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REGULATION OF THE CELL CYCLE BY A NEGATIVE GROWTH FACTOR IN YEAST

by

FRED CHANG

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

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Regulation of the Cell Cycle by a Negative Growth Factor in Yeast.

Fred Chang

ABSTRACT

This thesis addresses the question of how a negative growth factor, α-factor, causes cell cycle arrest at G1 in the yeast cell. This work approaches the general problem of how environmental factors, such as growth factors, regulate the cell cycle. The α-factor response pathway consists of a cascade of events which involve a G-protein coupled receptor, protein kinases, a transcription factor, and "arrest" genes such as *FAR1* , which culminate in the inhibition of cell cycle genes, the G1 cyclins, to cause cell cycle arrest. The most significant aspect of this work has been to link up a signal transduction pathway with the cell cycle, giving the first overview of such a pathway from beginning to end. This work may give insight into how growth is controlled in higher eukaryotes and how uncontrolled growth in diseases such as cancer arises.

FAR1 (*f*actor *ar*rest) was identified as a gene necessary for cell cycle arrest in response to α-factor but not for other responses to α-factor, such as morphological or transcriptional induction responses. A deletion allele of *FAR1* is defective in α-factor arrest, and is not affected in mitotic division or other START controls, suggesting that *FAR1* does not have a role in more general cell cycle regulation. The nucleotide sequence of *FAR1* reveals no homologies with known proteins.

Genetic evidence demonstrates that *FAR1* acts in response to α-factor to inhibit one of the three G1 cyclins, CLN2, and provides evidence that α-factor causes arrest by inhibiting the three G1 cyclins, CLN1, CLN2, and CLN3. FAR1 inhibits CLN2 either by regulating the synthesis, stability, or activity of the CLN2 protein.

FAR1 is regulated in at least different three ways: first, transcription of *FAR1* is induced five-fold in response to α-factor; second, FAR1 is phosphorylated in response to α-factor; third, FAR1 protein is only expressed in G1 of the cell cycle and may be regulated on the level of protein stability.

FAR1 also has another function in mating, which is in directing polarity of the cell towards the mating partner during the mating process.

Ira Herskowitz
4/30/91

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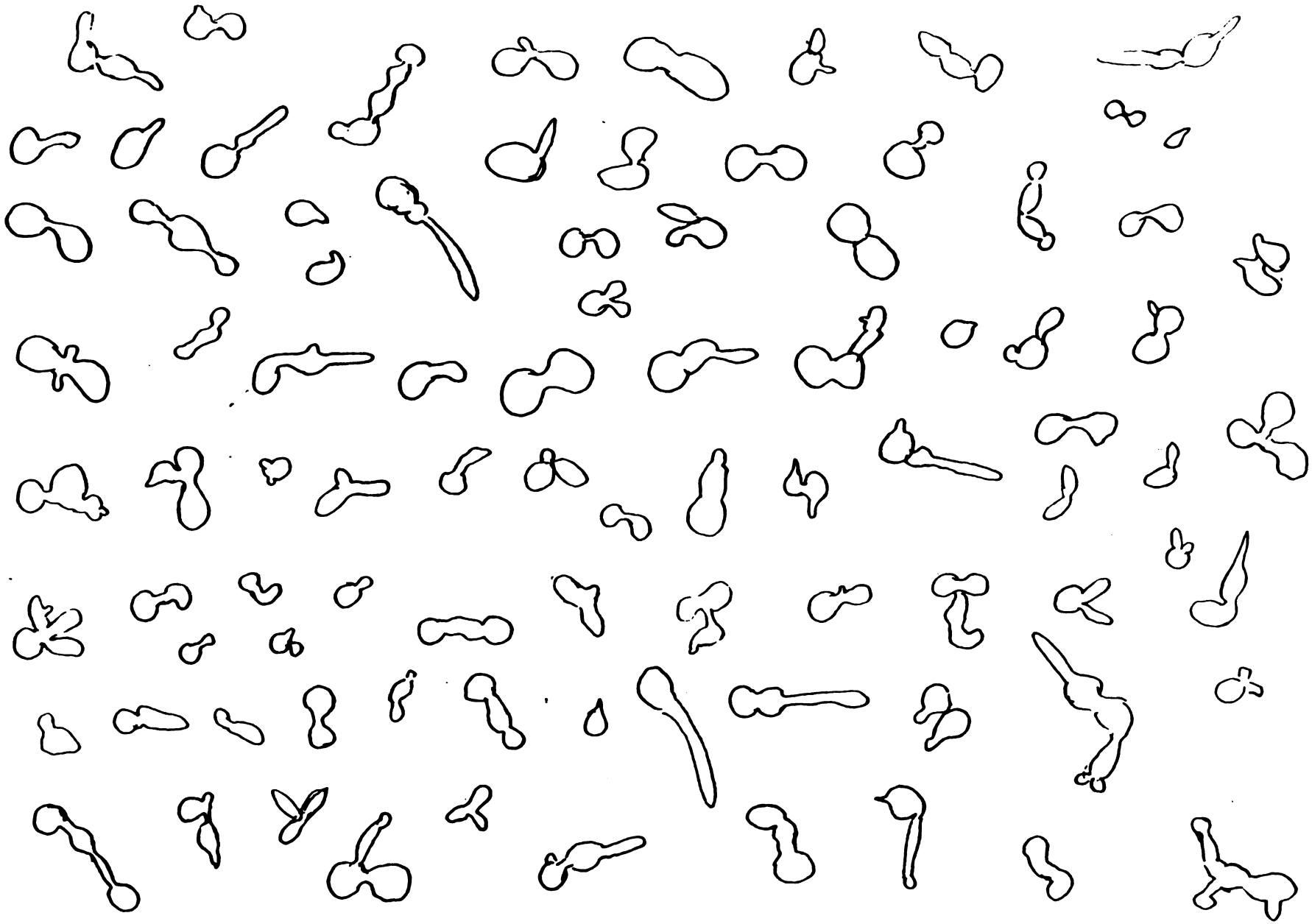
"One must have the time to look, the patience to 'hear what the material has to say to you,' the openness to 'let it come to you.' Above all, one must have a 'feeling for the organism.' One must understand 'how it grows, understand its parts, understand when something is going wrong with it. An organism isn't just a piece of plastic, it's something that is constanly being affected by the environment, constantly showing attributes or disabilities in growth. You have to be aware of all of that. . .'"

Barbara McClintock

in *The Feeling for the Organism* by E.F. Keller.



INTRODUCTION

The mere existence of that cell should be one the great astonishments of the earth. People ought to be walking around all day, all through their waking hours, calling to each other in endless wonderment, talking of nothing except that cell.

Lewis Thomas, in The Medusa and the Snail.

Somewhere on a grape, a remarkable event of biology is taking place. Two yeast cells are engaged in a complex mating dance complete with conversation, courtship, commitment, and finally consummation by the fusion of the two cells to form the diploid zygote. For a brief moment in its life cycle, the yeast cell becomes a multicellular organism, two cells engaged in an intimate communication. In the study of this simple "two cell organism", the student has an opportunity to explore processes, such as differentiation and cell-cell communication, that are vital in the development of larger, complicated, multicellular organisms.

The mating process in yeast involves diffusible mating factors what bind to cell surface receptors and ultimately trigger a variety of responses in the yeast cell, including cell-cycle arrest in G1. A particular goal of my thesis is to understand the mechanism by which mating factors cause cell-cycle arrest. Although this process is fascinating in itself, I hope these studies help illuminate how cells regulate their growth in all eukaryotes and give insights into diseases such as cancer. It has been shown that components involved in regulating the cell cycle in yeast are conserved in organisms such as frogs and humans, fueling the idea these studies in yeast may be directly relevent to larger organisms.

To place the work described in my thesis in context, I describe in this introduction the mating factor response pathway and eukaryotic cell cycle.

MATING IN YEAST

The budding yeast *S. cerevisiae* has two haploid mating types, **a** and α. Cells of these two mating types mate with each other to form the **a**/α diploid. Under starvation conditions, the **a**/α diploid undergoes meoisis and sporulates to form four haploid spores. The cell type of the yeast cell, whether it is an **a**, α, or **a**/α, is determined by products encoded at the *MAT* locus (see Herskowitz, 1989 for a review).

The mating process begins when two cells of opposite mating type come into close proximity. The **a** and α cells communicate through secreted mating factors, which are used to signal the location of the cells and to prepare the other cell for mating. The **a** cell secretes **a**-factor, a twelve amino acid peptide containing a fatty acid group at its C-terminus (Anderegg et al., 1988), which activates the α cell by binding to receptors on the surface of the α cell. The α cell secretes α–factor, a thirteen amino acid peptide (Stotzler and Duntze, 1976), which is recognized by receptors on the surface of the **a** cell. This communication with mating factors culminates in the fusion of the cells and nuclei to form the diploid zygote (see Cross et al., 1988 for review).

The purification of α-factor (Stotzler and Duntze, 1976), its subsequent synthesis, and easy availability (from Sigma) has led to the detailed characterization of how **a** cells respond to α-factor in the absence of the α cell. The ease of adding peptide to yeast cultures makes it possible to analyze large, homogenous populations of cells responding to α-factor.

α-factor induces the terminal differentiation of the **a** cell into a mating cell, eliciting changes in the yeast cell which are very similar to the differentiation program of higher cells. The responses to α-factor include the transcriptional induction of many products involved in mating, cell cycle arrest at G1, and morphological changes.

Through the work of many investigators, outlines of pathways responsible for these changes in the responding **a** cell are emerging. One simple model for how α-factor produces these responses is that it sets off a signal transduction cascade, which activates a set of gene products, each of which mediates a specific task in mating, such as cell fusion or cell cycle arrest (Figure 1; see Cross et al., for additional review). Thus, the genes involved in the α-factor response may be divided into two classes: genes in the signal transduction pathway, and the mating genes that serve as the targets.

I. The Signal Transduction Pathway.

Most of the genes in the signal transduction pathway were identified as mutants which failed to exhibit cell-cycle arrest to α-factor(Manney and Woods, 1976; Hartwell, 1980; Whiteway et al., 1989) or as mutants which failed to mate (MacKay and Manney, 1974a, 1974b). These mutant hunts identified a set of *STE* (sterile) genes which are required for mating and response to α-factor. Null mutations in any of these *STE* genes abolish all responses to α-factor assayed to date (Hartwell, 1980; McCaffrey et al., 1987; FC, unpublished). The molecular characterization of these *STE* gene products has allowed us to organize these gene products into a signal transduction pathway beginning with the receptor and culminating in the activation of a transcriptional activator in the nucleus, which activates genes involved in the mating process (Figure 1; see Marsh and Herskowitz, 1988).

The receptor for α-factor is *STE2*, which has structural similarity to integral membrane receptors such as the beta-adrenergic receptor and rhodopsin, so called "serpentine receptors" (Burkholder and Hartwell, 1985; Nakayama et al., 1985). One line of evidence used to demonstrate that the STE2 product indeed bound α-factor was by showing that a STE2-related protein from a related yeast defined ligand specificity (Marsh and Herskowitz, 1988). *STE2* is expressed only in **a** cells (Hartig et al.,

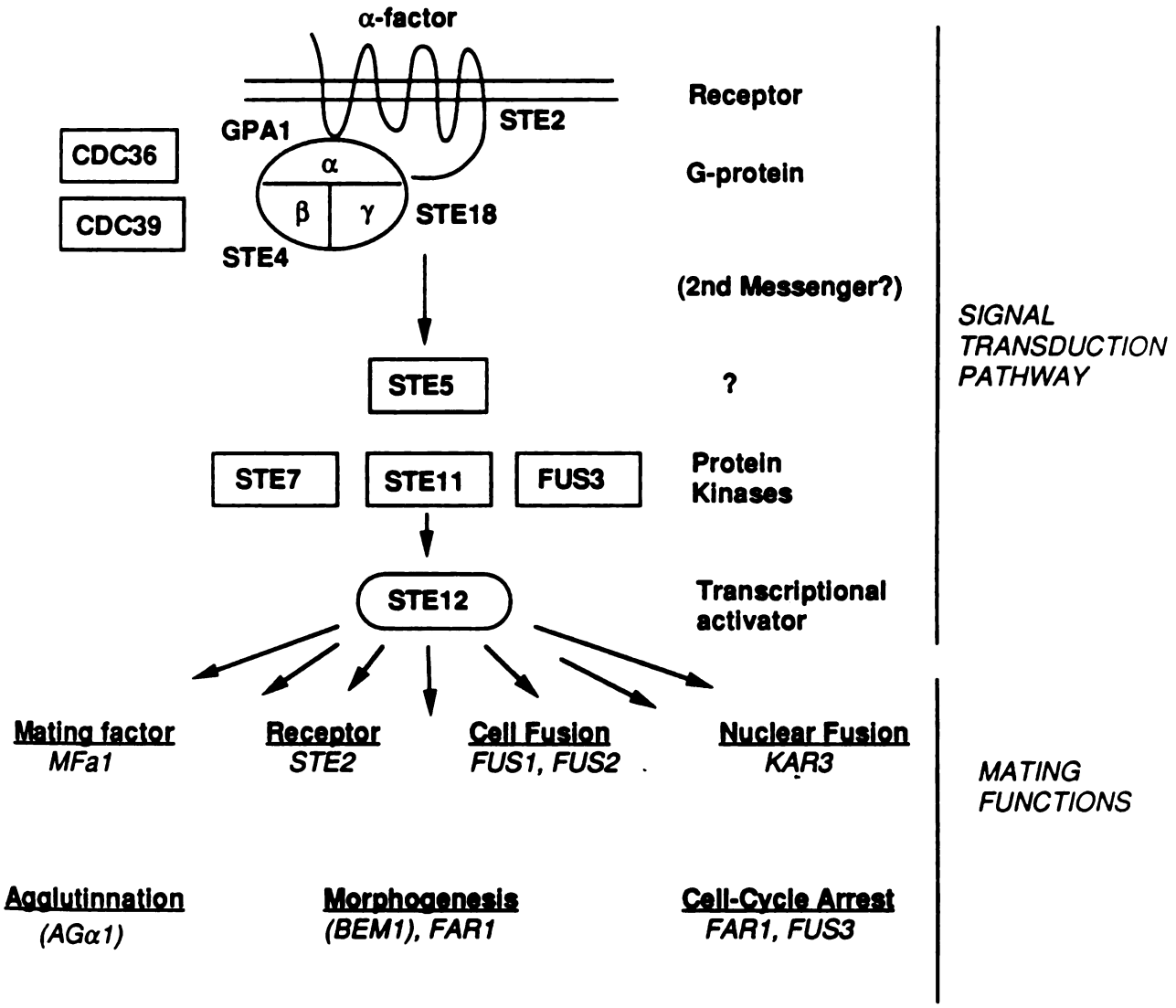


Figure 1: A model for the α-factor response pathway: a signal transduction pathway activates a set of mating function genes.
See text for details. The transcription regulation by α-factor of the mating function genes in parentheses (e.g. *BEM1*) has been proposed but has not been shown.

1986), and mutations in *STE2* affect mating of only **a** cells. The receptor for **a**-factor is *STE3*, which has a similar structure to *STE2* (Nakayama et al., 1985; Hagen et al., 1986) and is expressed in only α cells (Sprague et al., 1983).

The difference in the two cell types in their response to either **a**-factor or α-factor is determined primarily by which receptor is expressed. It has been shown that a *MATα* cell that is engineered to express the a-factor receptor *STE2* instead of *STE3* and to secrete **a**-factor instead of α-factor, now responds to α-factor and mates as an **a** cell (Bender and Sprague, 1987; Nakayama et al., 1987). This elegant experiment suggests that the response pathways to **a**-factor and α-factor are the same downstream of the receptor. This conclusion is further supported by the finding that genes in the pathway downstream of the receptor (described below) are expressed and required for mating in both mating types (see Marsh and Herskowitz, 1988).

Since many serpentine receptors have been shown to be coupled to G proteins, it was not surprising that a G protein was identified in the α-factor pathway. The Gα subunit, encoded by *GPA1*(*SCG1*), contains structural and functional similarity to the Gα subunit of mammalian cells (Dietzel and Kurjan, 1987; Miyajima et al., 1987; Jahng et al., 1987). A null mutation in *GPA1* results in the constitutive activation of the response pathway, suggesting that the normal role of the α-factor ligand is to inhibit the activity of *GPA1*. Recent *in vitro* studies have confirmed that the coupling of the GPA1 protein to the STE2 receptor is regulated by GTP hydrolysis (Blumer and Thorner, 1990).

STE4 and *STE18* appear to encode the two other subunits of the heterotrimeric G protein. *STE4* has sequence similarity to the β subunit of G proteins (Whiteway et al., 1989). and *STE18* has weak sequence similarity to the γ subunit (Whiteway et al., 1989). Epistasis tests have confirmed their location in the response pathway. *STE4* and *STE18* are necessary for pheromone response, suggesting that these subunits activate the next steps in the pathway. Overproduction of *STE4* causes constitutive activation of the pathway and the overproduction of *GPA1* prevents this constitutive activation by

STE4 (Whiteway et al., 1990; Cole et al., 1990; Nomoto et al., 1990). These observations suggest the model that free Gβγ subunits activate the pathway, and that in the absence of α-factor, Gα in the GDP form, may inhibit Gβγ by binding to them. The binding of α-factor to the receptor stimulates the binding of GTP of Gα, which frees Gβγ to activate the pathway.

We have found that two additional factors, *CDC36* and *CDC39* act to regulate the G protein in some way (Neiman et al., 1990; de Barros Lopes et al., 1990) Mutations in *CDC36* and *CDC39*, result in the constitutive activation of the pathway and cell cycle in G1, which suggest that the wildtype products may act to inhibit activation of the G protein in the absence of mating factor.

In mammalian G protein systems, the G protein usually regulates enzymes which govern levels of a small second messenger molecule, such as cAMP. No such second messenger or an obvious second messenger enzyme such as a cyclase or lipase has yet been identified in the yeast mating pathway. Candidate second messengers such as cAMP and phospholipids have been shown not to be involved (G. Casperson, unpublished observations; K. Matsumoto, personal communication).

Downstream of the G protein are *STE5* and a set of genes which act probably as protein kinases, coded by *STE7*, *STE11*, and *FUS3*. The molecular role of *STE5* is not known, although it may be a nuclear protein (J. Thorner, personal communication). *STE7*, *STE11*, and *FUS3* have sequence similarity to protein kinases (Teague et al., 1986; Rhodes et al., 1990; Elion et al., 1990). Rhodes et al. (1990) have also demonstrated that immunoprecipitations of STE7 contain protein kinase activity *in vitro*. *FUS3* may have an additional role in cell-cycle arrest (Elion et al., 1990; also Chapter 1), which is described in Chapter 2.

At the end of pathway is *STE12*, which encodes a transcriptional activator. The STE12 product binds the DNA sequence TGAAACA, which has been termed the "pheromone response element"(PRE) (Dolan et al., 1989; Errede and Ammerer, 1989). The PRE

sequence is found upstream of many genes which are transcriptional induced by α-factor (Van Arsdell et al., 1987; Trueheart et al., 1987) and is responsible for α-factor induction (Kronstad et al., 1987). The behavior of protein fusions of *STE12* with *GAL4* has demonstrated that *STE12* itself has the ability to activate transcription in response to α-factor (S. Fields, personal communication). In some genes, upstream sequences which confer regulation by α-factor have only weak similarity to the PRE consensus and contain binding sites for other transcription factors such as α1 and MCM1 (Errede and Ammerer, 1989). Thus, STE12 may bind with these other factors in regulating activating transcription at these promoters. The overproduction of STE12 has recently shown be sufficient to activate its target genes (e.g. *FUS1*) in the absence of α-factor. This activation is not dependent on *STE7* or *STE11*, showing that *STE12* works downstream of these kinase genes (Dolan et al., 1990). A model is that one or more of the protein kinases in the pathway, such as STE7 and STE11, activate the STE12 protein by phosphorylation in response to α-factor. The STE12 protein has recently been shown to be phosphorylated in response to α-factor (Song et al., 1991).

Clearly, there are many gaps in our understanding of this signal transduction pathway. The role of gene products in the middle of the pathway such as *STE5* and the kinases as well as the nature of the putative second messenger remain unclear. Genetic epistasis experiments, the identification of possible additional genes, the molecular characterization of the components, and the eventual development of *in vitro* systems will provide a more complete view of the pathway.

II. Targets of the Signal Transduction Pathway.

The signal transduction pathway culminates in the activation of a set of target genes which enact the mating program. Transcription of these genes are induced by varying

degrees (from 2- to 1000-fold) by α-factor. In general, the transcription of these genes is greatly reduced in **a**/α cells and is dependent on components of the signal transduction pathway for expression. For instance, expression of these genes is reduced in cells deleted for *STE4*, the Gβ subunit. The target genes are not essential for cell growth and generally appear to function specifically in the mating process.

Cell-cell communication. One set of targets of the signal transduction pathway include the mating factors and components of the pathway itself. Expression of the **a**-factor gene *MFa1* increases approximately 5-fold in response to α-factor in **a** cells(Kubo and Michaelis, unpublished observation). Components of the signal transduction pathway are also induced. For instance, the expression of the receptor gene, *STE2*, is induced 3-fold by α-factor (Hartig et al., 1986). This induction of receptor and mating factor heightens communication between the two cells as the mating process progresses.

The basal level of expression of some of the components of the pathway, such as the *STE2* gene, are dependent on the *STE* genes for expression (Hartig et al., 1986; see Marsh and Herskowitz, 1988). One experimental complication posed by this interdependence is that a mutation in a *STE* gene not only affects the one step of the pathway the *STE* product performs, but affects the expression of the whole pathway.

Agglutination. Early in the mating process, cells of opposite mating type stick together in large aggregates, in a process known as agglutination. This agglutination is mediated by specific **a** and α agglutinins, which are thought stick the cells together by binding to each other. The α-agglutinin gene has been cloned and has been shown to induced by **a**-factor (Lipke et al., 1989). An α agglutinin mutant demonstrates that the α-agglutinin is not required for mating under solid conditions (agar plate) but does aid mating when the cells are in culture. The complementary interaction between the two

agglutinins should serve as useful model for how specific cell-cell interactions occur, for instance, in the immune system.

Cell fusion. Two genes, *FUS1* and *FUS2,* are required specifically for fusion of the two cells during mating (Trueheart et al., 1987; McCaffrey et al., 1987). When **a** and α *fus1 fus2* mutants are mixed with each other, the cells form zygote-like structures but do not fuse. *FUS1* is a plasma membrane protein located at the shmoo tip and at the juncture between two cells in zygotes and is hypothesized to mediate fusion of the membranes of the two cells (Trueheart et al., 1987). The *FUS1* transcript is not detectable in the absence of α-factor and is induced (greater than 100-fold) by α-factor (Trueheart et al., 1987; McCaffrey et al., 1987). This large induction makes *FUS1* a useful gene to assay for the transcriptional induction of genes by α-factor.

Nuclear fusion. Even before cell fusion, the nuclei migrate to the juncture between the two cells, with the extracellular microtubules emanating toward the fusion site (Byers and Goetsch, 1975). Upon cell fusion, the microtubules quickly mediate the joining and fusion of the nuclei at the spindle pole body. Genes necessary for nuclear fusion, such as *KAR1*, *KAR3*, and *BIK1*, affect microtubule function (Rose and Fink, 1987; Meluh and Rose, 1990; Berlin, 1990). Rose et al. (1986) showed that α-factor induces functions required for efficient nuclear fusion. The α-factor induction of the *KAR3* gene could explain the requirement of α-factor for nuclear fusion. *KAR3,* a gene necessary for nuclear fusion, is thought to be a microtubule motility motor which drives the two nuclei together (Meluh et al., 1990). The regulation of *KAR3* is different from other target genes in that it is still expressed at low levels in **a**/α cells and is required for normal growth of cells.

Morphogenesis. At high concentrations of α-factor, cells arrest and form the pear-shaped shmoo, which has many of the characteristics of the changes in morphology seen during the mating process. At lower concentrations of α-factor, cells form structures in the shape of peanuts. Morphological changes are not dependent on cell cycle arrest, since *far1* mutants, which do not arrest, still exhibit dramatic morphological changes (see Chapter 1). Thus, the morphologic changes should be regarded as a separate response to α-factor.

Genes necessary for several different aspects of morphogenesis have been identified. Chenevert et al. (1991) have a identified a gene, *BEM1*, which is necessary for polarization during the mating process. Mutants carrying special alleles of *BEM1* bud normally but when treated with α-factor, grow as unpolarized balls. Phenotypes of a deletion allele of *BEM1* show that the gene also has a role in polarity outside of mating. The sequence of *BEM1* reveals sequence similarity to other actin-binding proteins, suggesting that *BEM1* may regulate the actin cytoskeleton in response to mating factor. The upstream region of *BEM1* does contain potential binding sites for *STE12*, although the regulation of the transcript has not yet been shown. As described in Chapter 4, the *FAR1* gene is not necessary for polarization per se, but is necessary for determining the *direction* of polarity of the mating cell toward the proper site (Chapter 4). Another factor contributing to morphogenesis may the cell wall component chitin, whose synthesis and localization in a broad band around the shmoo tip is induced by α-factor (Schekman and Brawley, 1979). How α-factor may activate chitin synthases is not known.

Cell-Cycle Arrest. α-factor causes cell-cycle arrest at G1 (Bucking-Throm et al., 1973). The mechanism of the cell-cycle arrest is the primary focus of this thesis. At the outset, I hypothesized that one target of the α-factor response pathway could be a gene which has a specific role in cell-cycle arrest. This hypothesis has led to the

identification of the *FAR1* (factor arrest) gene, which is necessary primarily for cell-cycle arrest. *FAR1* shares the same type of regulation as other mating function genes in that it is induced 5-fold in response to α-factor and is not expressed in **a**/α diploids or a *ste12* mutant (Chapter 1).

