solutes dissolve. Add 10ml of a 25mM solution of KCl. Adjust the pH to 7.0 with 5N NaoH (.2ml). Adjust the volume of the solution to 1 liter with deionized H_2o. Sterilize by autoclaving for 20 minutes at 121^{0}C on liquid cycle. Allow it to cool to 60^{0}C or less and then add 20ml of a sterile 1M solution of glucose. Just before use, add 5ml of sterile solution of 2M $MgCl_2$.

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).

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