Why is the cell-cycle arrest useful for mating? It can imagined how the synchronization of the cell-cycle in the two mating partners might by facilitate events following the fusion of the cells. For instance, fusion of a 1N nucleus in G1 with a 2N nucleus in M phase could result in mitotic disaster, or at best a triploid nucleus, which would fail in meiosis. In addition, the mechanism of mating may require functions which can operate only in G1. Reid and Hartwell (1977) have shown that only cells in G1 have the ability to mate. Cells arrested in other parts of the cell cycle do not mate. One mating function which may restricted to G1 may be morphogenesis. The cell cycle restriction in morphogenesis is suggested by the fact that *DAF1-1* mutants (a dominant allele of *CLN3*), which have very small or no G1 period and do not arrest when treated with α-factor, do not express any morphological changes in response to α-factor and do not mate (FC, unpublished observations; Cross, 1988). This putative cell-cycle restriction of shmoo morphogenesis to G1 may be related mechanistically to the cell-cycle restriction of bud formation to G1/S.

REGULATION OF THE CELL CYCLE

The Universal Cell-Cycle Oscillator

Studies on the cell-cycle in organisms such as budding yeast, fission yeast, fruit flies, sea urchins, star fish, frogs, and humans have recently converged on a common set of regulators of the cell cycle and have led to the startling conclusion that the fundamental mechanisms of the cell-cycle may be conserved in all eukaryotes. The

central player is a protein kinase, known as cdc2/CDC28/p34, which is conserved both functionally and structurally in many eukaryotes (see reviews Murray and Kirschner, 1989a; Nurse, 1990). This protein kinase was identified in fission yeast as *cdc2*, a gene necessary for both entry into mitosis and into S-phase (Nurse and Bisset, 1981), and in budding yeast as *CDC28*, a gene necessary for entry into S-phase (Hartwell, 1973). (CDC28 has recently been shown to have a role also in the entry in mitosis (Reed and Wittenberg, 1990)). The kinase was elevated to the status of a "universal cell-cycle regulator" when it was also discovered in Xenopus to be the catalytic subunit of MPF, M phase-promoting factor (Dunphy et al., 1988; Gautier et al., 1988).

The cdc2/CDC28/p34 protein kinase has been best characterized in its role as an inducer of mitosis (reviewed by Murray and Kirschner, 1989). The injection of MPF into protein-arrested frog embryos or oocyte extracts causes these oocytes or extracts to enter mitosis (Masui and Markert, 1971; Wasserman and Masui, 1976). The MPF and the activity of cdc2 kinase oscillates every cell-cycle, peaking at M-phase (Gerhart et al., 1984). The cdc2 kinase is thought to trigger mitosis by phosphorylating a set of proteins involved in mitosis. Scores of proteins which change in activity and behavior during mitosis are hypothesized to be regulated by cdc2 and have been shown to phosphorylated by purified cdc2 kinase *in vitro* (see Moreno and Nurse, 1990). For example, cdc2 kinase phosphorylates residues on nuclear lamins, which result in the disassembly of the nucleus during mitosis (Peter et al., 1990; Ward et al., 1990).

The activity of the cdc2 kinase is regulated by a set of proteins known as cyclins. Cyclin was first identified as a protein in clam embryos which accumulates during mitosis and is abruptly degraded at metaphase (Evans et al., 1983). It has since been identified in the *Xenopus* system as a component of MPF (Lohka et al., 1988; Draetta et al., 1989; Gautier et al., 1990). Cyclin binds to the cdc2 kinase and triggers its activation in a series of steps involving phosphorylation and dephosphorylation of residues of cdc2 (Solomon et al., 1990). Destruction of cyclin appears to be necessary

for exit from mitosis (Murray et al., 1989). The abundance of cyclin protein, which peaks each cell cycle at metaphase, has been shown in *Xenopus* to be regulated by protein stability via the ubiquitination pathway (Starnart et al., 1987; Glotzer et al., 1991).

In addition to cyclin, the cdc2 kinase is also regulated by three other proteins, cdc25, wee1, and suc1, which were first identified in fission yeast. cdc25 activates cdc2 by the dephosphorylation of tyrosine 15 of cdc2 (Gould et al., 1990). wee1, which has sequence similarity to protein kinases, functions as an inhibitor of cdc2 and may phosphorylate cdc2 (Russell and Nurse, 1986). The function of wee1 itself is regulated by another protein kinase, nim1 (Russell and Nurse, 1987b). suc1/p13 is necessary for entry into mitosis and binds tightly to cdc2 and cyclin, although its role is still not well defined (Brizuela et al., 1987; Moreno et al., 1989; Booher et al., 1989). Homologs of (G2) cyclins, cdc25, and suc1 have recently identified in *S. cerevisiae* and may play analogous roles in activating CDC28 in the regulation of mitosis (Russell et al., 1989; Hadwiger et al., 1989; S. Reed, K. Nasmyth, personal communications). In summary, the onset of mitosis in eukaryotes has been found to be regulated by a complex of conserved proteins which act to phosphorylate critical proteins involved in mitosis.

Genetic evidence from *S. cerevisiae* and *S. pombe* suggest that in addition to its role in mitosis, cdc2/CDC28 also regulates entry into S phase (Bisset and Nurse, 1981; Hartwell et al., 1973). In particular, budding yeast cells carrying temperature-sensistive mutations of the *CDC28* arrest in late G1 of the cell cycle. However, evidence for the role of the cdc2 kinase in regulating the G1/S transition in higher eukaryotes has been slim, although the human homologue of the cdc2/CDC28 kinase can provide the G1 function of CDC28 when introduced into *S. cerevisiae* (Wittenberg and Reed, 1989). By analogy to its role in mitosis, cdc2/CDC28 might drive a cell into S phase by phosphorylating proteins such as the spindle pole body and DNA replication initiation proteins (Figure 2). According to this model, the substrate specificity of cdc2/CDC28 kinase in G1/S may be different from its M-phase state. This difference in substrate

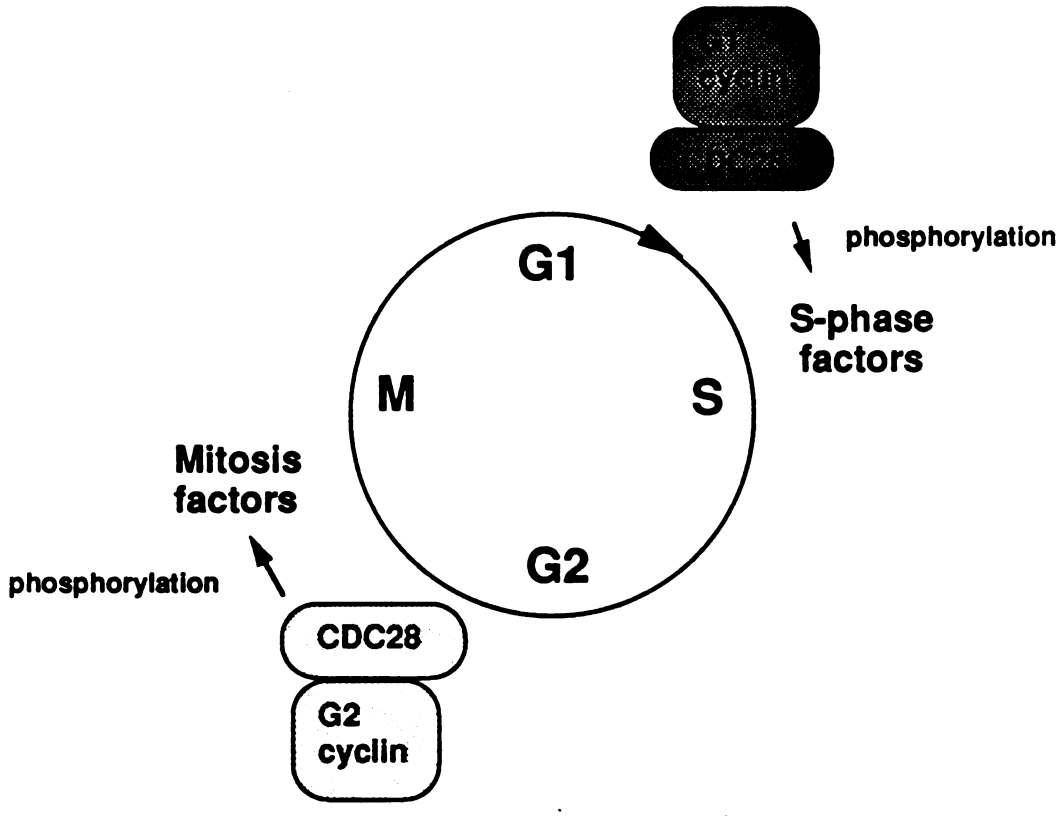


Figure 2: A model for the CDC28 protein kinase and the cyclins in the regulation of the cell cycle.
In G1, the protein kinase CDC28 associates with G1 cyclins and drives entry into S phase. In G2, CDC28 associates with G2 cyclins and drives entry into M phase. See text for details.

specificity may be imparted by differences in proteins which are associated with CDC28/cdc2 or by difference in the modification state of cdc2 itself.

Proteins termed "G1 cyclins", which might regulate CDC28 during G1, have been identified in *S. cerevisiae*. *CLN3* was identified by a dominant mutation which caused cells to be small (Nash et al., 1988) and to be resistant to arrest by α-factor (Cross, 1988). *CLN1* and *CLN2* were identified as genes which rescued growth of a temperature-sensitive *cdc28* mutant strain when overexpressed on a high copy plasmid (Hadwiger et al., 1989). The three *CLN* genes possess weak similarity (about 20% identity) to mitotic cyclins and contain conserved "cyclin" boxes (Nash et al., 1988; Hadwiger et al., 1989) . In addition, *CLN1* and *CLN2* are highly similar to each other, especially in the N-terminal regions, and both *CLN1* and *CLN2* are no more similar to *CLN3* than a clam cyclin. A deletion mutation in any one of the *CLN* genes does not produce a strong phenotype. However, when all three are deleted, the cells arrest at G1, indicating that the *CLN* genes do play an essential role at the G1/S transition and are functionally redundant (Richardson et al., 1989). In addition, the transcripts of *CLN1* and *CLN2* and the *CLN2* protein have been shown to be expressed only during G1 (Wittenberg et al., 1990). CLN2 has also been shown to coimmunoprecipitate with CDC28 kinase (Wittenberg et al., 1990). In analogy to the properties of mitotic cyclins, it is hypothesized that the *CLN* products might function to bind and activate CDC28 kinase activity for progression at START.

Thus in *S.cerevisiae*, and presumably in other organisms, there are two classes of cyclin-like molecules: the mitotic cyclins, which are expressed in G2/M and are necessary for entry into mitosis (S. Reed, K. Nasmyth, personal communications), and the G1 cyclins, which are expressed in G1/S and are necessary for entry into S-phase . Since both classes of cyclins may function to activate the CDC28 protein kinase (although this has not yet been demonstrated for any of the *S. cerevisiae* cyclins), one model for cell-cycle regulation is that CDC28 complexed with G1 cyclins drives entry

into S phase, and CDC28 complexed with a mitotic cyclin drives entry in M phase (Wittenberg et al., 1990; Figure 2). However, the simple model that the cyclins alone specify whether CDC28 performs its G1 or G2 function may be not true, since a *S. cerevisiae* G2 cyclin is capable of supplying CLN (G1 cyclin) activity when present on a high copy plasmid (P. Leopold, personal communication). Recently, numerous candidate G1 cyclins from humans and fission yeast which have *CLN* function in *S.cerevisiae* and have sequence similarity to *CLN3* have been isolated (D. Beach, P. Nurse, and S. Reed personal communications).

Regulation of the Cell Cycle

Having identified some of the components which control cell-cycle progression, we can now begin to examine in molecular detail how the cell-cycle may be regulated by environmental and intracellular factors. The proliferation of almost all cells are controlled in some aspects by factors in their environment, such as by contact with other cells, growth factors, and nutritional conditions. Both positive and negative growth factors have been found to regulate the proliferation of mammalian tissue culture cells at G1 of the cell cycle (Pardee, 1989; Moses et al., 1990).

The proliferation of budding yeast cells is regulated at a point in G1 termed START. At START, the cell receives signals from its environment, such as mating factor or nutrition, and chooses one of three fates: to commit to a mitotic cell cycle, to arrest by nutritional arrest (and enter meoisis and sporulation if it is a diploid), or to arrest and mate . Once a cell has committed to a mitotic cell cycle, it will not arrest with either α-factor or by nutritional signals until the next cycle (Nurse, 1981)

Cell cycles are also regulated by internal checks. Cell size is also somehow evaluated at START, and a cell will pause in G1 prior to START until it grows large enough (Hartwell and Unger, 1977). Cells also check that DNA replication is complete

before starting mitosis, and that spindles are properly assembled before anaphase ensues. If these conditions are not met, the cell-cycle is halted until the defects are fixed. Mutants which are defective in these check-points define genes, such as *RAD9* (Weinert and Hartwell, 1989) and the *MAD* genes (R. Li, personal communication), which might arrest the cell-cycle in response to the state of DNA or spindle.

A cell cycle might be regulated in many different ways. With the discovery of cdc2/CDC28 and associated factors, it is very attractive to think that a cell cycle could be regulated by regulating the activity of this protein kinase. Potential targets for regulation include the cdc2/CDC28 kinase, cyclins, cdc25, suc1, and wee1. Inhibition of the synthesis or activity of the cyclins, for instance, could halt the cell cycle. However, it is also possible that the direct targets for cell cycle regulation could be structural components, such as the spindle, which are necessary for cell cycle progression.

Evidence is accumulating which indicate that events at START in *S. cerevisiae* might involve the regulation of the CDC28 kinase. The first indication came with the finding that α-factor treatment or starvation, which cause cell cycle arrest at START, causes decrease in the the kinase activity of CDC28 (Mendenhall, 1987; Wittenberg et al., 1988). Effects of mutated CLN products on START regulation suggest that the G1 cyclins, which are thought to regulate the activity of CDC28, may be more directly involved in aspects of regulation at START. First, the control of cell size is altered in *CLN* mutants. Dominant alleles of *CLN3* or *CLN2*, which have been proposed to encode hyperstable or hyperactive versions of the CLN proteins, result in reduction in cell size. Examination of the cell cycle in these mutants reveal that the G1 period of the cell-cycle is very short or absent. A deletion of one or two of the *CLN* genes causes the opposite phenotype: cells are larger than wildtype and have a longer G1 period (Cross, 1988; Nash et al., 1988; Hadwiger et al., 1989). These findings suggest that the CLN products are rate limiting in progression through G1.

A dominant allele in *CLN3*, caused by a C-terminal truncation, also made cells resistant to arrest by α-factor (Cross, 1988; Nash et al., 1988). This α-factor resistance phenotype led to the original hypothesis that α-factor might cause cell-cycle arrest by inhibiting the CLN products (Cross, 1988). This dominant mutation of *CLN3* was proposed to render the CLN3 product resistant to inhibition by α-factor (Cross, 1988). (See Chapter 2 for further developments and discussion). A C-terminal truncation allele of *CLN2* confers a defect in G1 arrest in response to nitrogen starvation (Hadwiger et al., 1989), suggesting that the nutritional state of the cell may also regulate the CLN products.

In 1986, when I first began these studies, I had very few preconceptions of how α-factor might arrest the cell-cycle at G1. I considered that α-factor might inhibit one of the CDC products, or a component of the DNA replication machinery, or perhaps a component of the spindle pole body. One goal in my thesis research was to try to determine what part of the cell was responsible for this arrest. The results were surprisingly simple but complex at the same time.

CHAPTER 1

IDENTIFICATION OF A GENE NECESSARY FOR CELL CYCLE ARREST: *FAR1*

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INTRODUCTION

α–factor, a peptide of thirteen amino acids secreted by yeast α cells, is a negative growth factor that induces differentiation in its target, **a** cells (reviewed by Cross et al., 1988; Herskowitz, 1989). α–factor stimulates **a** cells to prepare for mating by causing them to arrest in the G1 phase of the cell cycle, to undergo morphological changes, and to induce expression of many genes involved in mating. α-factor arrests cells at a point in G1 (termed START) that appears to be a key decision-making point in the cell cycle, at which cells either commit to a mitotic cycle or undergo differentiation leading to meiosis or mating (Nurse, 1981; Pringle and Hartwell, 1981).

We wish to define the sequence of events that leads to cell-cycle arrest, a process that begins with binding of α-factor to its cell-surface receptor and culminates with arrest in G1 (reviewed by Marsh and Herskowitz, 1988). Many of the genes involved in the signal transduction pathway (*STE2*, *STE4*, *STE5*, *STE7*, *STE11*, *STE12*, and *STE18*) have been identified as mutants that are resistant to growth inhibition by α-factor (Hartwell, 1980; Whiteway et al., 1989). The *STE2* gene codes for the α-factor receptor, a member of the integral membrane protein family with seven presumptive membrane-spanning regions (Burkholder and Hartwell, 1985; Nakayama et al., 1985). This receptor appears to function by communicating with a heterotrimeric G protein, composed of Gα, Gβ, and Gγ subunits coded by the *SCG1 (GPA1)*, *STE4*, and *STE18* genes respectively (Dietzel and Kurjan, 1987; Miyajima et al., 1987; Whiteway et al., 1989). Downstream of the G protein (Nakayama et al., 1988; Blinder et al., 1989)

lies *STE5*, whose function is unknown, and *FUS3*, *STE7*, and *STE11*, which appear to be protein kinases (Elion et al., 1990; Teague et al., 1986; B. Errede, personal communication). The STE12 protein appears to be a transcription factor that functions further downstream in the pathway: STE12 protein binds at a nucleotide sequence (termed the PRE, "pheromone response element"), located in the upstream regions of many α–factor-inducible genes, that confers inducibility by α-factor (Dolan et al., 1989; Errede and Ammerer, 1989). Induction of gene expression by α-factor is hypothesized to result from activation of the STE12 protein in some way, perhaps due to action of the protein kinases earlier in the pathway (see Dolan and Fields, 1990).

How the mating-factor response pathway causes cell-cycle arrest is not known. Mutants defective in the components of the pathway described above (*STE2, STE4* etc.) are defective for both cell-cycle arrest and gene induction. Since expression of a gene such as *FUS1* is not necessary for arrest, and arrest is not necessary for *FUS1* expression, we imagined the pathways leading to gene induction and cell-cycle arrest might be separate at some point. Figure illustrates our initial models for how the α-factor response pathway might be organized. The first model is that the pathway leading to arrest branches before transcriptional induction. The *STE* gene products are necessary for portions of the pathway before the branch point (the DNA-binding properties of STE12 were not known at the time). The second model is that the transcriptional induction contributes to arrest by increasing the synthesis of an effector protein responsible for arrest. In either model, genes in the arrest-specific branch of the pathway could be identified as mutants (defective in a step indicated by X in Figure 3,)which do not exhibit cell-cycle arrest but are proficient for transcriptional induction of *FUS1*. In this chapter, we describe mutants of this type, which identify a new component of the α-factor response pathway, *FAR1*. *FAR1* appears to be an effector for α-factor arrest, which acts at the end of the α-factor response pathway as a link between the signal transduction pathway and the cell cycle.

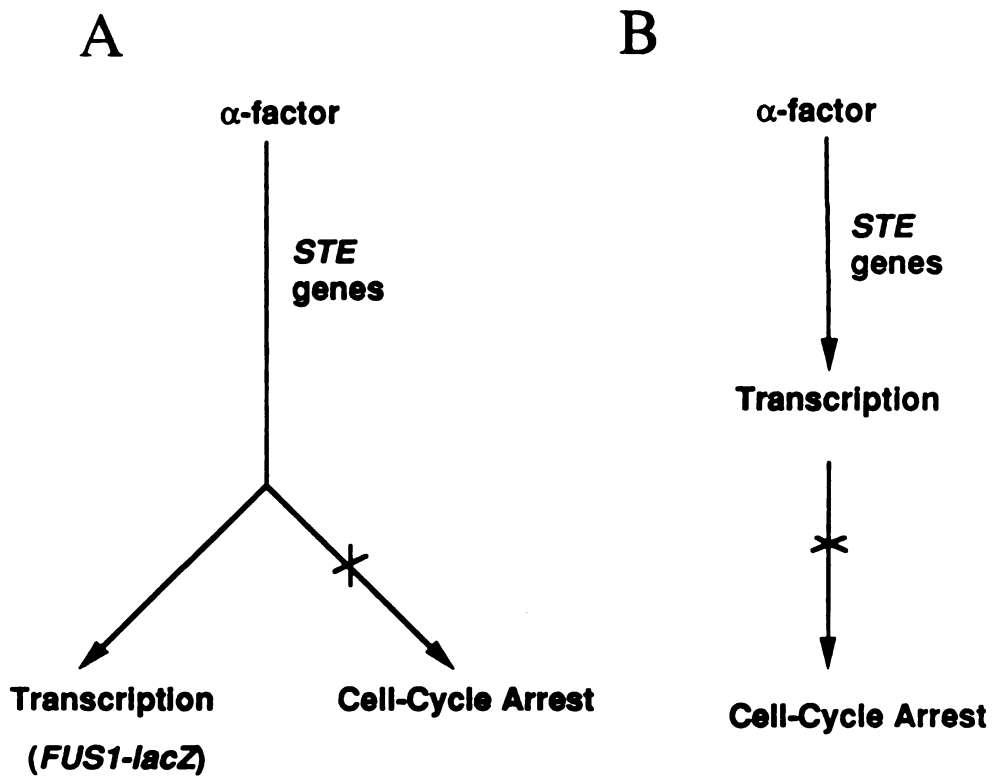


Figure 3: Two initial models for the organization of the α-factor response pathway.
See text for details. The X repesents a mutation in a gene in the cell-cycle branch of the pathway, which would block cell cycle arrest but not the transcriptional induction of *FUS1-lacZ*.

RESULTS

Isolation Scheme

The known components of the signal-transduction pathway affect both transcriptional and cell-cycle responses to mating factors. To identify new components that affect only the cell-cycle response, we screened for mutants that are specifically defective in cell-cycle arrest induced by α-factor but proficient in transcriptional response. We monitored transcriptional response by expression of the *FUS1* gene using a *FUS1-lacZ* gene fusion (Trueheart et al., 1987): β-galactosidase activity is very low in uninduced cells and is induced 1000-fold by α-factor. Strains carrying the *FUS1-lacZ* gene and a mutation in *STE2, STE4, STE5*, or in any of the other *STE* genes in the signal-transduction pathway cannot be induced by α-factor (McCaffrey et al., 1987) and are expected to form white colonies on media containing α-factor and the chromogenic substrate X-gal. We anticipated that a mutant specifically defective in arrest would form a blue colony under these plating conditions.

The mutant hunt had two steps. First, α-factor-resistant mutants were isolated from unmutagenized and lightly mutagenized cultures of a strain FC140A (which carried FUS1-lacZ; see Experimental Procedures) by selecting for colony formation on plates containing α-factor. These mutants were then screened for those that exhibited transcriptional response (induction of *FUS1-lacZ* to form blue colonies) and other responses to α-factor (such as changes in cell morphology). Approximately 90% of the α-factor-resistant colonies were white and presumably contained mutations in the known *STE* genes. The blue α-factor-resistant colonies were further analyzed for cell morphology, mating ability, a-factor production, and β-galactosidase activity after exposure to α-factor. Approximately 10% of these mutants exhibited distinctive cell morphology and produced wild-type levels of *FUS1-lacZ* activity in response to α-

factor. Six independently-isolated mutants with essentially identical phenotypes were obtained.

Spheroplast fusions of the mutants to a wild-type **a** strain and to each other demonstrated that the mutations were recessive and comprised a single complementation group. Three of these mutations were also confirmed to be allelic by segregation analysis. We have named this gene *FAR1* because it is necessary for "factor arrest".

A second screen yielded five independent mutants (see Experimental Procedures). Four appeared to be mutations in *FAR1* because they were complemented by pFC1. The fifth contained a mutation unlinked to *FAR1* and defined a separate gene, *FAR2*, which was subsequently shown to be identical to *FUS3* (Elion et al. 1990; E. Elion and F. Chang, unpublished observations).

FAR1 Mutants Exhibit Multiple Responses to α-factor but do not Arrest

far1 mutants were tested for a variety of responses to α-factor and for their behavior in the absence of α-factor. Most of the phenotypes chronicled below are for an **a** strain (FC204) carrying the null mutation *far1::URA3*, which replaces most of the putative *FAR1* open reading frame with *URA3* (Figure 7). In some studies, one of the original mutations (*far1-1*) was used; its phenotype was similar to that of the *far1::URA3* mutation.

The inability of *far1* mutants to arrest in response to α-factor was seen by several different assays. *far1*⁻ mutants initially grew in the presence of high concentrations of α-factor at a similar rate as cells growing in the absence of α-factor (as assayed by the confrontation assay; see Experimental Procedures), although subsequently they grew slower, probably because of the morphological aberrancies induced by α-factor (see below). Additional assays for G1 arrest, by determining the percentage of unbudded cells after addition of α-factor in culture (Figure 12) and by scoring growth inhibition of lawns around an α-factor source on a plate (Figure 11), also showed that *far1*⁻ mutants

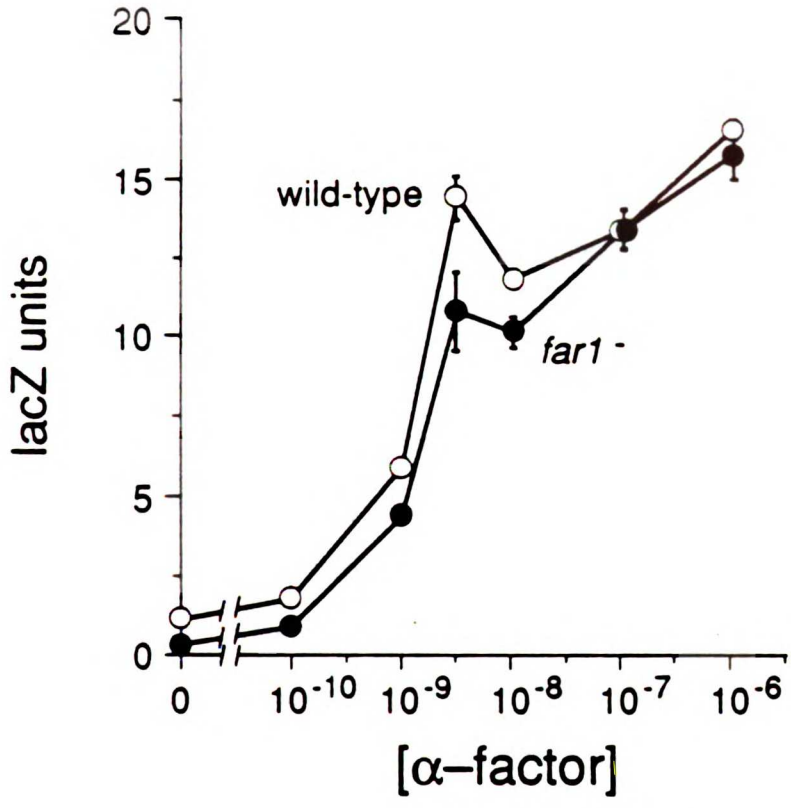


Figure 4: FUS1-lacZ induction in far1⁻ and wild-type cells

FAR1⁺ and far1⁻ strains growing exponentially in YEPD medium at 30°C were incubated for 30 min with α-factor at the indicated concentrations (M) for 30 minutes in YEPD at 30°C and then harvested and assayed for ß-galactosidase activity. Assays were carried out in triplicate; standard deviations are indicated. Strains were FC312 (a bar1-1 FAR1⁺ FUS1-lacZ) and FC311 (a bar1-1 far1::URA3 FUS1-lacZ).

failed to arrest in response to α-factor. Although they do not arrest, several observations show that *far1*⁻ mutants still respond to α-factor. First, they exhibited a normal transcriptional response to α-factor: induction of the *FUS1* gene (assayed as β-galactosidase activity from the *FUS1-lacZ* gene or as *FUS1* RNA by Northern blotting; data not shown) was very similar in wild-type and *far1*⁻ cells at different doses of α-factor (Figure 4). The *far1-1* mutant also exhibited normal induction of *STE2-lacZ* (data not shown).

Second, α-factor induced a distinctive morphological change in *far1*⁻ mutants. After exposure to α-factor, wild-type cells arrested and exhibited the pear-shaped "shmoo" morphology by 3 hours (Figure 5E), whereas *far1*⁻ cells appeared primarily as two large cells or shmoos stuck together. After six hours of exposure to α-factor, wild-type cells remained arrested and often possessed multiple shmoo tips (Figure 2F). *far1*⁻ cells at this time exhibited unusual morphologies (Figure 5C) in which large lobes were connected by tubes and formed projections. Some lobes or cells appeared to have similar morphology to cells arrested in mitosis. DAPI staining showed that most of the cells contained multiple nuclei. These unusual morphologies might result from expression of α-factor-induced morphological changes that normally occur in an arrested cell but here occur in a dividing (*far1*⁻) cell. In wild-type a cells, α-factor induces chitin synthesis and distribution in a broad, diffuse band around the shmoo neck (Schekman and Brawley, 1979). *far1*⁻ cells exhibited a similar response, exhibiting broad, diffuse bands around the connections between lobes and around the cellular projections (Figure 6). This increase in chitin could cause at least some the morphological defects seen in the far1- mutants, since vegetative cells exposed to calcofluor, which induces chitin synthesis, exhibit remarkably similar morphologies; specifically, these calcofluor-treated cells form chains of lobes connected by tubes. The observation that *far1*⁻ mutants undergo morphological changes even though they do not arrest indicates that

Figure 5: Morphology of *far1* and wild-type cells treated with α-factor

Exponential phase culture of a cells growing in YEPD at 30°C were treated with 1 μM α-factor for 0, 3, and 6 hours, then fixed with 70% ethanol and stained with DAPI. Photomicrographs are Nomarski and fluorescence double exposures.

A: *far1*, no α-factor
B: *far1*, α-factor for 3 hours
C. *far1*, α-factor for 6 hours
D: *FAR1*$^+$, no α-factor
E. *FAR1*$^+$, α-factor for 3 hours
F. *FAR1*$^+$, α-factor for 6 hours

The *far1* strain was FC204 (which carries the far1::URA3 mutation); the *FAR1*$^+$ strain was the isogenic strain FC140. DAPI staining of the cells in panels B and C shows that most cells contain multiple nuclei. Panel E shows the standard shmoo morphology.

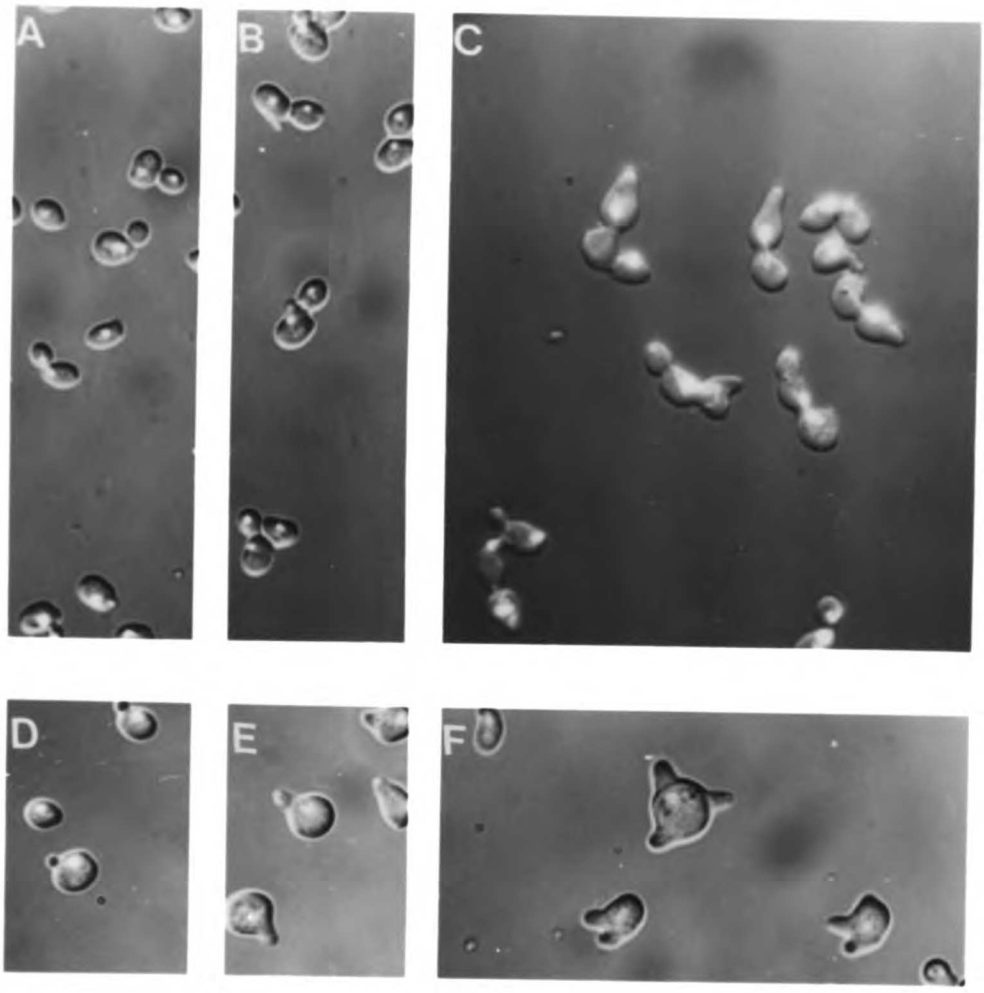

A
B
C
D
E
F

Figure 6: Chitin distribution in the *far1* mutant

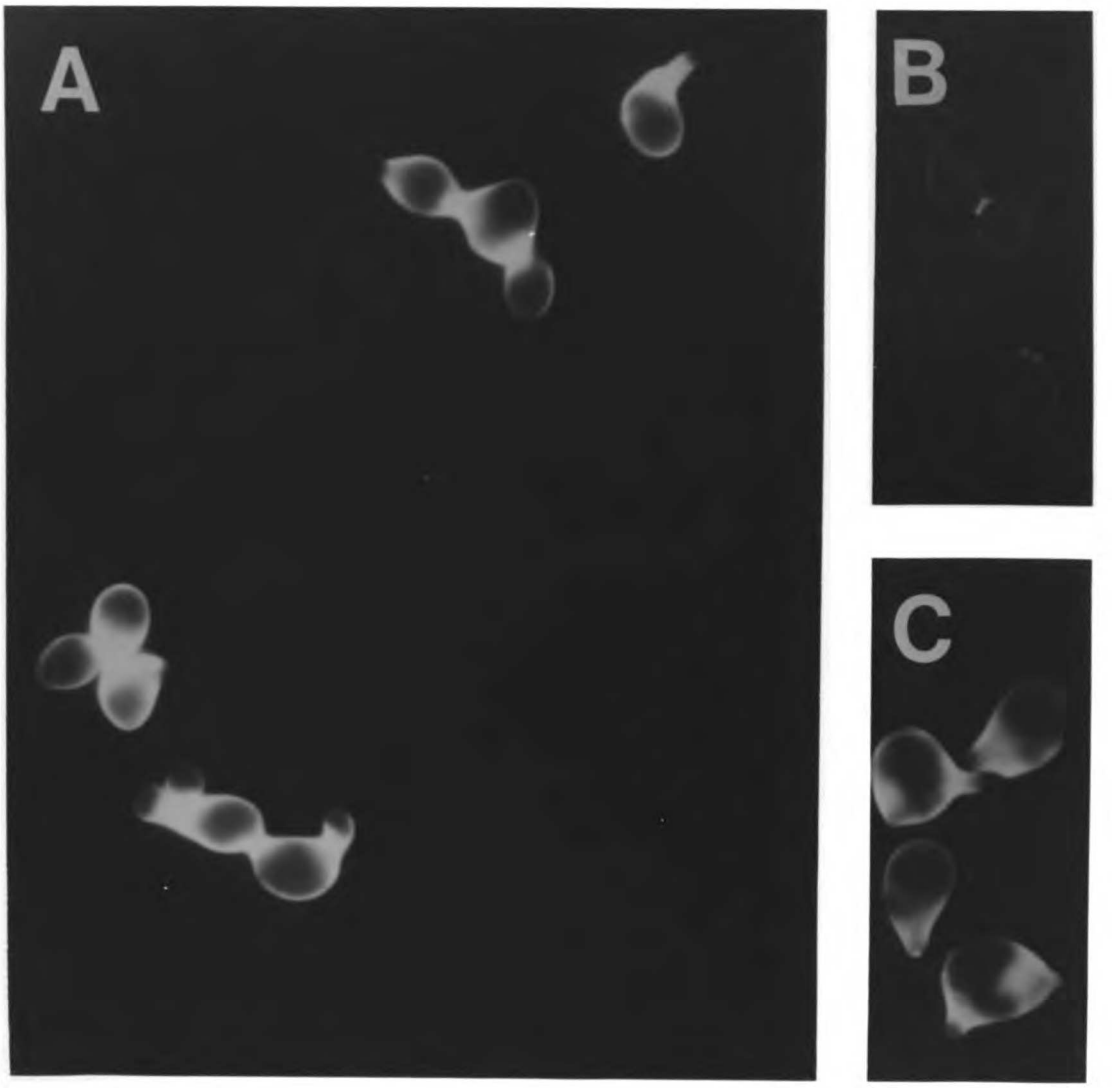
Exponential phase culture of **a** cells growing in YEPD at 30°C were treated with 1 μM α-factor for 0, or 6 hours, then fixed with 3.7% formaldehyde and stained for chitin with calcofluor.

A: *far1*, α-factor for 6 hours

B: *FAR1*$^+$, no α-factor

C: *FAR1*$^+$, α-factor for 6 hours

The *far1* strain was FC204 (which carries the far1::URA3 mutation); the *FAR1*$^+$ strain was the isogenic strain FC140.


A
B
C

these changes are a primary effect of α-factor and not a secondary consequence of cell-cycle arrest.

far1⁻ mutants also exhibited two other responses to α-factor: induction of agglutinins and competence for nuclear fusion (data not shown; see Experimental Procedures). We have also observed that treatment of an **a** *far1-1* strain with α-factor induced formation of **a**/**a** diploids in approximately 10% of the population (see Experimental Procedures). These diploids may result from fusion between two nuclei within a multinucleate cell.

Mutants defective in *FAR1* exhibited a defect in mating. An **a** *far1*⁻ strain (FC204) mated with a wild-type α partner at 7% the efficiency of an isogenic a *FAR1*⁺ strain (Table 1). A defect was also exhibited by an α *far1*⁻ strain mating with a wild-type partner. The severity of the mating defect was greatly enhanced when both partners were *far1*⁻: mating of the **a** *far1*⁻ strain FC204 to an **a** far1- partner was reduced an additional 10^4-fold. The observation that the *far1*⁻ mutation exhibited a mutant phenotype in both **a** and α cells suggests that the FAR1 gene plays a similar role in both cell types.

In summary, the only phenotypes exhibited by *far1*⁻ mutants were those affecting mating-factor response and the ability to mate. *far1*⁻ mutants grew at normal rates, exhibited normal budding morphologies and normal cell size in the absence of α-factor, and they produced normal levels of **a**-factor and α–factor. In addition, they arrested normally as unbudded cells in G1 in response to starvation, exhibited normal survival in stationary phase cultures, and they sporulated normally (data not shown). *FAR1* thus appears to act specifically in response to α-factor (and presumably to **a**-factor as well) and not in more general cell-cycle control.

Mapping the position of FAR1 with respect to GPA1 and CDC28 by epistasis.

TABLE 1: Mating ability of *far1*⁻ mutants

Genotypes of Mating Partners	Mating Frequency
1. a *FAR1*⁺ X α *FAR1*⁺	0.18
2. a *FAR1*⁺ X α *FAR1*⁺	0.46
3. a *far1*⁻ X α *FAR1*⁺	0.023
4. a *FAR1*⁺ X α *far1*⁻	0.0046
5. a *far1*⁻ X α *far1*⁻	$<10^{-7}$

Table I: Mating ability of *far1*⁻ mutants

Strains were mated by a filter mating assay, as described in Experimental Procedures. The mating frequency is calculated as the number of diploids obtained divided by the number of total cells present at the end of the mating. Frequencies are averages of mating assays performed in triplicate. Strains used were the following: (1) FC140 X IH1793; (2) IH1792 X FC139; (3) FC204 X IH1793; (4) IH1792 X FC296; (5) FC204 X FC297.

far1 gpa1 and *far1 cdc28* double mutants were constructed in order to test where in the pathway *FAR1* functions. A deletion allele of *GPA1*, the Gα subunit, causes constitutive cell cycle arrest, transcriptional and morphological responses and therefore dies (Miyajima, et al.1987; Dietzel et al., 1987 ; Jahng et al., 1988). *far1 gpa1* double mutants were obtained by first constructing a diploid heterozygous for both *far1-1* and *gpa1-* and sporulating the diploid. The segregation of haploid spore phenotypes was consistent with *gpa1 FAR*$^+$ haploids as dead, and *gpa1 far1-1* haploids as alive. The *gpa1 far1-1* double mutants grow slowly and posses the same abnormal morphologies as a *far1* mutants treated with α-factor for long periods of time, suggesting that double mutants exhibit constitutive morphological changes but are not arrested for growth. The finding that *far1* mutation blocks the cell cycle arrest caused by the *gpa1*$^-$ mutation suggests that *FAR1* functions downstream of *GPA1*. A similar analysis showed that *FAR1* also functions downstream of two factors which regulate the G protein, *CDC36* and *CDC39* (Neiman et al., 1990).

We next tested whether the *far1* mutation could bypass the need for *CDC28*. A *far1 cdc28* double mutant was constructed by a cross. A *cdc28-9* mutant arrests as unbudded cells when raised to restrictive temperature (Hartwell, 1974). A *far1-1 cdc28-9* double mutant also arrested as unbudded cells at restrictive temperature, showing that the *far1* mutation does not block arrest by the *cdc28* mutation. Thus *FAR1* does not act downstream, and may work upstream of *CDC28* .

Cloning of FAR1

The *FAR1* gene was cloned by complementation, screening for plasmids that were able to correct the severe mating defect of *far1* mutants when mated with other *far1*$^-$ mutants (see Experimental Procedures). Eight plasmids identified in this manner (pFC1 and others) all carried the same DNA insert. Plasmid pFC1 (Figure 7A) fully complemented the mating defect of the *far1-1* strain but did not restore cell-cycle arrest in response

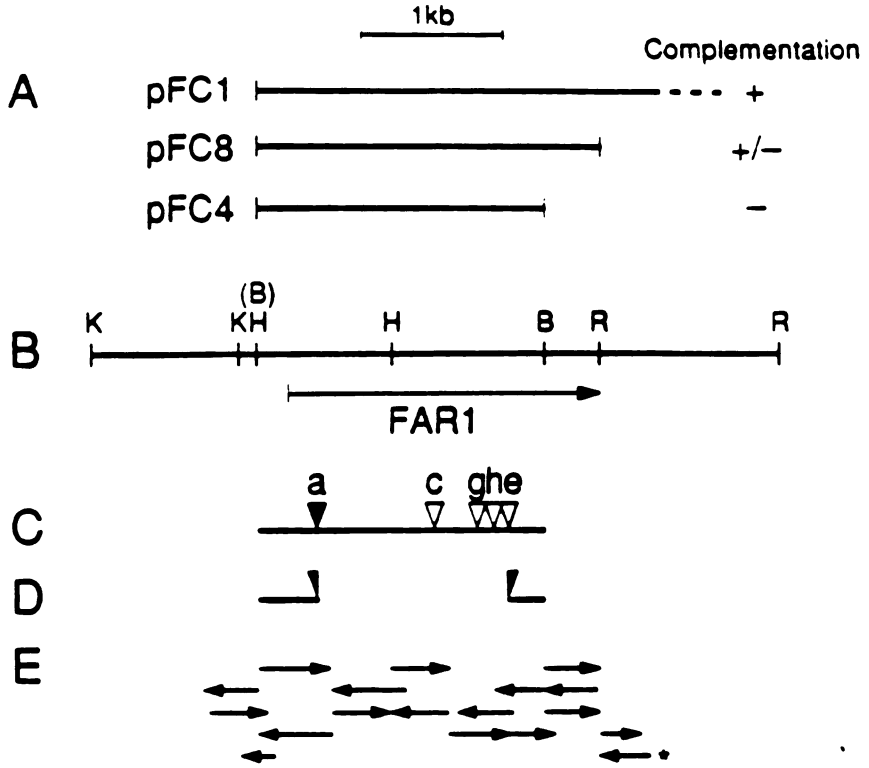


Figure 7: Maps of restriction sites, insertions, and deletions, and the sequencing strategy for the *FAR1* gene

A. Physical maps of inserts contained in different plasmids. Complementation was assayed by transforming the indicated plasmid into *far1-1* recipient strains FC-D1 and FC1002-2D. +, restoration of mating; +/-, partial restoration of mating; -, no restoration of mating.

B. Restriction map of insert in plasmid pFC15 containing *FAR1*. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; K, *Kpn*I. The arrow labled FAR1 indicates the position of the FAR1 open reading frame and the direction of transcription. (B) indicates a *Bam*HI site present in pFC1 but not in the genome.

C. Inverted triangles indicate positions of insertions of the Tn3::*URA3* element. The filled triangle represents an insertion that produced a defect in both arrest and mating; open triangles are insertions that resulted in a defect only in mating.

D. The *far1* deletion (*far1*::*URA3*) was constructed by joining the two outermost deletions (a and e) *in vitro* by cleaving the element with *Eco*RI and then ligating.

E. Sequencing strategy. Fragments were cloned into M13mp18 and M13mp19 and nucleotide sequences determined as described in Experimental Procedures. Arrows indicate direction of sequencing.

to α-factor. We isolated another FAR1 plasmid, pFC15, by colony hybridization using the insert from pFC1 as probe. pFC15 fully complemented the mating and cell-cycle-arrest defects of a *far1-1* strain. It carries approximately 6 kb of DNA to the left of *FAR1* and 7 kb to its right (Figure 7B). In contrast, pFC1 contains the full *FAR1* open reading frame (described below) but appears to be lacking some upstream regulatory sequences. The cloned segment was confirmed to carry the *FAR1* gene by showing that a plasmid containing part of the original insert from pFC1 integrated at the *FAR1* locus (data not shown; see Experimental Procedures). Subcloning revealed that a 2.5 kb HindIII-EcoRI fragment partially complemented the *far1-1* mating defect, whereas a 2.0 kb BamHI-BamHI fragment did not. To localize the *FAR1* complementing activity further, we carried out transposon mutagenesis in *E. coli* using the *Tn3-URA3* transposon (Seifert et al., 1986; see Experimental Procedures). The resulting mutations were then introduced into the genome of strain FC140 by gene replacement (Rothstein, 1983; see Experimental Procedures). Insertions over a 1.3 kb span (insert **a** on the left and insert **e** on the right) inactivated the complementing ability (Figure 3C). A deletion allele of *FAR1* was constructed by splicing together these inserts to form the *far1::URA3* allele (Figure 7D; see Experimental Procedures).

The nucleotide sequence of the 2.5 kb fragment and surrounding regions showed a single large open reading frame encoding a putative polypeptide of 780 amino acid residues (Figure 4). Searches of data banks (using dfastp and dfasta to search GENbank) have not revealed any significant similarities to known proteins nor to any motifs indicative, for example, of protein kinases. The open reading frame does contain a cysteine-histidine rich region in the N-terminus which has similarity to the LIM motif. If cysteines and histidines are interchanged, the spacing of the amino acids in *FAR1* show a suggestive similarity to the LIM consensus sequence (Figure 9). The LIM sequence is most often adjacent to the homeobox domain in several homeoproteins such as *lin-11* (Freyd et al., 1990) but is also found in proteins which do not contain homeoboxes, such

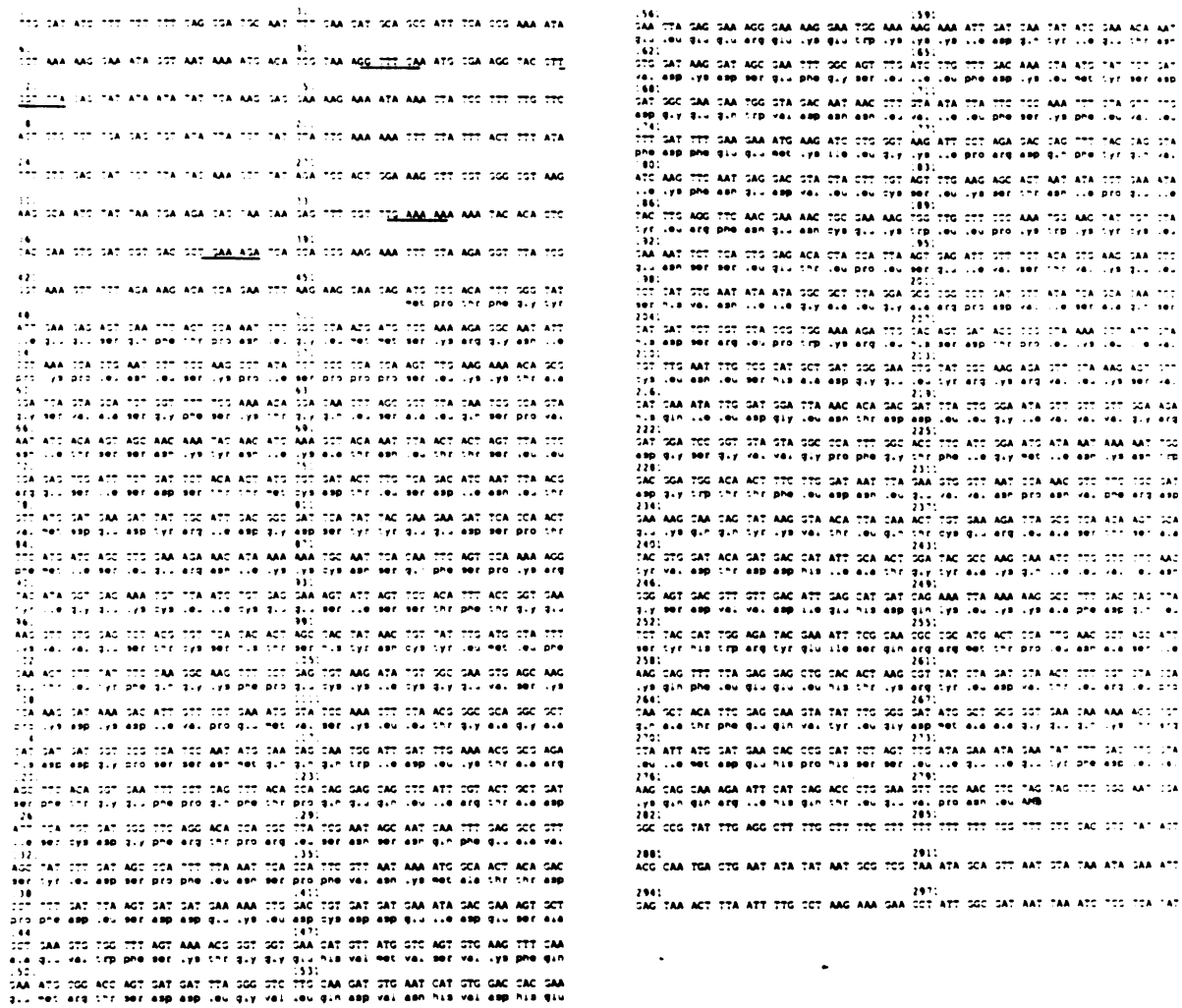


Figure 8: Sequence and putative ORF of *FAR1*

Underlines indicate 6/7 matches to the pheromone response element consensus, TGAAACA. No matches to the a1-α2 consensus sequence (Miller et al., 1985) were found.

```
NECAACAQ PILDRYVFTVL GKCWHQSCLRCCDCRAPM    SMTCFSFDGLILCKTD   lin-11
SLCVGCGN QIHDQYILRVSPDLEWHAACLKCAECNQTLDE  SCTCFVRDGKTYCKRD   Isl-1
NKCNCCN  QIYDRYIYRMD NRSYHENCVKCTICESPL    AEKCFWKNGRIYCSQH   mec-3
KGCAGCNRKIKDRYLLKAL  DKYWHEDCLKCACCDCRLGE VGSTLYTKANLILCRRD   rhombotin

ORCAGCDGKLEKEDLVRRAR DKVFHIRCFQCSVCORLLDTGDQLYIMEGNRFV CQSD   lin-11
IKCAKCSIGFSKNDFVMRAR SKVYHIECFRCVACSFQLIPGDEFALREDGLF  CRAD   Isl-1
HRCAGCKKGVSPTDMVYKLKAGLVFHVECHCCSLCGRHLSPGEQILVDDTMMTVSCMSH  mec-3
GNCAACSKLIPAFEMVMRAR DNVYHLDCFACQLCNQRFCVGDKFFLKNNMIL  CQMD   rhombotin

PKCPKCDKEVYFAERVTSLG  KDWHRPCLKCEKCGKTLTSGG   HAEHEGKPYCNHPC  CRIP

  CXXC     X 17-19       HXXCXXCXXC      X 15-19       C      consensus


EKCLICEESISSTFTGEKVVEST  C SHTSHYNCYLMLFE    TLYFQGKFPECKIC   FAR1
  C  C     X 17          C  H  H  C      X 16          C
```

Figure 9: FAR1 has similarity to the LIM motif.

See text for explanation.

as rhombotin (Boehm et al., 1990). The function of the LIM sequence is unknown in any protein, but it has been proposed to mediate protein-protein interactions or to bind metals.

The upstream region contains four imperfect (6/7) matches to the pheromone response element (PRE) consensus sequence (TGAAACA), which is found upstream of many α-factor-inducible genes such as *FUS1* (Trueheart et al., 1987; Van Arsdell et al., 1987). These elements may be responsible for transcriptional induction of FAR1 by α-factor (discussed below).

Regulation of FAR1 Transcription

Northern analysis with a *FAR1* probe revealed a single major message of approximately 3 kb, which was absent from the *far1::URA3* mutant (Figure 10, lane 5). Northern blots showed that the *FAR1* transcript is highly regulated: *FAR1 RNA* was present at low levels in uninduced a and α cells (Figure 10, lanes 1 and 2) and is induced 4- to 5-fold by α-factor (Figure 10, lanes 3 and 4). Transcription was dependent on genes in the mating-factor-response pathway: the *FAR1* transcript was absent or much reduced in *ste12* mutants (Figure 10, lane 6), as well as in *ste4* and *ste5* mutants (data not shown). The *FAR1* transcript was not observed in a/α cells (Figure 10, lane 5). *FAR1* is thus a member of a group of genes, which includes *FUS1* (Trueheart et al., 1987; McCaffrey et al., 1987) and FUS3 (Elion et al., 1990), that are expressed only in haploid **a** and α cells and are induced by α-factor in **a** cells.

DISCUSSION

The studies described here identify a new gene, *FAR1*, that is necessary for arrest of the cell cycle in response to a negative growth factor. *FAR1* was identified in a screen

Figure 10: Regulation of the synthesis of the *FAR1* transcript.

Total RNA was isolated form the strains listed below and subjected to Northern blot analysis using either a *FAR1* probe, which extends from the putative AUG (at position 45) to the internal HindIII site at position 1202 (**upper panel**), or a *LYS2* probe as control (**lower panel**). Lane 1; FC139 (α *FAR1*$^+$), no mating factor added; lane 2: FC140 (**a** *FAR1*$^+$), no mating factor added; lane 3: FC140 (**a** *FAR1*$^+$), treated with α-factor for 75 min; lane 4: FC140 (**a** *FAR1*$^+$), treated with α-factor for 120 min; lane 5: FC209 (**a**/α), no mating factor added; lane 6: FC204 (**a** *far1::URA3*), no mating factor added; lane 7: IH1934 (**a** *ste12*), treated with α-factor for 120 min. α-factor was used at a concentration of 1 uM.

α
a
a
a
a/α
far1
ste1
αf - - + + - - +
1 2 3 4 5 6 7

for mutants that are defective in arrest but are competent for transcriptional responses. Since all known *STE* genes in the α-factor response pathway affect both cell-cycle arrest and transcriptional responses, our screen was designed to avoid identifying loss-of-function mutations in these genes. We identified mutations in two genes, *FAR1* and *FAR2*, the latter corresponding to *FUS3* (Elion et al., 1990). A key observation concerning *FAR1* is that null mutations block arrest but do not affect other responses to α-factor such as transcriptional induction and morphological changes. It thus is likely that *FAR1* functions at the end of the mating-factor response pathway to inhibit essential functions necessary for cell-cycle progression. Since *FAR1* is only expressed in haploid cells, and cells deleted in *FAR1* show no defects in vegetative growth, *FAR1* does not appear play essential functions in the normal mitotic cell cycle.

The Position of FAR1 in the Pathway of Response to Mating Factors

As described in the Introduction, the gene products of the response pathway can be arranged into a single pathway with four main steps: a receptor (*STE2* or *STE3*, depending on whether the cell is an **a** or α cell), a G protein (with *GPA1/SCG1*, *STE4*, and *STE18* subunits), a group of protein kinases (STE7, STE11, and FUS3), and a transcriptional activator *STE12*). A simple view of the pathway is that it leads to production of an activated transcriptional activator, the STE12 protein (Errede and Ammerer, 1989; Dolan and Fields, 1990), which then stimulates transcription of a diverse group of genes, some of them (such as *STE2* and *GPA1*) involved in signal transduction itself, others (such as *FUS1*) necessary for the mating act. Because transcription of *FAR1* is induced 4- to 5-fold by α-factor and its upstream region contains sequences similar to a STE12 binding site, it is simplest to view *FAR1* as another target of STE12 that resides at the end of the pathway.

We have considered two models for where in the pathway *FAR1* acts. The simplest model, the "effector" model, is that *FAR1* does not function in the signal transduction

pathway between the receptor and STE12, but functions as an specific effector of cell cycle arrest. This model is supported by the observations that null mutants of *FAR1* exhibit near normal transcriptional induction.

The other model, the "leaky signal" model, stipulates that in a *far1*⁻ mutant, the signal leading to *STE12* is attenuated so that the signal is sufficient for *FUS1* induction, but is not strong enough for cell cycle arrest. Although *FAR1* is not an essential component of the signal transduction pathway, in this model, it might act as an amplifier or modulator for the signal. This model is supported by the fact that while the induction of *FUS1-lacZ* in a *far1* strain is substantial, it is usually 10-20% less than in a wild-type strain. One prediction of the leaky signal model is that a higher concentration of α-factor may be necessary for cell-cycle arrest than for full *FUS1-lacZ* induction. This prediction is not true, since the two functions have very similar K50 values in dose response curves (data not shown). Another prediction is that mutations which reduce but do not knock out the activity of known components of the signal transduction pathway, the *STE* genes, could give a similar phenotype. In the screen for *far* mutants, the *far1* mutants formed a distinct class of mutants which did not include mutants in the *STE* genes (with the exception of the allele of *FUS3*, which may be a special case - see Discussion of Chapter 2). The strongest evidence against this model, however, is the genetic interaction of *FAR1* with the cell cycle gene, *CLN2* , which is described in the next chapter.

Uncoupling differentiation from cell-cycle arrest.

In many differentiating cells, differentiation is coupled to cell-cycle arrest. The relationship between these two processes is unclear: is cell cycle arrest a prerequisite for differentiation, or is differentiation required for arrest? The *far1* mutant uncouples differentiation from cell-cycle arrest, and thus illustrates how these processes may be coupled in yeast. Transcriptional induction of genes in response to α-

factor occurs in a *far1* mutant in the absence of cell cycle arrest, showing that this response does not require arrest. More surprising is that *far1* cells also exhibit morphological changes in the absence of arrest, suggesting that the morphological changes induced by α-factor are not a consequence of the cell-cycle arrest, as previously thought, but constitute a separate response. Thus, many aspects of differentiation are not dependent on cell-cycle arrest.

Differentiation appears to be necessary for arrest. Expression of an putative effector for cell cycle arrest, *FAR1*, is dependent on the same pathway (the STE genes) which induces expression of the specialized genes, such as *FUS1.* Cell-cycle arrest thus may be considered in this example as an extension of the differentiation program.

EXPERIMENTAL PROCEDURES

Media and genetic methods: YEPD (complete) medium, SD (minimal) medium, and supplements are described by Hicks and Herskowitz (1976) and X-gal plates by Sternberg et al. (1987). α-factor plates contained 4 μg of α-factor (Sigma) in 0.2 ml YEPD, which was spread on 25-ml YEPD agar plates four hours prior to use. Standard yeast genetic techniques were utilized (Sherman et al., 1982). Yeast transformations were performed by the lithium acetate procedure (Ito et al., 1983).

Strains: FC140 was constructed by crosses among several strains from our laboratory collection, which yielded FC139, of genotype *MATα HMLa HMRa.* The *HML* and *HMR* alleles were confirmed by Southern blot analysis and by their phenotype after introduction of the HO gene on a plasmid (Jensen et al., 1983). The mating type of FC139 was switched by introduction of a plasmid carrying HO to produce an isogenic a strain, FC140, which was used in the construction of several other isogenic strains. Gene disruptions were performed with plasmids pFC13 (*far1::URA3*) and with a

bar1::LEU2 plasmid provided by V. MacKay (ZymoGenetics Corporation) (MacKay et al., 1988). FC311 (*far1::URA3 FUS1-lacZ*) and FC312 (*FAR1⁺ FUS1-lacZ*) are segregants of a cross between FC140 which had been transformed with pSB286 (an integrating *URA3+* plasmid carrying *FUS1-lacZ;* from J. Trueheart) and FC296 (a *far1::URA3*). Other information on strains is given in the text and in Table 2.

Construction of insertion and deletion mutations of FAR1: A 0.9 kb HindIII-HindIII fragment (Figure 3) was inserted into a HindIII site in pHSS6 and subjected to insertional mutagenesis by a *Tn3::URA3* minitransposon (Seifert et al., 1986). The plasmids and *E. coli* strains used for this shuttle mutagenesis were a gift of F. Heffron. pFC13 was constructed by joining fragments from two different plasmids that contained the **a** and **e** inserts (Figure 3). The fragments were linked at an EcoRI site located within the Tn3::URA3 insert and resulted in substitution of *FAR1* sequences by one copy of the Tn3::URA3 element. For carrying out one-step gene replacement (Rothstein, 1983), plasmids were cleaved with NotI prior to transformation. In initial studies, the *FAR1* gene was disrupted in an a/α diploid strain. Sporulation of these strains yielded four viable segregants, which demonstrated that *FAR1* is not an essential gene. Insertion and deletion mutations of *FAR1* constructed by gene replacement were confirmed by Southern blot analysis (data not shown). Standard DNA manipulations were performed according to Maniatis et al. (1982). DNA fragments were purified using Geneclean (Bio-Check).

Mutant isolation and initial characterization: The parent strain, FC140, contained a mutation in the *BAR1* gene, which does not significantly affect mating (Sprague and Herskowitz, 1981) but reduces the concentration of α-factor needed to elicit cell-cycle arrest and thus greatly facilitated the isolation and characterization of α-factor resistant mutants. FC140 also carries silent **a** cassettes at both the *HML* and

HMR loci. Thus sir⁻ mutants, which express the silent cassettes and thereby exhibit the phenotype of an a/a diploid, will not be obtained among α-factor resistant mutants (Hartwell, 1980). Screen 1: In screen 1, the parent strain was FC140A, which carries pSB234, a high-copy number (2μ) plasmid containing *FUS1::lacZ* (Trueheart et al., 1987), kindly provided by J. Trueheart. Initial screens, which yielded the mutant FC-D1 (*far1-1*) were performed without mutagenesis. A subsequent screen used cultures that were lightly mutagenized with EMS (>50% survival). Approximately 10^7 cells, grown to saturation in SD-ura medium to maintain the plasmid, were spread on α-factor YEPD plates. α–factor resistant colonies were obtained at a frequency of approximately 10^{-5}. These colonies were replica printed to X-gal plates containing α-factor and screened for blue colonies (indicative of *FUS1-lacZ* expression). These colonies were purified by streaking and tested for (1) *FUS1-lacZ* expression by colony color on X-gal plates and by quantitative b-galactosidase assays of cultures; (2) a-factor production by halo assay; (3) mating by plate assay; (4) cell-cycle arrest in response to α-factor by monitoring cultures for percent unbudded cells and for morphology in cultures containing 1 uM α-factor in YEPD. Screen 2: In screen 2, Ty insertion mutagenesis was employed. FC140 carrying pJEF1105 (pGTyH3-neo; a gift of J. Boeke) was induced for Ty transposition (Boeke et al., 1988), and α-factor resistant colonies were selected by spreading the cells on α-factor YEPD plates. α–factor resistant colonies were further screened for a-factor synthesis, mating ability, and cell morphology as described above. Although three of the six putative far⁻ mutants were resistant to 50 ug/ml G418, indicating that they contained at least one TyH3-neo insert, tetrad analysis demonstrated that the G418 resistance determinant was unlinked to the far⁻ determinant. The possibility that these far mutations are due to an endogenous Ty element was not pursued. Complementation and dominance tests were carried out as follows: a *far1*⁻ mutants obtained in screen 1 were fused to a FAR1+ strain FC1001-1C (a leu1 trp1 ade5 met bar1) and to a *far1-1* mutant (FC1002-1B,

a *ura3 leu2 his3 lys bar1*) by spheroplast fusion, according to the method of Rose et al. (1986), except that the a strains were first induced with 1 uM α-factor in YEPD. Diploids (which are a/a) were selected from this mix as prototrophs.

Assays for mating and other phenotypes: Standard plate assays for mating and mating-factor production by halo assay were used (Sprague and Herskowitz, 1981). *far1* mutations were routinely scored in crosses and in transformants by their characteristic weak mating defect when mated to a wild-type tester strain and a strong mating defect when mated to a *far1*$^-$ strain (for example, strain 1002-2D) as assayed by plate mating assay. Quantitative mating assays were modified from the procedure of Reid and Hartwell (1977), in which 5 x 10^6 cells of each mating type were filtered and incubated on a YEPD plate for six hours at 30°C. Assays for nuclear fusion were performed by measuring efficiency of spheroplast fusion as described by Rose et al. (1986) and and for cytoductants (Dutcher and Hartwell , 1982). Agglutination was assayed as described by Michaelis and Herskowitz (1988). Cell-cycle arrest in response to α-factor was assayed as follows: in the confrontation assay (Duntze et al., 1970; as modified by Hicks and Herskowitz, 1976; modification of budding assay, Chant and Herskowitz, 1991), log phase a cells were sonicated lightly and plated on YEPD 4% agar slabs at dilute concentrations so that individual cells were were well spaced. α cells (strain IH1793) freshly grown on a YEPD plate were spread in a broad streak onto the slab with a toothpick. The slabs were then incubated at 30°C on a coverslip in a moist chamber. Division and morphology of the a cells adjacent to the α-factor source or on a different slab were monitored every 2-3 hours. Cells in culture were tested for cell-cycle arrest by adding α-factor at different concentrations to log phase cells growing in YEPD at 30°C, which were then incubated aerobically by shaking at 30°C. Samples were taken periodically, lightly sonicated, and then usually fixed with 3.7% formaldehyde and assayed for morphology and percent unbudded by phase-contrast

microscopy (Bucking-Throm et al., 1973). Growth inhibition by α-factor was also determined by halo assays (Fink and Styles, 1972), using approximately 10^4-10^5 log-phase cells per plate. Ploidy of cells after treatment with α-factor was ascertained by inspection of cells from approximately 100 individual colonies under bright field microscopy to determine if they have the characteristics of diploid cells--more oval and larger than haploid cells. All of the polyploid colonies remained phenotypically a. A few of these colonies were confirmed to be a/a diploids by introducing a MATα plasmid, which allowed sporulation and yielded four viable haploid segregants per tetrad. DAPI straining of DNA and Calcofluor staining of chitin were performed as recommended by Pringle et al. (1989). Photomicroscopy was performed using a Nikon Optiphot camera with a 100X Zeiss Plan objective. Induction of *FUS1-lacZ* in cultures was assayed as follows: log phase cells grown in YEPD were distributed into tubes containing α-factor at different concentrations and incubated at 30°C. Cells were harvested at different times, collected on ice, and assayed for β-galactosidase as described by Stern et al. (1984).

Cloning FAR1: FAR1 was cloned by complementation of the mating defect exhibited by *far1-1* strains. The strains used for these purposes (FC1002-2D and FC1011-2C) were chosen because their efficiency of mating with each other is $<10^{-9}$, which is considerably lower than for other **a** *far1*$^-$ X α *far1*$^-$ matings presumably due to modifier mutations. FC1011-2C (*a1*$^-$ *ura3 far1-1*) was transformed to Ura+ with a YCp50 yeast genomic library (a gift of M. Rose). Transformants were pooled and frozen for further screening. After thawing, transformants were allowed to undergo one cell division in YEPD, then mixed with α *far1-1* cells (strain FC1002-2D) (approximately 10^8 cells of each mating partner in 0.2 ml fresh YEPD), and spread on SD + leucine plates to select for prototrophs. The resultant diploids were *MATα/mata1*$^-$ *far1-1/far1-1* and presumably contained plasmids containing FAR1, which allowed mating by

the FC1002-2D parent. These colonies were rescreened for their Far+ phenotype by testing mating ability to an **a** *far1-1* tester. The plasmids were recovered from these transformants using the procedure of Hoffman and Winston (1987) and transformed into E. coli for further analysis. Plasmid pFC1 was obtained in this manner. A second plasmid containing *FAR1* (pFC15) was isolated by colony hybridization (Ausubel et al., 1989) using a segment from pFC1 to probe a YEp24 yeast genomic library (constructed by M. Carlson and provided by E. Shuster).

Nucleotide sequence analysis: Restriction fragments shown in Figure 3 were subcloned into M13 vectors (M13mp18 and M13mp19) and their nucleotide sequence determined by the dideoxy chain-termination method (Sanger et al., 1977) using Sequenase (USB). The putative open reading frame was subjected to a search for similarities to other proteins by dfastp (Biomathematics Computation Lab) to the Genbank, Mark Goebl, and Genentech databases.

Southern and Northern blot analysis: Total RNA was isolated using a glass bead preparation method and run on a hepes-formaldehyde 1% agarose gel for 40 hours at 36 amps, blotted to a nylon filter by electroblotting, and subjected to Northern blot analysis (Jensen et al., 1983). The *FAR1* probe was generated by random priming (Feinberg and Vogelstein, 1983) of a *FAR1* fragment that extends from the putative AUG (at position 451) to the internal HindIII site at position 1202. The *LYS2* probe was generated by random priming of plasmid *LYS2*/pBR328 (Barnes and Thorner, 1986). Southern blot analysis was performed as described by Ausubel et al. (1989) using aqueous hybridization conditions. Hybridization to *E. coli* colonies carrying the yeast genomic library was carried out on filter using aqueous hybridization conditions (Ausubel et al., 1989). The probe was prepared from a 0.9 kb HindIII-HindIII FAR1 segment by random priming.

CHAPTER 2

FAR1 INHIBITS A G1 CYCLIN, CLN2

CHAPTER 2

FAR1 INHIBITS A G1 CYCLIN, CLN2

INTRODUCTION

α-factor arrests cells at a point in the cell-cycle in G1 known as START. It is possible, in principle, that in response to α-factor, the FAR1 product causes cell-cycle arrest by inhibiting synthesis or function of a cell division cycle (CDC) gene product that is necessary for cells to pass through START. One of the *CDC* genes necessary for traversal through START is *CDC28*, the budding yeast homologue of the p34/cdc2 protein kinase, which has been shown to regulate the transition from G2 to M in a wide variety of eukaryotes and the transition from G1 to S in budding and fission yeasts (Lewin, 1990). The hypothesis that α-factor inhibits CDC28 receives support from the observation that treatment of a cells with α-factor causes activity of the CDC28 protein kinase to decrease (Mendenhall et al., 1987; Wittenberg and Reed, 1988).

Richardson et al. (1989) have recently demonstrated that three additional genes -- *CLN1*, *CLN2*, and *CLN3*-- are also required for progression through START. The *CLN* genes have been termed G1 cyclins because they are required for the cell to progress from G1 to S and because they possess structural and functional similarities to mitotic cyclins. Like mitotic cyclins, the CLN2 product associates with the CDC28-cdc2 class of protein kinases, and is hypothesized to be requred for the activity of CDC28 kinase. Also, like mitotic cyclins, the level of CLN2 protein varies in the cell-cycle, though unlike the mitotic cyclins whose level peaks in G2, the G1 cyclin CLN2 protein peaks around G1. The *CLN* genes have been found to be functionally redundant: a *cln1*$^-$ *cln2*$^-$

cln3⁻ mutant arrests in G1, whereas single and double mutants are viable (Richardson et al., 1989).

Since a deletion of the three *CLN* genes results in an arrest at the same point in the cell-cycle as α-factor arrest, it is formally possible that α-factor might cause arrest by inhibiting the activity or synthesis of all three *CLN* genes. A link between α-factor arrest and the CLN proteins first came from the finding of a dominant mutation in *CLN3* (known as *DAF1-1*) which has a phenotype similar to a *far1* mutant: the *DAF1-1* mutant is resistant to α-factor and is competent in the transcriptional induction of *FUS1*. This mutant produce a C-terminal truncation of the CLN3 protein which has been proposed to be insensitive to inhibition by α-factor.

In this chapter, I describe experiments showing that *FAR1* is primarily responsible for inhibiting only one of the three G1 cyclins, *CLN2*. These studies provide a strong evidence that α-factor arrests the cell-cycle by regulating the G1 cyclins.

RESULTS

FAR1 is an inhibitor of *CLN2*

The FAR1 product is necessary for cell-cycle arrest and thus might be act by inhibiting the *CLN* genes or their products. Because there are three CLN products, any one of which suffices for cell-cycle progression, we could imagine two formal possibilities: FAR1 might inhibit all three CLN products, or it might inhibit only one or two of them. We have therefore genetically analyzed interactions between *FAR1* and *CLN1,2,3* genes. The analysis described next indicate that *FAR1* specifically inhibits *CLN2*.

We constructed strains that are simultaneously defective in *FAR1* and in one or two of the *CLN* genes. In all cases, strains carried null mutations in the appropriate genes, and isogenic strains (constructed by gene replacements; see Experimental Procedures) were

Figure 11: Deletion of CLN2 restores arrest to far1⁻ mutants (halo assays).

Lawns of a strains of different genotypes were spread onto an agar plate, and a disk containing 1 ug of α-factor was placed in the center. Plates were incubated for 2 days at 30° C and then photographed. The zone of inhibition ("halo") indicates that growth of the cells in the lawn has been arrested by the mating factor. The strains used for this analysis are an isogenic set constructed by crosses of strains transformed with deletion ations of the *FAR1*, *CLN1*, *CLN2*, and *CLN3* genes. Strains were as follows: wild-type, FC279; *far1*, FC280; *cln2*, FC291; *far1 cln1*, FC289; *far1 cln2*, FC310; *far1 cln3*, FC329; *far1 cln1 cln2*, FC290; *far1 cln1 cln3*, FC319; *far1 cln2 cln3*, FC322. Additional information on these strains is given in Table 2 and in Experimental Procedures.

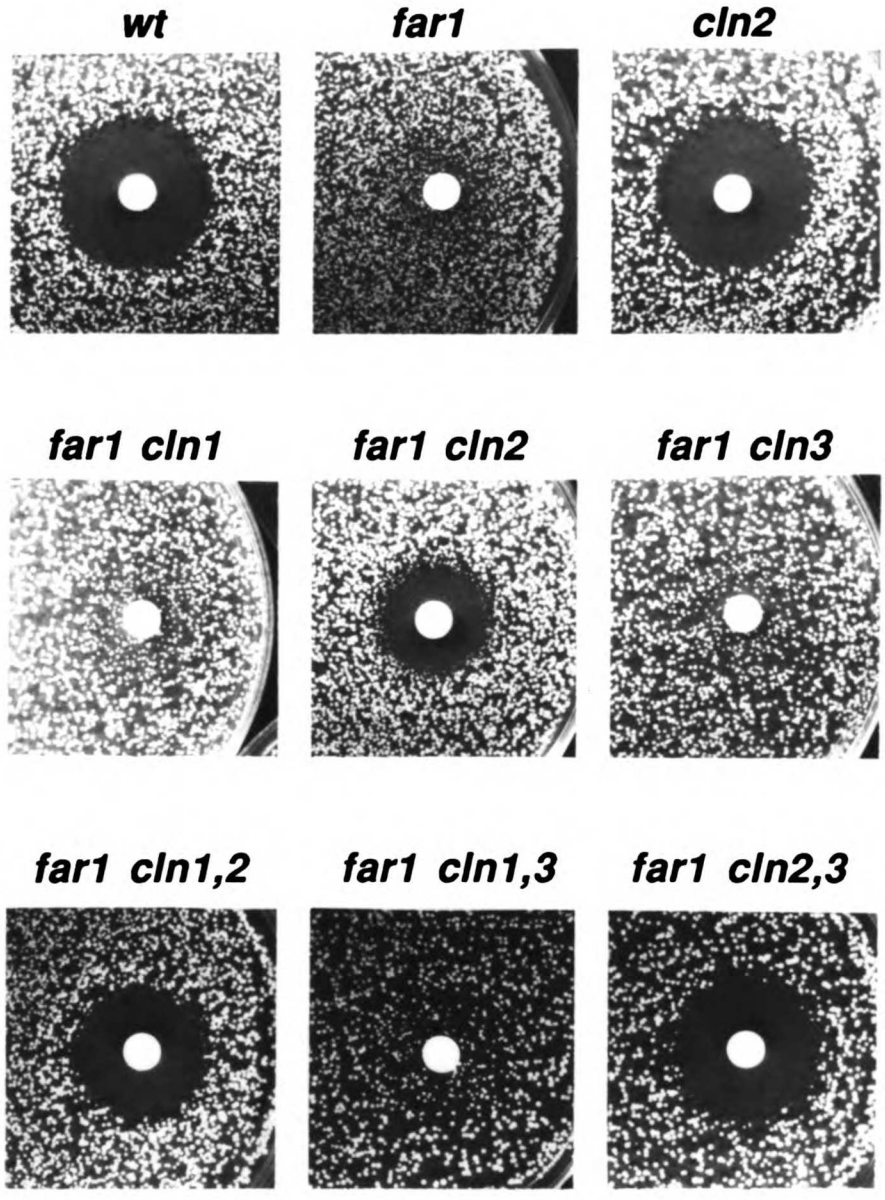
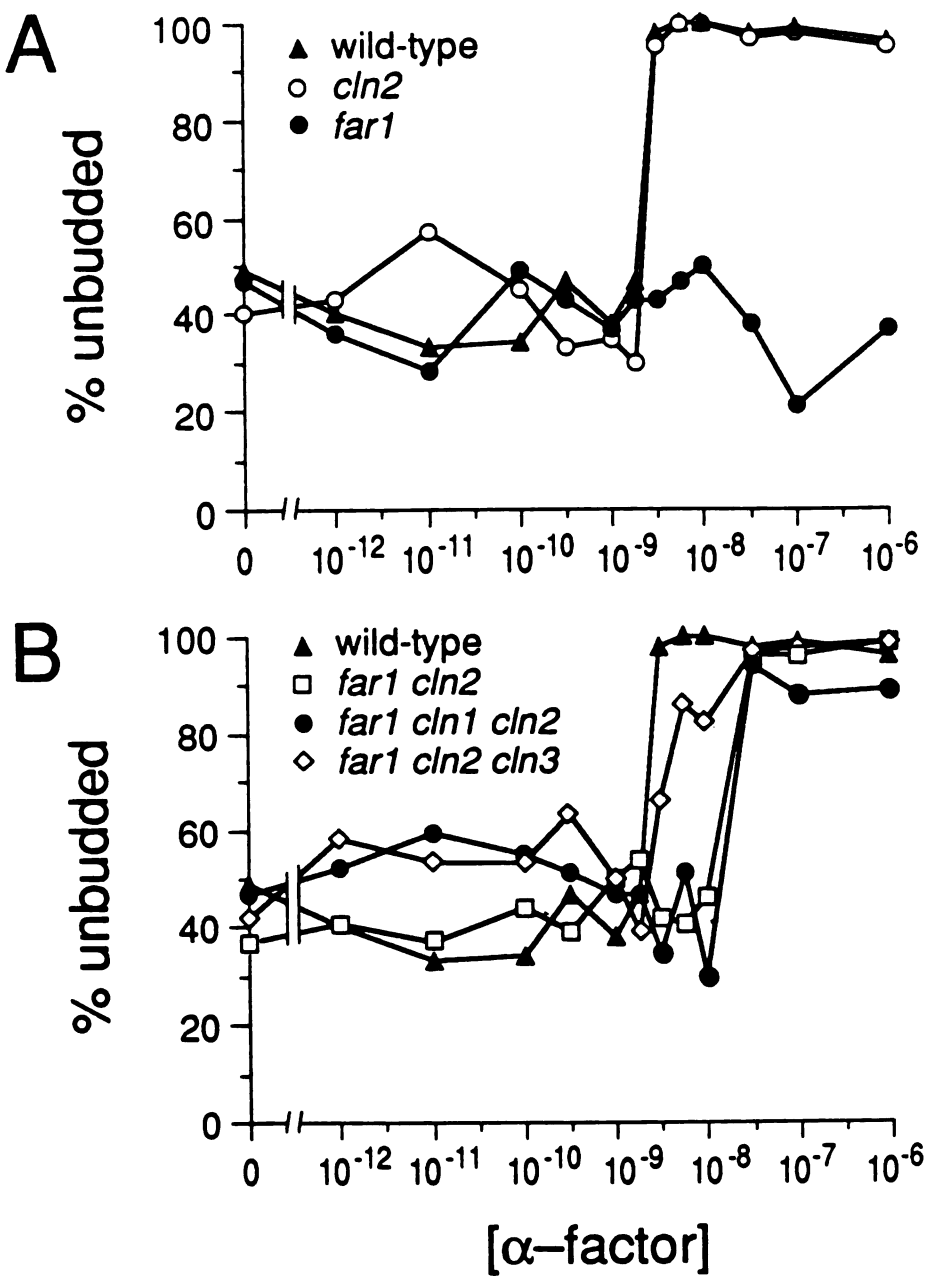

wt
far1
cln2
far1 cln1
far1 cln2
far1 cln3
far1 cln1,2
far1 cln1,3
far1 cln2,3

Figure 12: Deletion of CLN2 restores arrest to far1- mutants (assays in culture)

α-factor, at the indicated concentrations, was added to cultures of cells growing exponentially in YEPD medium at 30°. After three hours of incubation at 30°, samples were taken, fixed with formaldehyde, and scored to determine percent unbudded cells, a measure of cells arrested in G1. Strains are the isogenic set described in the legend to Figure 6. Six different strains were tested at the same time and plotted in two panels for purposes of presentation. The same data for the wild-type strain are plotted in both panels. Data for other strains analyzed at the same time


A
% unbudded
100
80
60
40
20
0
wild-type
cln2
far1
0
10-12
10-11
10-10
10-9
10-8
10-7
10-6
B
% unbudded
100
80
60
40
20
0
wild-type
far1 cln2
far1 cln1 cln2
far1 cln2 cln3
0
10-12
10-11
10-10
10-9
10-8
10-7
10-6
[α–factor]

used for analysis. As shown in Figure 11(top row), α-factor inhibited growth of wild-type a cells and a *cln2* cells, producing a halo surrounding the source of α-factor, but it did not inhibit growth of cells that carry a *far1*⁻ mutation. The *far1 cln2* mutant (Figure 11, middle row) exhibited a striking behavior: it produced a substantial halo, indicating that this mutant arrested in response to α-factor. Thus, inactivation of *CLN2* restored α-factor arrest in a strain lacking *FAR1*. In contrast, inactivation of *CLN1* and *CLN3* or both genes (Figure 11, middle and bottom rows) did not restore α-factor arrest to the *far1* mutant.

Assaying cell-cycle arrest by scoring the percentage of unbudded cells growing in culture reinforced the major conclusions from the plate assay and provided quantitative information on the sensitivity of strains to different concentrations of α-factor (Figure B). Wild-type and *cln2*⁻ cells both arrested at a concentration of 5 x 10^{-9}M α-factor. Mutants defective in FAR1 did not arrest at any concentration (up to 10^{-6}M). However, if they carried a mutation in *CLN2*, they exhibited full arrest at an α-factor concentration of 5 x 10^{-8}M: >95% of the cells formed normal, unbudded shmoos.

These studies show that although *FAR1* is required for arrest of cells by α-factor, it is not required in the absence of *CLN2*. We explain these observations by proposing that the primary function of *FAR1* is to inhibit synthesis or activity of *CLN2*: in a *far1* mutant, *CLN2* is present even when cells are exposed to α-factor. In a *far1*⁻ *cln2*⁻ double mutant, when both *FAR1* and *CLN2* are absent, cells arrest in response to α-factor because the other two *CLN* products are still inactivated by α-factor.

Although the a *far1*⁻ *cln2*⁻ strain arrested in response to α-factor, it required a ten-fold higher concentration of α-factor (5x10^{-8}M) then the wild-type strain for full arrest (Figure 12). Inactivation of the *CLN3* gene, to produce a *far1 cln2 cln3* strain, improved arrest at 5x 10^{-9}M α-factor to near wild-type behavior. Mutations in *CLN1* had no effect. These observations may indicate that *FAR1* has a second role, as an inhibitor of *CLN3* at low α-factor concentrations. Another explanation of these

observations is that mutations in *cln3* make cells more supersensitive to α-factor (data not shown).

Effect of *FAR1* and α–factor on *CLN2* mRNA

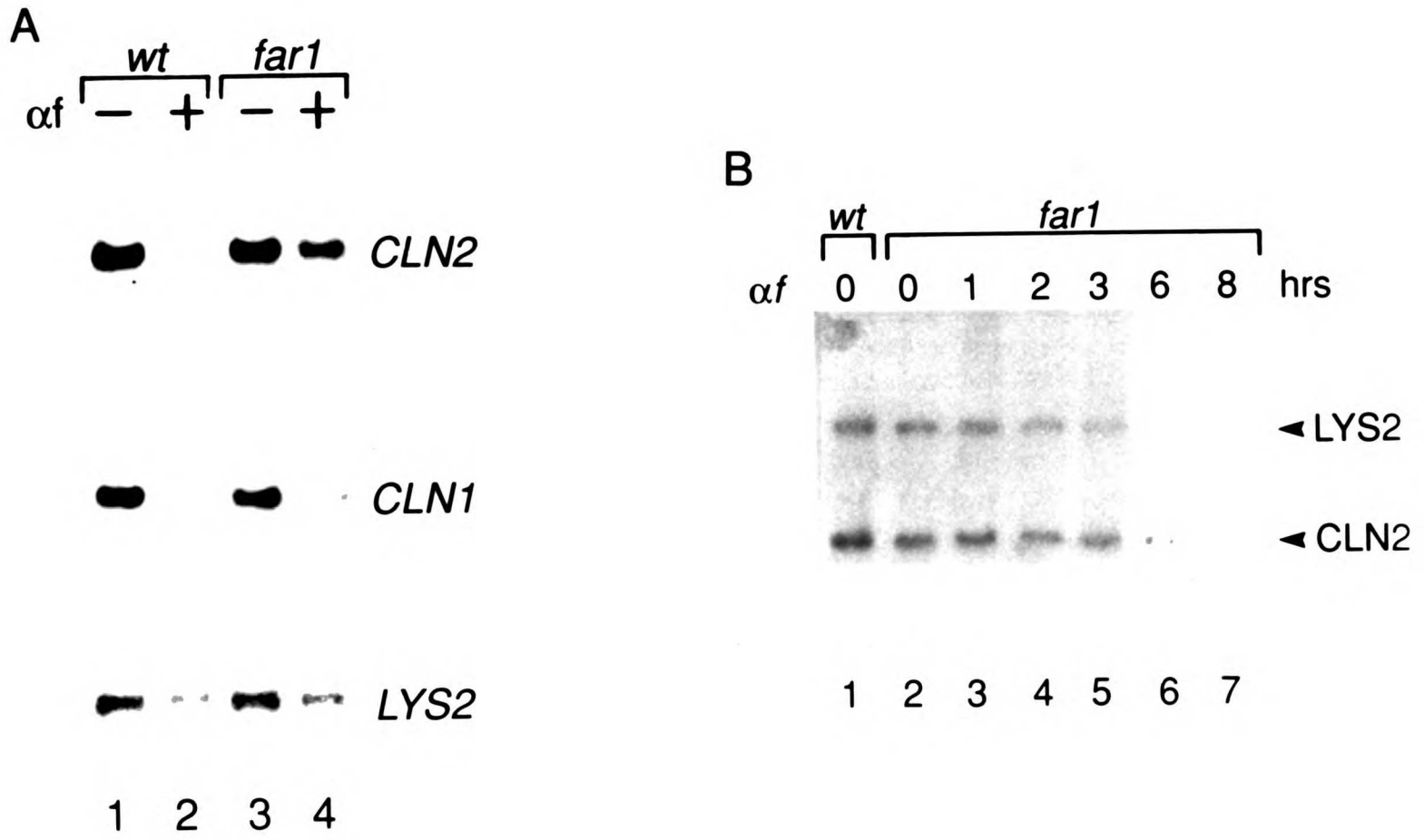
Genetic arguments in the previous section showed that α-factor, working via *FAR1*, acts somehow to inhibit the function of *CLN2*. We wanted to determine the molecular mechanism of this inhibition in order to define a molecular role for *FAR1*. First, we sought to define the effects of α-factor on *CLN2* mRNA and protein. Wittenberg et al. (1990) showed that α-factor causes a decrease in both *CLN1* and *CLN2* mRNA but not in *CLN3* mRNA. The decrease in *CLN2* mRNA occurs gradually over a 2 hour interval. In contrast, CLN2 protein, as assayed by immunoblot, disappears abruptly 30 minutes after the addition of α-factor. No changes in the mobility of CLN2 protein, which might indicate ubiquination or changes in phosphorylation, are seen before the protein disappears. (It has not been possible to examine the effect of α-factor on CLN1 or CLN3 proteins since no usable antibodies to CLN1 or CLN3 have been reported.) Since CLN2 is an unstable protein even in the absence of α-factor, Wittenberg et al.(1990) have proposed that the primary action of α-factor is to decrease *CLN2* mRNA levels, which then results in a decrease in CLN2 protein synthesis and in CLN2 protein levels. According to this hypothesis, α-factor might act to inhibit transcription of *CLN2* or to destabilize *CLN2* mRNA.

Since *FAR1* is necessary for the inhibition of *CLN2*, we tested the effect of α-factor on *CLN2* mRNA in a *far1*⁻ strain. Northern blots show that α-factor causes *CLN2* mRNA to decrease in wild-type strain (Figure 13; Panel A). In a *far1::URA3* strain, *CLN2* mRNA is present at the same level as in a wild-type strain and is not altered by treatment with α-factor for 1 to 8 hours. (Figure 13; Panels A and B). It was also found that the CLN2 protein does not decrease or change in mobility on Western blot in response to α-factor in a *far1::URA3* strain (Wittenberg and Chang, unpublished

Figure 13 : Effect of α-factor on *CLN1* and *CLN2* mRNA in wild-type and a *far1::URA3* mutant.

Panel A. Wild-type **a** cells (FC279) and **a** *far1::URA3* cells (FC280) were grown in YEPD without α-factor(lanes 1 and 3) or were treated with 10^{-6}M α-factor for 90 minutes (lanes 2 and 4). Total RNA was extracted and analyzed by Northern blotting using probes recognizing *CLN1*, *CLN2*, and *LYS2* (see Exp. Procedures for details). *CLN2* and *LYS2* were probed on the same blot. *CLN1* was probed on another blot in parallel using the same RNA samples. The bands were also quantitated by densitometry. The densitometry readings of each band normalized to the LYS2 control are presented in the parentheses: lane 1- CLN2 (141), CLN1 (78); lane 2- CLN2 (11). CLN1 (0); lane 3 - CLN2 (177), CLN1 (210); lane 4 - CLN2 (210), CLN1 (60).

Panel B. Northern blot showing levels of *CLN2* and *LYS2* mRNA in **a** *FAR+(FC279)* or **a** *far1::URA3* cells (FC280) were grown in YEPD with 10^{-6}M α-factor for the indicated periods of time. The bands were not quantitated by densitometry.


A
wt
far1
αf
– + – +
CLN2
CLN1
LYS2
1 2 3 4
B
wt
far1
αf 0 0 1 2 3 6 8 hrs
◄ LYS2
◄ CLN2
1 2 3 4 5 6 7

observations). These data are consistent with the model that α-factor, working via FAR1, inhibits *CLN2* mRNA, either by inhibiting *CLN2* transcription or by destabilizing *CLN2* mRNA.

The genetic arguments in the previous section indicate that FAR1 is not necessary for the inhibition of CLN1 activity. Northern blots demonstrate that *CLN1*mRNA, unlike *CLN2*, does decrease in α-factor-treated *far::URA3* cells, although not as much as in a wildtype cell (Figure 13; Panel A). Thus, *FAR1* is not necessary for the drop in *CLN1* mRNA levels.

An Additional Mode of Regulation for CLN2

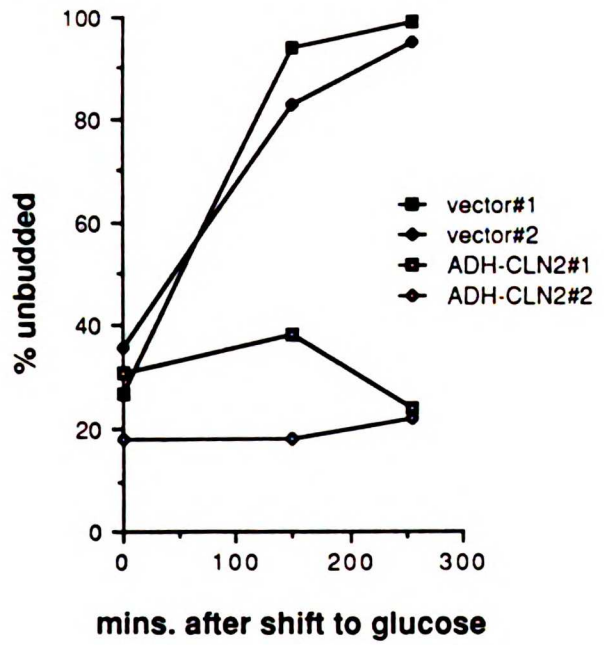
If α-factor acts primarily to inhibit *CLN2* transcription, then replacing the *CLN2* promoter with a promoter which is not responsive to α-factor would make the cell resistant to α-factor arrest.

A p*ADH-CLN2* construct, in which the strong, constitutive ADH promoter is fused to the *CLN2* open reading frame at the first AUG codon, was obtained from Pierre Leopold. This construct was shown to express functional *CLN2* by showing that it complements a strain deleted for *CLN1, CLN2,* and *CLN3.* The complementation was tested in YPL1, an a strain which contained deletions of the three *CLN* genes and a CLN2 gene under the control of a *GAL1* promoter (obtained from P. Leopold). When YPL1 is shifted from galactose media to glucose media, expression of *CLN2* from the p*GAL-CLN2* is turned off, and the cells arrest in G1 just like a *cln1⁻cln2⁻cln3⁻* cells (Richardson et al., 1989). In the presence of the p*ADH-CLN2* construct, the YPL1 cells do not arrest on glucose media, but continue budding and form colonies on YEPD plates (Figure 14). Another indication that the p*ADH-CLN2* supplies *CLN2* function is that when it is introduced in wildtype cells, the cells are small or elongated and appear similar to cells carrying a p*GAL-CLN2* construct (Hadwiger et al., 1989).

Figure 14 : The p*ADH-CLN2* construct encodes a functional CLN product.

Panel A: p*ADH-CLN2* prevents G1 arrest in a *cln*$^-$ strain. YPL1 (**a** *cln1*$^-$*cln2*$^-$ *cln3*$^-$ *GAL-CLN2* strain) was transformed with either DB20 (an ADH vector control) or p*ADH-CLN2*. Two transformants (#1 and #2) of each plasmid were grown on Sgal-ura (galactose) and then shifted into YEPD (glucose)media (which represses expression of *GAL-CLN2*). Cells were assayed for cell cycle arrest in G1 by % unbudded cells at the indicated times. **Panel B.** p*ADH-CLN2* allows growth of a *cln*$^-$ strain. Transformants of YPLI1 (α *cln1*$^-$*cln2*$^-$*cln3*$^-$ *GAL-CLN2*) with the indicated plasmids were grown on a Sgal-ura plate and then streaked on a YEPD plate, grown for three days at 30°C, and photographed. The top 4 streaks are DB20 (vector)transformants, and the bottom 4 streaks are p*ADH-CLN2* transformants. A similar result was also seen on SD-ura plate. All the transformants produced colonies of similar size on Sgal-ura plates.

A


100
80
60
40
20
0
% unbudded
0
100
200
300
mins. after shift to glucose
vector#1
vector#2
ADH-CLN2#1
ADH-CLN2#2

B


vector
ADH-CLN2

We tested if α-factor acts to inhibit primarily transcription of *CLN2* by seeing if p*ADH-CLN2* affected α–factor arrest. Wildtype cells transformed with the p*ADH-CLN2* plasmid were grown in culture and treated with α-factor. Strikingly, >95% of the cells arrested as unbudded shmoos and stayed arrested for 24 hours (Figure 15; Panel A). This observation was repeated in multiple transformants in two different strain backgrounds. Cells transformed with a *GAL-CLN2* plasmid also exhibit a similar behavior (C. Wittenberg, personal communication). Thus, α-factor can inhibit *CLN2* even when *CLN2* is expressed from a heterologous promoter. This observation suggests that α-factor may not inhibit CLN2 solely by transcriptional control.

When the cells carrying p*ADH-CLN2* were tested for α-factor arrest by halo assay on a plate, the cells did not form a clear halo, but formed colonies around the source of α-factor after an incubation of 2 days (Figure 15; Panel B). When examined by confrontation assay, all the cells initially arrested, and four hours later, a small fraction (10%) of the cells resumed dividing and formed minicolonies. Thus, p*ADH-CLN2* promotes recovery from α-factor at least in a fraction of the cells. However, the same cells treated with α-factor in liquid culture did not exhibit any recovery for up to 24 hours (Figure 15;Panel A). The difference in recovery observed for these two conditions is not understood.

We next assayed levels of CLN2 mRNA in cells carrying p*ADH-CLN2* that are arrested by α-factor. Primer extensions (Figure 16) show that the CLN2 transcripts from the *pADH-CLN2* construct was not affected by α-factor. In the same cells, the transcript from the *CLN2* promoter was inhibited in response to α-factor. These data indicate that cells can exhibit cell-cycle arrest even in the presence of large quantities of *CLN2* mRNA.

The behavior of cells which carry the p*ADH-CLN2* construct demonstrates that although α-factor may act to inhibit *CLN2* mRNA levels in response to α-factor, this inhibition is not necessary for cell-cycle arrest. Thus, the primary action of α–factor

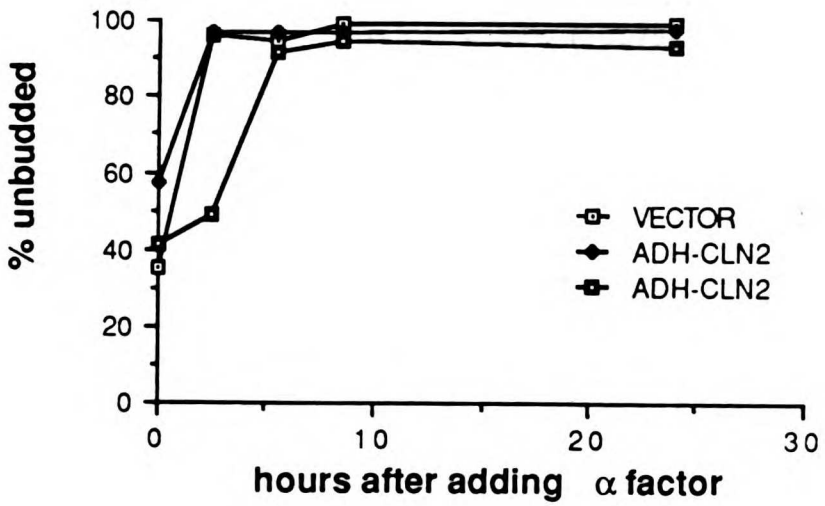
Figure 15: Cells carrying pADH-CLN2 exhibit a-factor arrest and recovery.

Panel A: Culture assay. DB20 (*URA3*$^+$ vector) and pADH-CLN2 (*URA3*$^+$)were transformed into FC279 (**a** *bar1::LEU2*). One transformant of DB20 and two independent transformants of pADH-CLN2 were grown in SD-ura at 30^o to exponential phase and then were treated with 1 uM α-factor. Aliquots were taken at indicated times after the addition of a-factor and counted for % unbudded cells by phase microscopy.

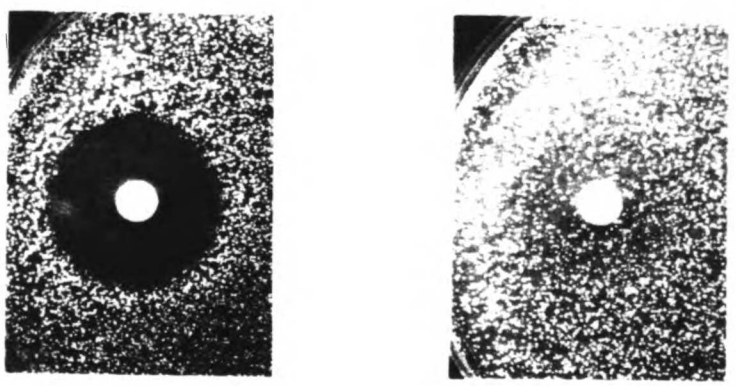
Panel B: Halo assays. Lawns of **a** strains were spread on SD-ura plates as a lawn, and paper disc containing 1 ug α-factor was placed on the lawn. The plates were incubated for 2 days at 30^o and photographed. In the left panel, the lawn is FC279 (**a** *bar1::LUE2*) transformed with pDB20. In the right panel, the lawn is FC279 transformed with pADH-CLN2.

A

ADH-CLN2 cells exhibit α-factor arrest



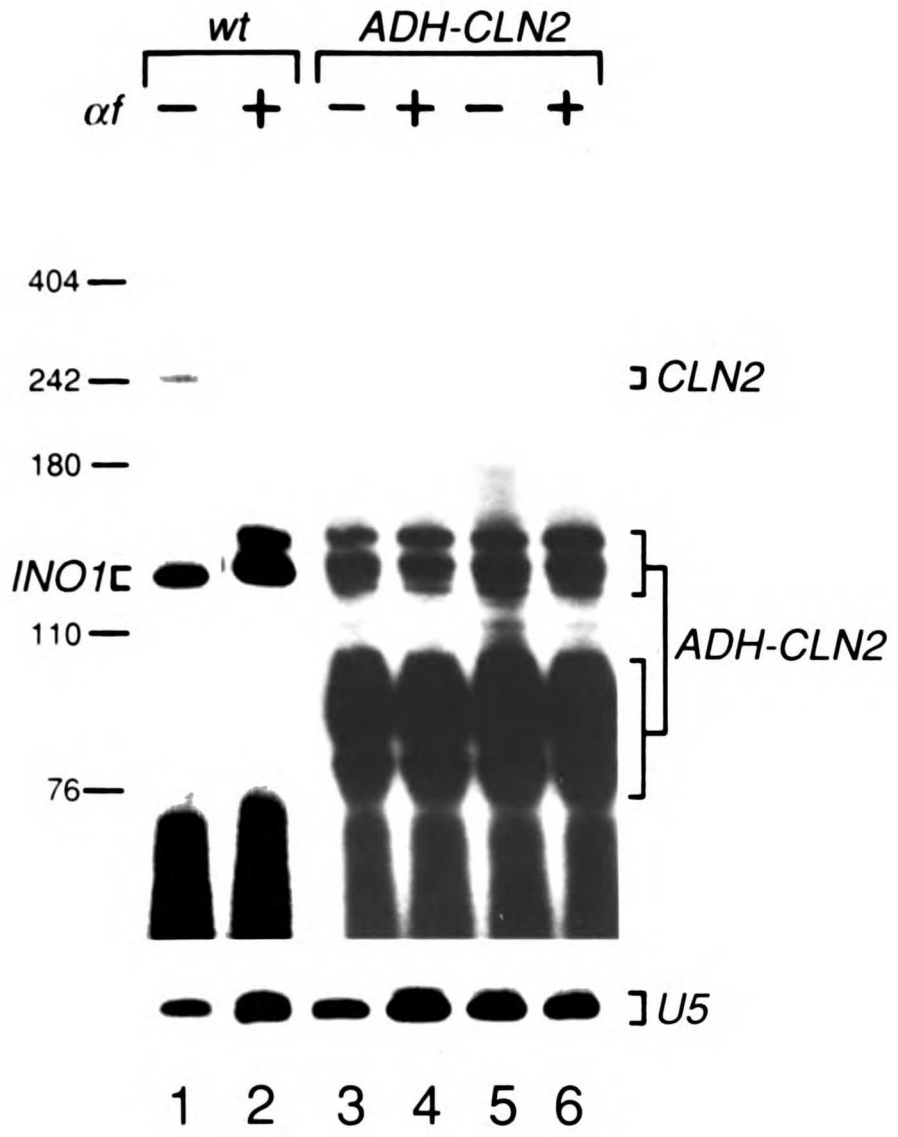
B



Vector

ADH-CLN2

Figure 16: Overexpression of *CLN2* mRNA in ADH-CLN2 cells which have been arrested with α-factor. Wild-type a cells (FC279) carrying a vector plasmid DB20 (lanes 1 and 2) or p*ADH-CLN2* (lanes 3 and 4, transformant 1; lanes 5 and 6, transformant 2) were grown in SD -trp and treated with 10^{-6}M α-factor for three hours. Cells carrying DB20 and p*ADH-CLN2* were arrested as unbudded shmoos. Total RNA was extracted and assayed by primer extension using oligonucleotide probes recognizing *CLN2*. *INO1* (upper panel) and *U5* (lower panel - separate primer extension reactions) oligonucleotide probes were also included as controls for the total amount of RNA loaded. The *INO1* probe did not efficiently primer extend in the *ADH-CLN2* strains for unknown reasons.


wt
ADH-CLN2
αf
− + − + − +
404
242
180
110
76
INO1
CLN2
ADH-CLN2
U5
1 2 3 4 5 6

and *FAR1* in inhibiting *CLN2* must be to affect some aspect of CLN2 protein, such as translation, protein stability, or activity.

DISCUSSION

Arrest of the Cell-cycle by Inhibition of G1 Cyclins

The three CLN products of yeast are functionally redundant--mutants arrest in G1 only if all three *CLN* genes are inactivated (Richardson et al., 1989). Thus it appears that any of the three CLN products is capable of driving the cell-cycle (Figure 17, panel A), presumably by activating CDC28 protein kinase (although this has not been experimentally demonstrated). If α-factor causes cell-cycle arrest by inhibiting the G1 cyclins, then presumably all three must be inhibited for cell-cycle arrest.

Our studies provide strong evidence that the α-factor causes cell-cycle arrest primarily by inhibiting the G1 cyclins. We have observed that FAR1 is largely dispensable for arrest when the CLN2 product is eliminated by mutation: *far1⁻ cln2⁻* mutants arrest in response to α-factor (Figure 17, panel B). It is this observation that indicates that the primary function of *FAR1* in cell-cycle arrest is to antagonize *CLN2*. The ability of *far1⁻* mutants to grow in the presence of α-factor is thus interpreted as resulting from the persistence of *CLN2*, which is sufficient to promote cell-cycle progression (Figure 17, panel C). The functional redundancy of the *CLN* products predict that other factors must regulate *CLN1* and *CLN3*. Thus, although the *CLN* genes perform similar functions, they are regulated by possibly three different regulators in response to α-factor.

The *FUS3* product is a strong candidate to be the factor that antagonizes *CLN3* (Figure 17) The *FUS3* gene was originally identified because *fus3* mutants are defective in cell fusion. They were subsequently found to be defective in cell-cycle arrest in response to mating factors (Elion et al., 1990). Different mutant alleles of *FUS3* exhibit

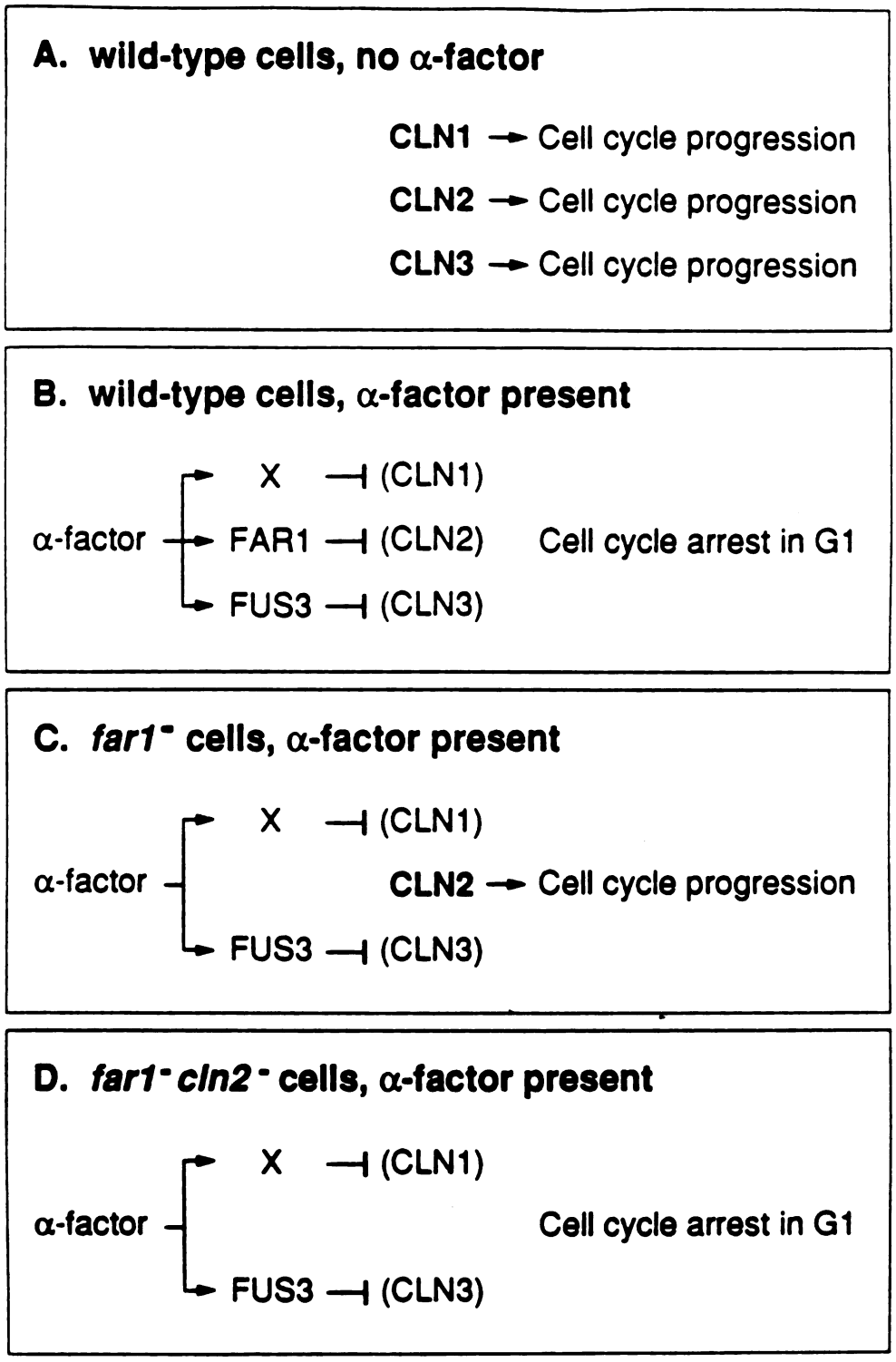


Figure 17: Trident regulation of cell cycle arrest by α-factor.

The panels depict an explanation for how α-factor affects a cells of different genotypes, either causing cell-cycle arrest or allowing cell-cycle progression. This hypothesis explains how the inactivation of the *CLN2* gene bypasses the requirement for *FAR1* for cell-cycle arrest induced by α-factor. Arrows indicate activation or stimulation; blunt arrowheads indicate repression or inhibition. Further explanation is provided in the text.

differences in their effect on transcriptional responses: deletion mutants of *FUS3* are uninducible for *FUS1* whereas as other mutations in *FUS3* (for example, *fus3-1*) allow near normal induction of *FUS1*. The *fus3-1* mutants exhibit a striking behavior: they arrest in response to α-factor if the *CLN3* gene is deleted. It thus appears that *fus3-1* mutants are able to inactivate *CLN1* and *CLN2* but not *CLN3*. These observations indicate that the FUS3 product has at least two functions: to activate transcription, possibly by acting on STE12, and to inactivate CLN3 (Elion et al., 1990). Since the nucleotide sequence of FUS3 predicts that it is a protein kinase, it may inhibit CLN3 activity by phosphorylation.

We predict that there is an additional product (denoted as *X* in Figure 17) that is specifically responsible for inhibition of *CLN1*. *X*⁻ mutants could be obtained among α-factor-resistant (*far*-like) mutants in strains lacking *CLN2* and *CLN3*.

Multiple Ways to Turn off CLN2.

In order to begin determining the molecular mechanisms of how *FAR1* inhibits *CLN2*, we examined the effect of α-factor and *FAR1* on *CLN2* mRNA. A key result is that cells carrying an p*ADH-CLN2* construct exhibit cell-cycle arrest in response to α-factor even when *CLN2* mRNA is overexpressed. This observation suggests that the primary effect of FAR1 to inhibit some aspect of the CLN2 protein, either its translation, stability, or activity.

Wittenberg et al. (1990) have demonstrated that both *CLN2* mRNA and protein levels decrease in response to α-factor. The decrease in mRNA is probably due to inhibition of transcription, since substitution of the upstream region of CLN2 for a constitutive pADH promoter results in constitutive expression of the transcript. We have found that *CLN2* mRNA does not decrease in response to α-factor in a *far1* strain. However, C. Wittenberg (personal communication)has recently shown that *FAR1* is not necessary for the decrease in the *CLN2* transcript if cells are arrested at G1. The *CLN2* transcript is

present in cells arrested at a *cdc28* block (as well as in a *cln1⁻ cln2⁻ cln3⁻* block) but decreases when the *cdc28* -arrested cells are treated with α-factor (C. Wittenberg, F. Cross, personal communications). This decrease in *CLN2* mRNA to α-factor is also observed in a *far1 cdc28* mutant, showing that in this case, *FAR1* is not necessary for the inhibition of the *CLN2* transcript. These observations suggest that the inhibition of *CLN2* transcription is very complex: the inhibition appears to require both cell-cycle arrest and an additional signal from α-factor, which is not dependent on *FAR1*. Given the requirement for cell-cycle arrest, the persistence of *CLN2* mRNA in *far1⁻* cells may be a consequence of the fact that *far1⁻* mutants do not arrest.

One model for the complex regulation of *CLN2* transcription is that α-factor might drives cells into alternate cell-cycle state: a Gmating state, which is analogous to a G0 state (see Pardee, 1989). This putative Gmating state would be distinct from a cdc28 block, but entry into the Gmating state would require first an arrest in G1.

In summary, α-factor and *FAR1* act primarily to inhibit some aspect of the CLN2 protein-- working either to inhibit its synthesis or activity or to stimulate its destruction. After CLN2 is inactivated and as cells arrest in G1, regulators in addition to FAR1 inhibit transcription of *CLN2*, which might insure that the cell remains arrested during the mating process. Examination of CLN2 protein in cells carrying the *pADH-CLN2* construct, where the protein can be assayed independently of changes in mRNA, should further direct us in determining how *FAR1* functions.

EXPERIMENTAL PROCEDURES

Strains and plasmids: The *far1⁻ cln⁻* strains used in Figures A and B were constructed by introduction of *far1:URA3* and *bar1::LEU2* mutations into the BF264-15D background (Richardson et al., 1989) by gene replacement (Rothstein, 1983) and by crosses from the cln⁻ mutant strains which were kindly provided by H. Richardson. YPL1 (**a** *cln1:TRP1 cln3Δ cln2::GAL-CLN2 - LEU2*), which was gift from P. Leopold, has most of the chromosomal CLN2 gene replaced by a construct containing a GAL-CLN2 fusion and a LEU2 gene (P. Leopold, unpublished). The plasmids pDB20 (ADH vector reference?) and pADH-CLN2 were also generously provided by P. Leopold.

Primer extensions: Primer extension reactions (McKnight and Kingsbury, 1982). were performed using total RNA extracted from cells (Jensen et al., 1983) and AMV reverse transcriptase (Promega). The sequnce of the oligonucleotide (synthesized by Operon) used to probe CLN2 mRNA is CTCAATCGGATAGTAGTCCGG, which hybridizes to CLN2 50 bp downstream of the putative start AUG . The sequence of the oligonucleotide used to probe INO mRNA is GCTGTCTTCGTAACTACAGC, which hybridizes at 111bp downstream from start of the mRNA. Labelled U5 probe was obtained from Jim Umen. The oligonucleotides were endlabelled with ^{32}P as described in Ausubel et al., 1989.

CHAPTER 3

REGULATION OF FAR1 ACTIVITY BY α-FACTOR

CHAPTER 3

REGULATION OF FAR1 ACTIVITY BY α-FACTOR

INTRODUCTION

Observations described in the previous chapters have led to the model that FAR1 is a protein necessary for the inhibition of *CLN2* in response to α-factor. Since *CLN2* is active in the absence of pheromone (Richardson et al., 1990), one attractive model is that FAR1 is inactive in the absence of α-factor and is activated by α–factor to inhibit *CLN2.* This section will detail experiments concerned with how the α-factor response pathway might affect FAR1.

One way that α-factor regulates FAR1 is by inducing the transcription of *FAR1.* As shown in Chapter 1, *FAR1* mRNA is present in low levels in the absence of α-factor and is induced 5-fold in the presence of α-factor. Both the basal and induced levels are dependent on components of the response pathway, such as *STE4* and *STE12.* One possibility is that the transcriptional induction of *FAR1* is sufficient for inhibition of *CLN2.* I show in this section that the transcriptional induction is not sufficient. Furthermore, I find that FAR1 is phosphorylated in response to α-factor. These observations suggest the model that α-factor may activate FAR1 by inducing its synthesis and by phosphorylation.

RESULTS

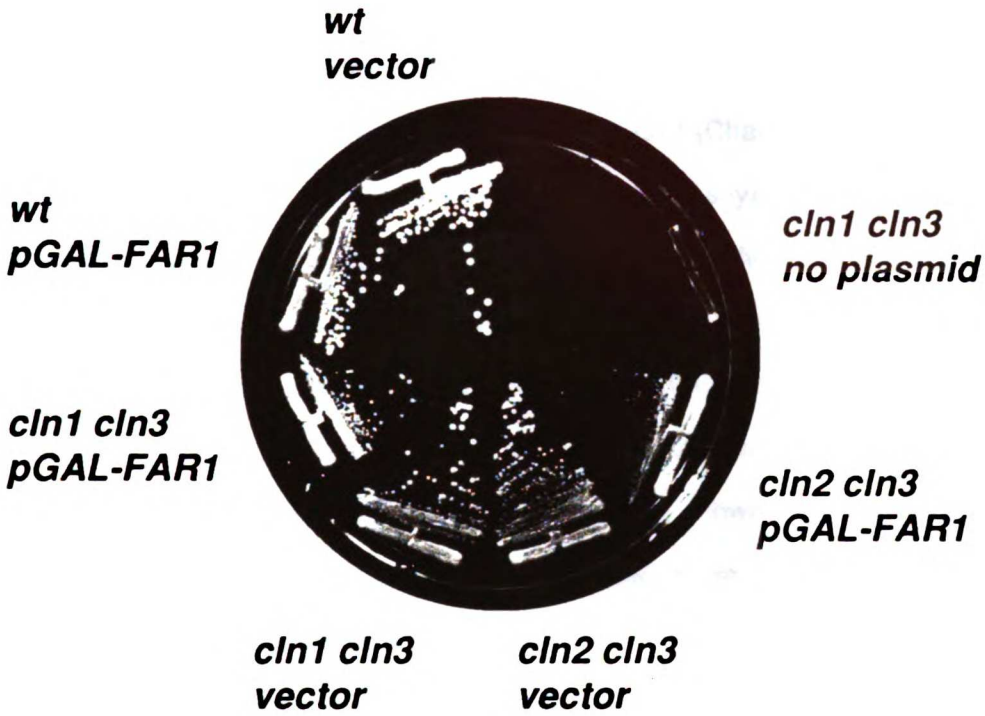
Effect of FAR1 Overexpression.

I wanted to test if the transcriptional induction of *FAR1* was sufficient for its activation. If this were true, then high levels of FAR1 expression under the control of a strong heterologous promoter might inhibit CLN2 activity in the absence of α–factor. The *FAR1* open reading frame was fused to the *GAL1* promoter on a centromere-based plasmid (pFC24; see Experimental Procedures for details). This construct was shown to contain a functional *FAR1* gene which was inducible by galactose by demonstrating that it fully complemented a *far1::URA3* mutation for α-factor arrest and mating ability at galactose media and not on glucose media (data not shown). Immunoblots (Figure 20; lanes 3 and 4) show that the *GAL-FAR1* construct expresses FAR1 protein at higher levels than the α-factor-induced levels of FAR1 in wild-type cells. (However, FAR1 expressed from the *GAL-FAR1* construct appears to run as heterologous smear of bands which run at predominantly slightly faster mobilities than the native FAR1 protein on SDS PAGE (Figure 20; lane 1). The faster mobility suggests that that FAR1 from the *GAL-FAR1* construct might be truncated, perhaps by proteolysis. However, as shown by complementation, the protein expressed from the *GAL-FAR1* construct is functional in the presence of α-factor,).

Overexpression of FAR1 had very little effect on the growth of wild-type cells, as assayed by colony size (Figure 18), growth curves, and recovery from starvation (data not shown). Mating frequency, arrest to α-factor, shmoo formation, and budding pattern were not affected (data not shown). In particular, cells expressing *GAL-FAR1* were not supersensitive to α-factor for arrest or for *FUS1-lacZ* induction, as assayed by dose response curves in culture (data not shown). The only phenotype found for cells expressing *GAL-FAR1* is that they

Figure 18: Overexpression of *FAR1* does not inhibit CLN2 in the absence of α-factor.

Strains of the indicated genotype were transformed with either pFC24 (*GAL-FAR1*; *TRP1*) or pRS129 (*GAL* vector; *TRP1*). The transformants were grown on SD-trp plates, and then streaked onto Sgal-trp plates, incubated for 4 days at 30°, and photographed. Strains starting from the top left and listing counterclockwise are: FC385 (wild-type , pRS129); FC386 (wild-type, pFC24); FC387 (*cln1:TRP1 cln3:URA3*, pFC24); FC388 (*cln1:TRP1 cln3:URA3*, pRS129); FC389 (*cln2:LEU2 cln3:URA3*, pFC24); FC390 (*cln2:LEU2 cln3:URA3*, pRS129); FC294 (*cln1:TRP1 cln3:URA3*) with no *TRP1* plasmid. The size of colonies correlate with the growth rate of the cells on galactose-containing media.


wt
vector
wt
pGAL-FAR1
cln1 cln3
no plasmid
cln1 cln3
pGAL-FAR1
cln2 cln3
pGAL-FAR1
cln1 cln3
vector
cln2 cln3
vector

were slightly larger and more elongated than cells carrying the vector alone. The significance of this altered morphology is not known.

Since FAR1 is thought to affect primarily CLN2 , we did not expect that FAR1 would have significant cell cycle effects in a wild-type cell, since the other two CLN products can functionally substitute for an inactive CLN2 (Chang and Herskowitz, 1990; Richardson et al., 1990). Therefore, we assayed the effect of *GAL-FAR1* on CLN2 in a strain deleted for *CLN1* and *CLN3*. Inactivation of *CLN2* in this strain causes the cells to arrest in G1 and inhibits colony formation (Richardson et al., 1990; see Figure 14 - Chapter 2) We found that *cln1⁻cln3⁻* cells overexpressing FAR1 do not exhibit cell cycle arrest, as shown by the ability to form colonies (Figure 18) and divide in culture (data not shown). (The possibility of a transient arrest was difficult to test because the *cln1⁻cln3⁻* strain itself grows poorly on galactose media.) The continued division of *cln1⁻cln3⁻* cells carrying *GAL-FAR1* demonstrates that the overexpression of FAR1 is not sufficient for fully inhibiting CLN2. The *GAL-FAR1* construct however did cause the *cln1⁻cln3⁻* mutant cells to grow slightly slower when grown on galactose-containing minimal media, as shown by the formation of smaller colonies (Figure 18). Thus overexpression of *FAR1* may weakly inhibit the activity of CLN2. This weak effect on growth however was not specific to the inhibition of CLN2, since the expression of *GAL-FAR1* in a *cln2⁻cln3⁻* strain and a *cln1⁻ cln2⁻* also caused slower growth (Figure 18).

The inability of FAR1 overexpression to inhibit CLN2 in the absence of a-factor disproves the initial simple hypothesis that increased synthesis of FAR1 is sufficient to inhibit CLN2 and suggests two new models for how FAR1 might be activated: first, FAR1 may require post-translational modification for activation; second, FAR1 may work with other factors which require activation by α-factor in the inhibition of CLN2. These two models are not mutually exclusive.

Phosphorylation of FAR1

Consistent with the first model, we found that FAR1 does acquire post-translational modification in response to α-factor in the form of phosphorylation. Polyclonal antibodies were raised in rabbits against a TrpE-FAR1 fusion protein (see Experimental Procedures). Figure 3 shows an immunoblot of extracts from various *far* mutants probed with crude αFAR1 antisera. The antibodies recognized a major band of 97 Kd in wild-type yeast extracts (Figure 19; lane 5 and 6) which was absent in extracts from *far1::URA3* mutant strains (Figure 19; lanes 1 and 2) and is reduced and truncated in *far1-1* and in insertion mutants of *FAR1* (Figure 19; lanes 3,4, 7-12). Affinity purified antibody recognized predominantly the 97Kd band (Figures 20 and 23). This 97Kd band is thus the FAR1 protein. The molecular weight of 97Kd is consistent with the size of the *FAR1* open reading frame as predicted by the nucleotide sequence.

The FAR1 protein was detectable in wild-type cells in the absence of α-factor and upon exposure to α-factor, the amount of FAR1 increased (Figure 20: lanes 2 and 3; Figure 19 : lanes 5 and 6).

It is also observed that the mobility of the FAR1 protein changes and runs at an apparent higher molecular weight in cells exposed to α-factor. The shift in mobility was also seen in FAR1 expressed from the *GAL-FAR1* construct (Figure 20; lanes 4 and 5), demonstrating that the shift was not dependent on the FAR1 promoter and was a consequence of increased transcription of FAR1.

The shift in mobility was demonstrated to be due to phosphorylation by showing that treatment with alkaline phosphatase caused a reversal in the mobility shift seen with α-factor: FAR1 from α-factor-treated cells migrated at the same mobility after phosphatase treatment as the form found in cells in the absence of α-factor (Figure 21). The effect of the phosphatase was inhibited by a

Figure 19: Western blot showing the state of FAR1 protein in *far* mutant strains. Crude SDS extracts were separated on SDS-PAGE and immunoblotted using crude aFAR1 sera. Strains used: FC204 (far1::URA3), FC-D1 (*far1-1*), FC140 (FAR+), FC172 (*far1-c*), FC174 (*far1-g*), FC173 (*far1-e*), FC-G2 (*far2*). All strains are MATa (*bar1-1*) and isogenic to FC140. Cells in the even numbered lanes were treated with 1uM α–factor for 2 hr before extracted. The FAR1 protein appears as a 97kD band labelled as FAR1. The arrow labelled "far1-1" marks a band in lane 5 which is the putative product of the *far1-1* allele.

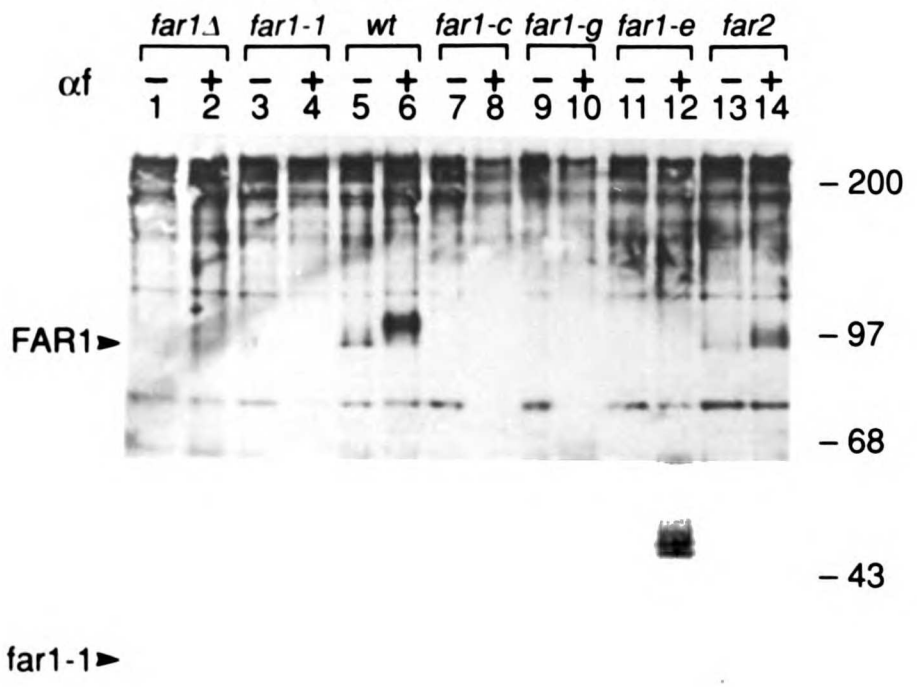

far1Δ
far1-1
wt
far1-c
far1-g
far1-e
far2
αf
– + – + – + – + – + – + – +
1 2 3 4 5 6 7 8 9 10 11 12 13 14
– 200
FAR1►
– 97
– 68
– 43
far1-1►

Figure 20: FAR1 protein in wild-type and *GAL-FAR1* strains in the absence and presence of α-factor. lane 1: FC280 (*far1::URA3*); lane 2 and 3, FC279 (*FAR*$^+$); lane 4 and 5 FC300 (*GAL-FAR1* in *far1::URA3*). Cells were grown S-gal with complete amino acids (lanes 1-3) or with lacking tryptophan (lanes 4-5) and were treated with 1uM α-factor for 2 hours (lanes 3 and 5). Crude SDS extracts were electrophoresed on SDS PAGE and immunoblotted using affinity purified αFAR1 sera.

αf
far1Δ
wt
GAL-FAR1
-
-
+
-
+
200
97
68
1
2
3
4
5

Figure 21: FAR1 is phosphorylated in response to α-factor. lane 1, FC280 (*far1::URA3*); lanes 2-6, FC279 (*FAR1*$^+$). Native extracts were prepared from cells which had been grown in exponential phase in YEPD and had been treated with 1μM α-factor for 2 hours (lanes 4-6). αFAR1 immunoprecipitates were treated with calf intestinal phosphatase (CIP; lanes 3 ,5,6) or mock treated (lanes 2,4). In lane 6, 10mM beta-glycerol phosphate (bgp) was added to the immunoprecipitate before addition of phosphatase. The immunoprecipitates were then boiled in SDS-Sample buffer, electrophoresed on SDS-PAGE, and visualized by immunoblotting with αFAR1 sera. The band in lane 2 comigrates with a 97Kd marker. See Experimental Procedures for details.

far1Δ
wt
wt + CIP
αf
αf + CIP
αf + CIP + βgp
1
2
3
4
5
6

Figure 22: *In vivo* ^{32}P labelling of FAR1.

lane 1, FC280 (*far1::URA3*); lane 2, FC300(*Gal-FAR1*); lane 3, FC300 treated with α-factor. FAR1 was immunoprecipitated from crude SDS extracts from ^{32}P *in vivo* labelled cultures, electrophoresed on SDS-PAGE, and exposed by autoradiography. See Experimental Procedures for details.

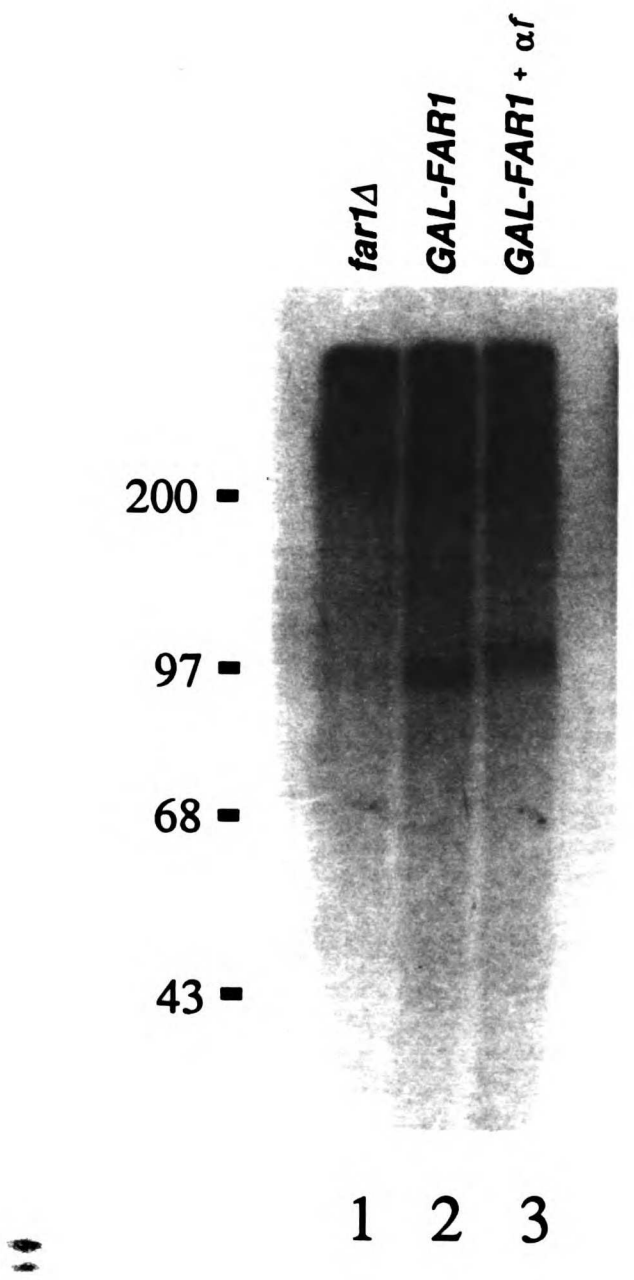

far1Δ
GAL-FAR1
GAL-FAR1 + αf
200
97
68
43
1
2
3

Figure 23: Time course of the effect of α-factor on FAR1 protein lane 1,FC280 (*far1::URA3*); lane 2-5, FC279(*FAR+*). Cells were treated with α-factor for different amounts of time, harvested on ice, and quickly extracted by SDS and boiling. Crude extracts were electrophoresed on SDS-PAGE and immunoblotted using affinity-purified αFAR1 antibody.


far1Δ
0
5'
15'
30'
1
2
3
4
5

phosphatase inhibitor, beta-glycerol phosphate, demonstrating that the effect of the phosphatase preparation was due to phosphatase activity and not due to contaminating proteases. These data demonstrate that α-factor causes FAR1 to be phosphorylated at sites which cause a change in mobility. The phosphatase had no effect on the mobility of FAR1 from cells without α-factor, suggesting that this form of FAR1 does not contain phosphorylations which alter its mobility.

We further confirmed that FAR1 is a phosphoprotein by *in vivo* ^{32}P labelling. αFAR1-immunoprecipitates of extracts from ^{32}P labelled cells contained one major ^{32}P-labelled protein which migrated at the same molecular weight as FAR1. This band was absent in extracts made from a *far1::URA3* mutant; it was present in wild-type (GAL-FAR1) cells; and it was shifted in α–factor-treated (GAL-FAR1) cells (Figure 22). Therefore this ^{32}P labelled band appears to be FAR1. Thus, FAR1 may be phosphorylated in the absence and acquire additional phosphorylations in the presence of α-factor, which are responsible for the shift in mobility. (This ^{32}P labelling experiment however has the caveat the FAR1 protein examined in this experiment is overexpressed, which may be phosphorylated differently from a non-overexpressed protein.)

A time course shows that the α-factor phosphorylations occur very rapidly in response to α-factor (Figure 23). Most of the protein displays a shift in mobility in extracts from cells which had been exposed to α-factor for only 5 minutes. At the 5 and 15 minute time points, an band of intermediate mobility is also seen. The multiple bands may indicate that FAR1 acquires phosphorylations on multiple sites in response to α-factor. Also, the increase in the amount of FAR1 protein is evident by 15 minutes.

CONCLUSION

FAR1 is affected by α-factor in at least two ways. First, transcription of *FAR1* is induced by α-factor approximately 4-5 fold. The transcriptional regulation of *FAR1* is probably mediated by STE12, which may bind at the *FAR1* promoter region at four putative PRE sequences (Chapter 1) Increased transcription of *FAR1*, however, is not sufficient for FAR1 to inhibit CLN2, since overexpression of FAR1 by a *GAL-FAR1* construct does not cause cell-cycle arrest in a *cln1⁻cln3⁻* cell in the absence of α-factor. Thus, FAR1 may require post-translational modifications necessary for its activation. Another possibility is that FAR1 may work with other factors which are dependent on α-factor for their activity.

We have found that FAR1 is phosphorylated in the absence of α–factor and receives additional phosphorylation(s) in response to α-factor. Although there is no proof that the α-factor phosphorylation is necessary for FAR1 activity, the finding that overexpression of FAR1 is not sufficient suggests that such a modification may be required. Proof will await the mapping and mutating of the phosphorylation site(s).

FAR1 is one of a group of proteins which have been found to be phosphorylated in response to α-factor. These proteins include STE2, STE4, STE12, glycogen synthase, and FUS1 (Reneke et al., 1988; Cole 1991?; Fields 1991?, Francois et al., 1991; Trueheart, personal communication). For STE2 and STE4, phosphorylation is thought to be involved in desensitization and thus may dampen the activity of these proteins (Reneke et al., 1988; Cole et al., 1991). It is unlikely that the phosphorylation of FAR1 mediates desensitization of FAR1 activity, since all detectable FAR1 is phosphorylated during the period when it is expected to be active. FUS1 is phosphorylated by α-factor; however, a mutation at the phosphorylation site does not affect FUS1 activity (J. Trueheart, personal communication). The phosphorylation of STE12, as with FAR1, is correlated with its activation (Fields personal communication).

Four protein kinases in the α-factor response pathway are candidates for the kinase which phosphorylates FAR1: STE7, STE11, and FUS3 (Teague et al., 1986; Rhodes et al., 1990; Elion et al., 1990). Identifying which kinase, if any of these, is responsible for phosphorylating FAR1 is difficult using genetic techniques. One preliminary result is that FAR1 shifts to only an intermediate level in a *far2* strain (an allele of *FUS3*)(Figure 3 Lanes 13 & 14). Although this change in mobility is consistent with FUS3 being responsible for a subset of the phosphorylations, this change may be an indirect effect of a leaky allele. One experimental difficulty is that null mutations in these kinase genes affect expression of other components in the pathway. Thus finding that FAR1 is not phosphorylated in a *ste7* mutant does not mean that STE7 acts directly on FAR1, but may reflect only the fact that the receptor *STE2* is not expressed in a *ste7* mutant. The kinase will be most readily identified by biochemical techniques. For instance, Rhodes et al. 1990 have shown that immunoprecipitations of STE11 contain active kinase activity, which can be tested for the ability to phosphorylate FAR1 *in vitro*.

The phosphorylation of FAR1 will be very useful as a tool to study the signal transduction pathway. The phosphorylation event assays earlier (or different) components in the pathway than *FUS1-lacZ*, and thus may be useful in distinguishing the early steps in the pathway from the later ones. It also may be useful in the development of an *in vitro* system to study this signal transduction pathway.

EXPERIMENTAL PROCUDURES

Strains and Media

All strains used were isogenic to 15Dau strain background (Richardson et al., 1989) and include FC279 (wt) FC280 (far1::URA3). α-factor was obtained from Sigma. Phosphate-free media. In the induction of GAL-FAR1 strains, cells were grown in culture overnight (> 12 hrs) in Sgal-trp media (containing 2% galactose). Sgal plates contained 2% galactose and 0.1% sucrose.

Plasmid construction of the GAL-FAR1.

pFC24 (GAL-FAR1) contains a 3.2 kb genomic DNA fragment containing the entire FAR1 open reading frame and 3' regions fused to the *GAL1* promoter on a centromere containing plasmid containing TRP1. A PCR fragment (0.8kb) containing the 5' part of FAR1 was created using an oligonucleotide (CGCTCGAGCAACAGATGCCCAC) which contains an engineered XhoI site and a fragment containing 8 bp upstream of the start AUG (underlined)of the FAR1 open reading frame and an oligonucleotide encoding a sequence internal to FAR1 at the Hind3 site (CCTGTGAAGCTTCTCGCCG) This PCR fragment was ligated along with a 2.8 kb Hind3 -Bgl2 fragment, which contained the rest of the FAR1 gene, into the polylinker XhoI-BamH1 sites of RS129 (constructed by R. Sikorski; gift from J. Li) in a directed triple ligation.

αFAR1 antibody

Rabbit antibodies were raised against a TrpE-FAR1 fusion protein. pFC27 (trpE-FAR1) was constructed by ligating a XhoI-Pvu2 fragment of pFC24, which contains the full length *FAR1* open reading frame in pATH 23 (gift of T.J. Koerner and A. Tzagaloff). The fusion protein was expressed in *E. coli* and extracted as described in Andrews and Herskowitz, 1989. The extracts were run on SDS-PAGE (8% acrylamide). A major 97kD degradation product of the 120 Kd fusion protein was isolated by excision from the gel, and either eluted protein or protein embedded in gel were used to immunize rabbits (BabCo, Inc., Berkeley, California). 3 mls of αFAR1 sera (3rd bleed) was affinity

purified (Craig Peterson, personal communication) by incubating the sera with a nitrocellulose strip containing a 90Kd glutathione transferase-FAR1 fusion protein (expressed from pFC28). The antibody bound to the strip was then eluted and concentrated to 0.3 ml.

Preparation of yeast extracts

Crude SDS extracts were prepared by growing 10 ml of cells to OD_{600} of 0.6-1.0. Cells were harvested, washed once in water, and resuspended in 100ul SDS sample buffer (100mM Tris pH6.8, 4% SDS, 10% glycerol, 20% beta-mecaptoethanol). The suspension was vortexed twice with glass beads for 30 sec., boiled for 5 min., incubated on ice for 5 min., vortexed for 10 sec., and centrifuged by microfuge for 5 min.. For Western blotting, 10-20 µl of the supernatants were loaded.

Native extracts were prepared by a mortar and pestle method (Sorger et al., 1987). 300 mls of cells were grown to an approximate OD_{600} 1.0. Cells were washed in water and then washed once and resuspended in 2 mls of Buffer D (50mM Tris pH 8.0, 20% glycerol, 50mM NaCl, protease inhibitors (.2 mM PMSF, 1 ug/ml leupeptin, 1 ug/ml pepstatin,.3 mg/ml benzamidine) and phosphatase inhibitors (5 mM EDTA, 50 mM NaF, 50 mM b-glycerol phosphate, 1 mM Na vanadate)(Andrews and Herskowitz, 1989)). The cell suspension was frozen by dripping into liquid N_2. The pellets were ground to a fine powder while frozen using a mortar and pestle. The powder was thawed, and the supernatant was collected following a 5 min microfuge centrifugation. The extracts were typically 5-7 mgs/ml, as determined by BioRad assay.

Western blots

Proteins were electrophoresed by SDS-PAGE (8% acrylamide, 0.21% bis-acrylamide) and were transferred from gel to nitrocellulose by electroblotting. Immunoblots were performed using the ECL Western blotting detection system

(Amersham) as recommended . For the primary antibody coupling , either crude αFAR1 rabbit sera (2nd bleed; 1/1000 dilution) or affinity purified sera (1/200 dilution) were used. Donkey α rabbit Ig-peroxidase (Amersham) was used as a secondary antibody at 1/1000 dilution.

Phosphatase treatment of FAR1

Prior to phosphatase treatment, FAR1 was immunoprecipitated in order to separate FAR1 away from endogenous yeast phosphatases present in the extracts. 20 ul of native yeast extract and 10 ul of affinity purified aFAR1 antibody in 200ul RIPA buffer (50 mM Tris pH8, .1% SDS, .5% deoxycholate, 150 mM NaCl, 1% Triton X-100, and protease and phosphatase inhibitors, as listed above) were incubated on ice for 90 min. and then were incubated with protein A-coupled Sepharose beads (Sigma) for 45-60 mins. on a nutator at 4°. The beads were washed 3x with RIPA, 2x with phosphatase buffer (50 mM Tris pH8.0 ,10% glycerol, protease inhibitors) and resuspended in 20 ul of phosphatase buffer. The immunoprecipitates were incubated at 37° for 30 minutes with 5 ul (25 units/ul) molecular biology grade calf intestinal phosphatase (Boeringer Mannheim) or mock treated with 5 ul of phosphatase buffer.. The protein was released from the beads by boiling in SDS sample buffer, electrophoresed on SDS-PAGE, and visulalized by Western blotting using crude αFAR1 sera.

***In vivo* ^{32}P labelling**

10 ml of cells were grown to OD_{600} 0.5-1.0 in Sgal media. Cells were washed 2x in phosphate-free media, incubated for 30 minutes at 37°, and washed 2x more in phosphate-free media. 0.5 mCi of ^{32}P phosphorus was added to the culture and incubated at 30° for 70 min. α-factor (1 uM) was added to one of the tubes 10 min after addition of the label. Following labelling, cells were washed 2x in PBS and subjected to disruption with glass beads and boiling as in the crude SDS extract, except

the cells were lysed in 100 ul of SDS lysis buffer (2% SDS, 100 mM Tris 7.5, 20% glycerol, and 1% β-mercaptoethanol, and protease and phosphatase inhibitors.). 20 ul of the extract was immunoprecipitated with affinity purified αFAR1 sera. Cold 50 mM Na phosphate was added to both the beads and the RIPA mix before the beads were added to reduce background of unincorporated label. The beads were washed with 2x with 1 ml RIPA and 1x Buffer A (2 M urea, 50 mM NaCl, 50 mM Tris, 0.1% deoxycholate, 0.1% Triton X100). Samples were boiled in SDS sample buffer and analyzed by electrophoresis on SDS-PAGE followed by autoradiography.

CHAPTER 4

THE ROLE OF *FAR1* IN MORPHOGENESIS DURING THE MATING PROCESS.

CHAPTER 4

THE ROLE OF *FAR1* IN MORPHOGENIC EVENTS IN THE MATING PROCESS

INTRODUCTION

Polarity in yeast is regulated both by intrinsic and extrinsic factors. The intrinsic program is reflected in the fact that yeast cells direct the sites for budding in a defined pattern: haploid α or a cells always bud next to site of the last bud -- this is termed " axial budding"; diploid a/α cells bud in a different pattern, forming buds at one of two ends of the cell -- this is termed " bipolar budding" Which of these two patterns the cell chooses is determined by the mating type locus, and is not dependent on ploidy or cues in its environment (Freifelder, 1960; see also Chant and Herskowitz, 1991).

Yeast cells also respond to extrinsic factors, which can override the intrinsic program of budding. When haploid cells of opposite mating type encounter each other, they begin a complex series of morphogenic events culminating in the fusion of the two cell membranes and fusion of the nuclei, forming the diploid zygote. In the early events of mating, the cells polarize toward each other. Actin dots form at the junction between the two cells, the spindle pole body turns to face the junction and emanates extranuclear microtubules toward the junction, and cell growth and secretion are directed toward the junction (Byer and Goetsch, 1975; Hasek et al., 1987; Field and Schekman, 1980) . Since cells can mate efficiently with each other regardless of where on the cell surface their partner touches them (see Jackson and Hartwell, 1989; Streiblova, 1970), it is likely that signals from the partner direct this polarization. In this process, the normal intrinsic pattern of axial budding is not followed. We have termed the change in the

direction of polarity from the axial position to a new site towards the mating partner "reorientation."

There is some evidence that the primary signal used to polarize cells towards each other are the mating factors, **a**-factor and α-factor. When purified α-factor is added to **a** cells, the cells form a shmoo, a polarized cell with actin, microtubules, and cell growth directed towards the shmoo tip . Rine (1980) has reported that **a** cells tend to bud or shmoo towards α cells placed at a distance; however, this phenomenon has been very difficult to reproduce (FC and J. Chenevert, unpublished observations), possibly because a sharp gradient of α-factor necessary for directed polarization of the **a** cell is difficult to establish if the **a** and α cells are not touching. I have found that uniform fields of a-factor can disrupt the axial budding pattern of a cells and cause budding (at low concentrations) and shmoo (at high concentrations) in random directions (see below; also L. Marsh, unpublished observations). Additional evidence showing that α-factor is involved in polarity is that mutants defective in the mating factor response pathway (a *ste4* mutant, for instance) continue normal axial budding in the presence of α-factor or potential mating partners (FC, unpublished observations). Jackson and Hartwell (1989; 1990) have shown that the pheromones are important how cells choose which cell to mate to. In a competition mating assay (described below), cells choose their mating partner by the cell secreting the most pheromone.

Genes governing polarity which may be involved in this polarization include the *CDC24*-class of genes and the *BUD* genes. The *CDC24*-class of genes include *CDC24*, *CDC42*, and *MYO2* (Sloat et al., 1981; Johnson and Pringle, 1990; Johnston et al., 1991). Mutations in these genes cause the cells to lack polarity, so that they exhibit delocalized actin distributions and delocalized cell growth, and grow as large balls. The *BUD* genes were identified as genes which affect bud site selection, so that haploid cells carrying a mutation in a *BUD* gene bud in a random pattern or a bipolar pattern but do not show gross defects in polarization per se (Chant et al., 1991; Chant and Herskowitz,

1991). Characterization of these genes has led to the hypothesis that the *BUD* gene products help position a complex of proteins, which include the *CDC24*-class of proteins, at a proper site on at the plasma membrane, and this complex directs the polarity of the cell at that site, in part by interactions with the actin cytoskeleton. We predict that a subset of these gene products will also function in polarization seen in mating and shmooing, and thus respond to the mating factor response pathway. For instance, *CDC24* has been found to be necessary for mating (Reid and Hartwell, 1977) and shmoo formation (FC, unpublished observations).

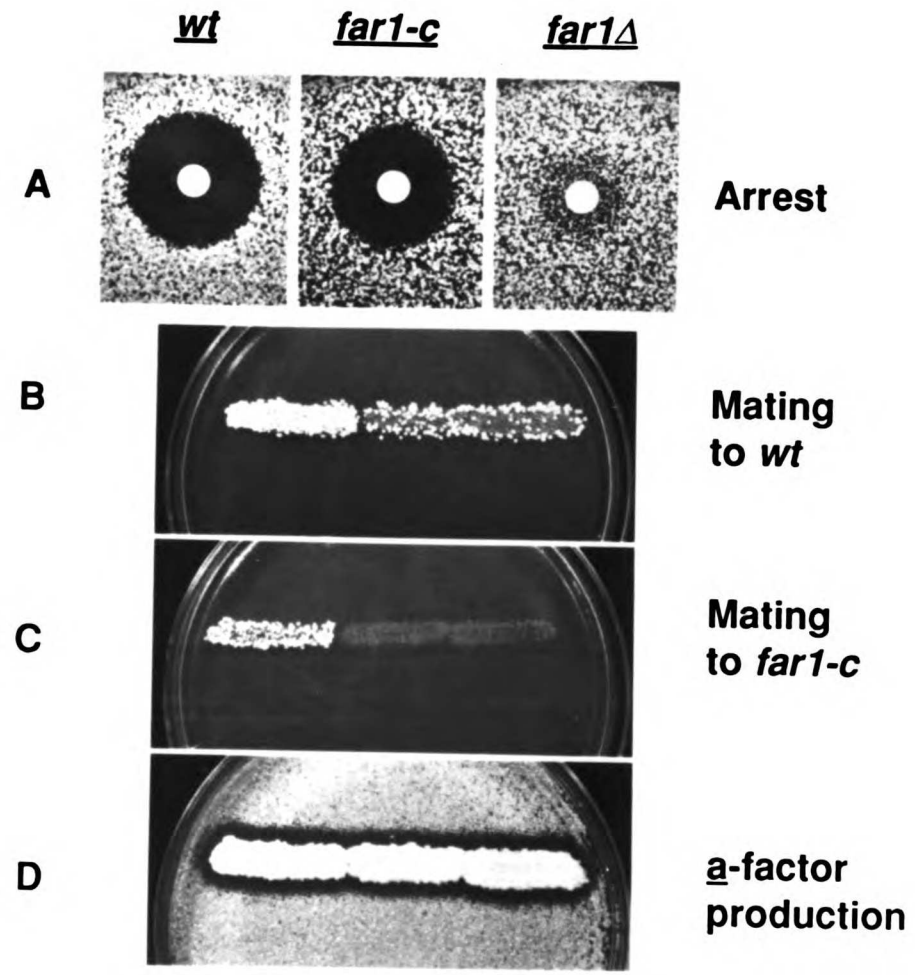
In this chapter, I describe evidence that *FAR1* has two separate functions: one in cell cycle arrest, as previously described, and a second function in this polarization during mating. Thus *FAR1* may play a role in regulating gene products dictating cell polarity in response to mating factors. *FAR1* is the first gene described affecting this step in mating, and thus helps to define and dissect events during this step.

RESULTS

FAR1 has an additional function in mating

Three genetic observations indicate that *FAR1* plays a role in mating in addition to its role in arresting the cell cycle. First, introduction of the *FAR1* plasmid pFC1, which may lack upstream regulatory sequences and may underexpress FAR1, complemented only the mating defect in a *far1-1* mutant, producing an Arrest- Mating+ phenotype. Second, the inactivation of *CLN2* and *CLN3* restored arrest to a *far1* strain, it did not restore mating, producing an Arrest$^+$ Mating$^-$ phenotype. Third, although the *far1::URA3* deletion has an Arrest$^-$ Mating$^-$ phenotype, certain *Tn3-URA3* insertion mutants (carrying insertions c,e,g, and h; see Figure) give an Arrest$^+$ Mating$^-$ phenotype (Figure). These mutants possesed the mating defect similar to *far1::URA3* but arrested and formed normal shmoos in the presence of α-factor. These insertions all

Figure 24: Arrest, mating, and production of mating factor in *FAR+*, *far1-c*, and *far1::URA3* cells. The strains were as follows: **a** *wt* (FC140) , **a** *far1-c* (FC172), a *far1::URA3* (FC204). **Panel A** shows a halo assay for α-factor arrest. Lawns of the indicated **a** strains were spread on YEPD, and a disc containing 1 ug α-factor was placed on the plate. The zone of inhibition indicates that growth of cells in the lawn have by arrested by α-factor. **Panel B** shows a assay for mating efficiency of the indicated **a** strains to an α wildtype tester strain (IH1793). Patches of the **a** strains were replica-printed onto a YEPD plate containing a lawn of the α strain. The plate was incubated for 7 hours and then replica plated to a SD plate. Growth of the diploid prototrophs on the SD plate assayed after 2 days incubation at 30° indicates successful mating. **Panel C** shows an assay for the mating efficiency of the indicated **a** strains to an α *far1-c* mutant, using a similar procedure described for Panel B, except an α *far1-c* strain was spread as the lawn. **Panel D** shows an assay for secretion of **a**-factor by the a cells. Patches of the indicated **a** cells were replica printed to a YEPD plate which contained a lawn of α *sst2* (IH993), which exhibit growth inhibition to **a**-factor. The plates were incubated at 30° for 2 days.


wt
far1-c
far1Δ
A
Arrest
B
Mating
to wt
C
Mating
to far1-c
D
a-factor
production

produce C-terminal truncations and allow expression of at least the N-terminal 400 amino acids of the FAR1 product. These observations suggest that the amino terminal segment of FAR1 may be sufficient for cell-cycle arrest and that the carboxy-terminal segment may be necessary for some other step in mating.

***far1-c* mutants have a defect in forming zygotes.**

In order to characterize this additional function of *FAR1*, we studied the properties of one of the *Tn3-URA3* insertion mutants, the *far1-c* mutant, in which the mating defect can be studied separately from the arrest defect. The *far1-c* mutant has a mating defect identical to mutants carrying a deletion of *FAR1*. They mate weakly to wild-type cells of the opposite mating type (at a frequency of approximately 1-5% of wild-type strains), and posses a strong mating defect ($<10^{-7}$) when mated to another *far1-c* mutant (Figure). This bilateral sterility is also a hallmark of mutants defective for cell fusion, specifically, *fus1fus2* mutants (Trueheart et al., 1987). We also found by quantitative mating assays that *far1-c* mutants also possess a strong mating defect to a *fus1fus2* mutant. **a** *far1-c* and α *far1-c* mutants have similar mating defects, suggesting that FAR1 is playing a similar role in both mating types.

far1-c mutants do not, however, possess obvious defects in response to mating pheromone. As shown for a deletion allele of *FAR1* (Figure), **a** *far1-c* mutants express wild-type or near-wild-type levels of *FUS1-lacZ* at a range of α-factor concentrations (data not shown). They exhibit normal G1 arrest in response to α-factor and form shmoos. They do require a slightly higher concentration (5x) of α-factor for full arrest, which may be due to poor expression of the truncated FAR1 protein. However, this arrest defect is not sufficient to explain the mating defect, since inactivation of *CLN3* allows the *far1-c* mutant to arrest at low concentrations of α-factor, but does not alter the defect in mating. The *far1-c* mutant also secretes normal levels of a-factor (Figure).

We next tried to determine what step of mating was defective in the *far1-c* mutant Two classes of mutants have been previously described which exhibit bilateral mating defects but can respond to mating factor. *fus1fus2* mutants, which are defective in cell fusion, form many zygotes which exhibit a thick septum by phase microscopy at the junction between the two cells where cell fusion has failed (Trueheart et al., 1987; McCaffrey et al., 1987). Mutants in nuclear fusion, which include mutants in KAR*1, KAR2, and KAR3,* form zygotes and perform cell fusion properly, but do not fuse their nuclei but instead, bud off cytoductants, which contain the genetic information of only one parent and the cytoplasmic information (such as mitochondria) from both parents (Conde and Fink, 1976; Polaina and Conde, 1982).

far1-c mutants do not resemble either *fus* or *kar* mutants. When a *far1-c* cells were mixed with α *far1-c* cells, no ($<10^{-3}$) zygotes or blocked zygotes were seen in the mating mix, unlike *fus* or *kar* mutants, which accumulate many zygotes. However, many shmoos did accumulate, showing that the mutants are secreting and responding to mating factors. In addition, when *far1-c* cells were mixed with *FAR*$^+$ cells, fewer zygotes formed than when *FAR*$^+$ cells were mixed with *FAR*$^+$ cells. Thus, the percentage of zygotes formed was roughly equivalent to the number of successful matings, suggesting that *far1-c* mutants have a primary defect in forming zygotes. We further confirmed that *far1-c* does not have a defect in nuclear fusion by showing that it did not have increased rates of cytoductant formation. Microscopic examination of individual pairs of cells also comfirmed a defect in zygote formation (see below).

Behavior of the *far1-c* mutant in the competition assay

Jackson and Hartwell (1989) have devised a competition assay for mating, which determines by an indirect mating assay how a cell chooses a mating partner(see also Jackson and Hartwell, 1990). In the mating assay, an α cell is asked to distinguish between an a test cell and a 50-fold excess of a competitor cells (see diagram in Table

test α cell	test **a** cell	competitor **a** cell	matings between test cells
far1-c	*wt*	*ste12*	244
		wt	223
wt	*wt*	*ste12*	2100
		wt	550

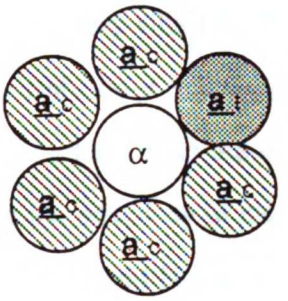


Table 2: Behavior of the *far1-c* mutant in a competition assay.

$6x10^5$ each of the α and **a** test cells and $3x10^7$ **a** competitor cells were applied on a filter, and after an incubation at 30° for 3 hours, were scored for number of matings between the test cells by number of prototrophs. Matings involving the competitor cells did not result in prototrophy and were not scored. The total number of test **a** cells in each mating was approximately equal (+/- 10%), showing that cells were successfully washed off the filter. The numbers of matings in the table represent 4% of the total matings on the filter. Strains used were as follows: test α cells: FC182 (*far1-c*), IH1784 (*FAR+*): test **a** cells: IH1793; competitor **a** cells: 1H1934 (*ste12:LEU2*), IH1783 (*STE+*).

The bottom diagram depicts a schema of the competition assay, in which the α cell is surrounded by **a** competitor cells (**a**c) and a small subset of **a** test cells (**a**t), and the α cell chooses which **a** cell to mate to.

). If the a test cell produces more a-factor than the a competitor cells, the α cell will efficiently mate with a test cells, indicating that the cells choose their partners by which cell secretes the highest concentration of pheromone (Jackson and Hartwell, 1989).

Using this competition assay, we have found a very interesting property of the *far1-c* mutant. When α *FAR*$^+$ cells were mated to wild-type tester a cell in the presence of a *ste12* mutants, which produce very little pheromone and does not compete for mating, the cells mated efficiently with the a wild-type tester cells. In contrast, when wild-type a competitor cells were added, then the α cells mated to both the a competitors and the a tester cells, and thus the efficiency of mating to the tester cells was decreased (as observed previously by Jackson and Hartwell, 1989). The a *far1-c* mutant showed a remarkable behavior: it mated at a similar frequency to a wild-type tester cells regardless of whether the competitor cells were a wild-type or a *ste12*. One interpretation is that a wild-type cell is not capable of competing for another wild-type cell for mating. Observations described below suggest that *far1-c* mutants may not choose their mating partner, but will mate with a wild-type partner if one is present in the right location.

***far1-c* mutants have subtle defects in shmoo formation and direction.**

Although *far1-c* mutants produce normal-shaped shmoos in the presence of α-factor, we next examined the *far1-c* mutants for more subtle defects. First, *far1-c* mutants exhibit a small delay in forming shmoo tips. Figure shows that although some *far1-c* mutants form shmoo tips quickly (at 2 hours) like wild-type, only after 4 hours in α-factor does the entire population form a shmoo tip. The cells arrested as unbudded cells with kinetics similar to wild-type cells, and accumulated as round, slightly swollen cells before forming a shmoo tip. *far1-c* mutants also were found to have defects in forming subsequent shmoo tips as well (data not shown).

far1-c mutants also show a difference in the direction in which they form shmoo tips. In these experiments, **a** cells were placed on an agar slab next to large numbers of α cells which secrete α-factor. Mother-daughter pairs of cells arrest and form shmoos, and are assayed for the direction in which the shmoo tips formed with respect to where it last budded. In analogy to budding, if a cell shmooed next to where it last budded (toward its mother or daughter), it was scored as an "axial" shmoo. If a cell shmooed away from where it last budded (away from its mother or daughter), it was scored as a "polar" shmoo. Wild-type **a** cells (*bar1*$^-$) in this assay produced both axial and polar shmoos, with a slight predominance of axial shmoos. Thus, cells in a uniform field of α-factor shmoo in largely random directions. (This observation has also been repeated using slabs containing purified α-factor as the source of α-factor.) At locations in the slab further away from the α cells, which have lower concentrations of α-factor, the cells continue budding, but instead of axial budding, they exhibit random budding patterns. These observations show that α-factor is capable of affecting the direction of polarity in budding and shmoo formation.

far1-c mutants show a striking difference in that after 2.5 hours exposure to α-factor, the vast majority (94%) of the shmoo tips were in the axial direction. However, at the 4 hour time point, cells exhibited a higher percentage of shmoos in the polar direction. (The absence of polar shmoos is not due to merely a delay in shmooing, since wild-type cells form polar shmoos at very early time points). The budding pattern of these mutants was also not randomized at low α-factor concentrations. Other *far1* insertion mutants (*far1-g, far1-e*) which also have an Arrest$^+$Mating$^-$ phenotype also exhibit a similar property in shmoo formation. These observations show that *far1-c* mutants primarily shmoo in the same direction as they would have budded, implying that they may utilize the mechanisms specifying axial budding for forming a shmoo tip.

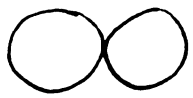
Shmoo tip direction	# individual cells *wt*	*far1-c*
AXIAL 	237 (59%)	185 (46%)
POLAR 	88 (22%)	12 (3%)
NO SHMOO TIP 	75 (19%)	203 (51%)

Table 3: *far1-c* mutants form shmoo tips predominantly in the axial position.

a *wt* (FC140) or a *far1-c* (FC172) cells were incubated in the presence of high concentrations of α-factor by a confrontation assay for 2.5 hours at 30°. 200 mother-daughter pairs (400 individual cells) were assayed by bright field microscopy for the presence of a visible shmoo tip and the direction of the shmoo tip. Shmoo tips facing towards the other cell or perpendicular to the other cell were scored as axial. Shmoo tips facing away from the other cell were scored as polar. Single cells (not a mother-daughter pair) were not scored.

Mating of *far1-c* is dependent on orientation of the mating partners.

One prediction of finding that *far1-c* mutants shmoo predominantly in the axial position is that they might mate more efficiently when the mating partner is located close to that position. I tested this prediction by performing cell-to-cell matings, in which pairs of α and **a** cells were positioned together by micromanipulation on an agar slab and assayed for zygote formation (see Experimental Procedures for details). The mating pairs were positioned in one of two configurations, using the bud of each cell as a marker for the cell's polarity: in the **cis** position, the cells were positioned with the buds pointing towards each other; in the **trans** position, the cells were position with the buds pointing away from each other (see Figure). The axial shmooing predicted that cells might mate more efficiently in the **cis** position.

Pairs of *FAR+* cells mated very efficiently in either position. When **a** *far1-c* cells were mated to a *FAR+* cells, the cells formed zygotes at approximately 50% efficiency when in the **cis** position, but formed zygotes at only 5% frequency when in the **trans** position. This striking result is consistent with the hypothesis that *far1-c* cells can only mate in the direction they can shmoo: in the axial position. An alternate model is the cells share more surface area in the **cis** position than in the **trans** position, which might create a stronger signal required for mating in the *far1-c* cell.

An additional observation in the two cell matings was that in the **trans** position, neither the **a** *far1-c* cell nor the α *FAR+* cell exhibited any morphological alterations, such as shmooing or changes in budding pattern, indicative of some response to α-factor. This lack of response is interesting given the fact that both partners secrete and respond to mating factors normally in other tests. The fact that even the α *FAR+* cell does not appear to respond suggests that the morphological response requires some cooperativity or commitment on the part of both mating partners.

The dependence on orientation for mating also suggests that factors involved in cell-cell communication, such as the receptors and the secretion of mating factors, are not

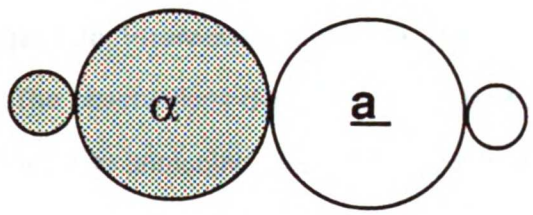
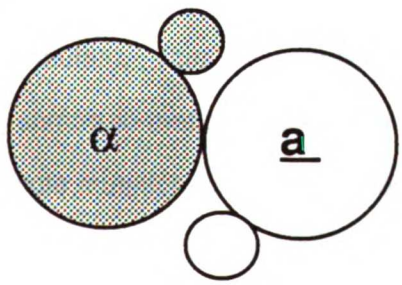
	fraction of pairs which form a zygote	
	a far1-c x α wt	a wt x α wt
CIS 	3 / 36	17 / 17
TRANS 	25 / 44	25 / 25

Table 4 Mating of *far1-c* cells is dependent on orientation: two cell matings.

Individual **a** and α cells were micromanipulated together on an agar slab in one of two configurations, as shown by the diagrams. (All the cells began with small buds.) The pairs were then incubated for 2.5 hours at 30° in a moist chamber, and assayed for zygote formation by visual inspection. The strains used were FC172 (**a** far1-c), FC140 (**a** wt), IH1792(α wt). The first number of each pair represents the number of pairs which formed a zygote, and the second number represents the total number of pairs assayed.

uniformly distributed on the cell surface, but may be localized to the area of the bud and bud neck in the absence of α-factor, and relocated to the site between the two mating partners when the cells initiate the mating process. This relocation may be defective in the *far1-c* mutant.

A genetic interaction between *FAR1* and *BUD5*

The observations described above lead to the hypothesis that *far1-c* mutants are restricted to shmooing and mating at the axial position. One possibility is that they utilize the machinery that determines the site of the next bud in shmooing and mating. *BUD5* is a gene necessary for axial budding, and *bud5* mutants bud in random sites (Chant et al., 1991). One prediction was that a *far1-c bud5* double mutant would no longer shmoo and mate only in an axial direction but would shmoo and mate in a random direction. Therefore a *bud5* mutation could, in principle, improve the mating efficiency of a *far1-c* mutant. Thus I examined the behavior of a *bud5* strain and a *bud5 far1-c* strain for shmoo formation and mating.

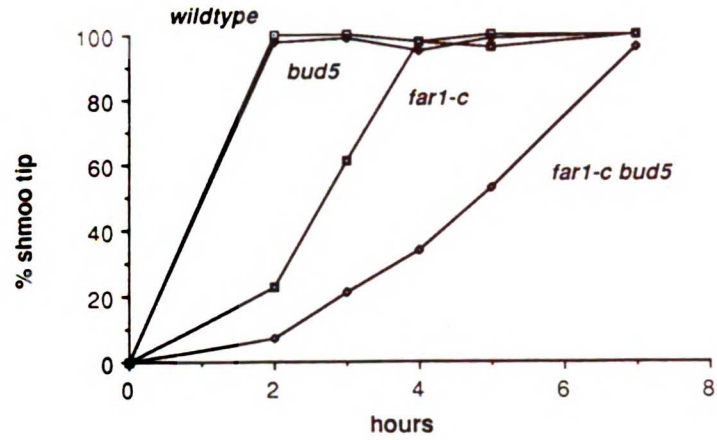
The single mutation in *BUD5* did not affect mating efficiency (to a either a *FAR+* or a *far1* strain) or shmoo tip formation (Figure). However, the *far1-c bud5* double mutant exhibited a dramatic defect in shmoo tip formation. In the presence of α-factor, the a *far1-c bud5* double mutant arrested primarily as swollen round cells (Figure , Panel B) and the shmoo tips that did form were blunt and small. Shmoo tips eventually formed in the majority of cells by 7 hours, showing that defect may be kinetic. As expected by the morphology, the mutation in *BUD5* did not increase mating efficiency in the *far1-c*, and slightly decreased it (data not shown). In summary, a mutation in *BUD5* exacerbated the mild defect in shmoo formation caused by the *far1-c* mutation.

The *bud5* mutation affects also the direction of shmoos. In wild-type cells, there is a mild predominance of shmoo tips forming in the axial position (Table Z). In the *bud5* cells, there are more cells which shmoo in the polar direction, making the distribution

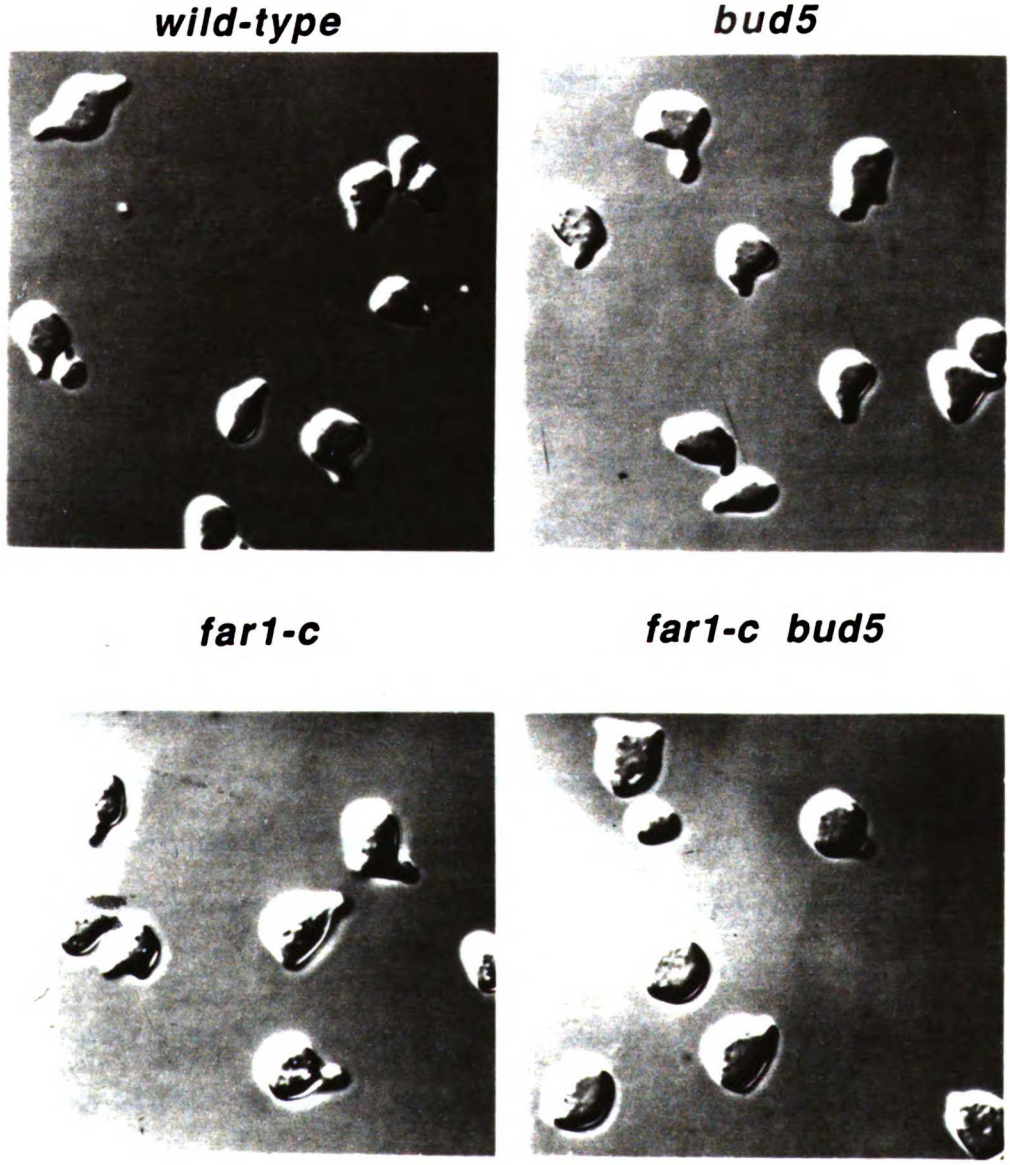
Figure 25: Defects in shmoo tip formation in *far1-c* and *far1-c bud 5* mutants.

Panel A. Exponential cultures of a *wt* (FC140), a *far1-c* (FC172), a *bud5* (FC224), and a *far1-c bud5* (FC225), were grown in YEPD at 30° and treated with 1μM α-factor for the periods of time, fixed with 3.7% formaldehyde, and assayed by phase microscopy for the percentage of cells exhibiting shmoo tip formation. All four strains were arrested as unbudded cells by 2 hours. **Panel B.** Normarski photomicrographs showing the morphology of the cells after 4 hours of exposure to 1μM α-factor. DAPI staining shows that the cells of all four strains only contain one nucleus per cell (data not shown). All the strains in the absence of α-factor exhibit normal budding morphology.

A


wildtype
bud5
far1-c
far1-c bud5
% shmoo tip
100
80
60
40
20
0
0
2
4
6
8
hours

B


wild-type
bud5
far1-c
far1-c bud5

between axial and polar more even. The few shmoo tips which form in the *far1-c bud5* cells (at 4 hours) are in random directions. These data suggest that *BUD5*, a gene involved in budding, may also be involved in shmoo site selection.

DISCUSSION

This chapter describes the characterization of defects in mating and shmoo formation in the *far1-c* mutant, which encodes a C-terminal truncation of the FAR1 product, and which is competent in cell cycle arrest but is defective in mating. The *far1-c* mutant has a novel defect in mating: it can respond to α-factor and form shmoos, but has defects in polarizing toward its mating partner. In this Discussion, I consider properties of the *far1-c* mutant and discuss implications of these observations for the role of the budding machinery in mating and for spatial requirements in cell-cell communication between two mating yeast cells.

Defects in *far1-c* mutants in polarization during mating.

During the mating process, yeast cells of the opposite mating type touch and polarize toward each other regardless of where on the cell surface their partner is positioned. The *far1-c* mutant has two types of defects in polarization. First, *far1-c* mutants are restricted in the site where they can polarize. Whereas *FAR*$^+$ cells are capable of forming shmoo tips and mating at many positions on the cell, *far1-c* mutants are restricted to shmoo and mate only next to the site where they last budded, at an axial position. Second, *far1-c* mutants also possess a weak defect in forming a shmoo tip. The implications of these properties for the role of FAR1 will be discussed in a subsequent section.

far1-c mutants were found to exhibit an interesting property in the competition assay in that the efficiency of mating in the *far1-c* mutant was affected equally by the

inclusion of wild-type or sterile (*ste12*) mutants. In light of the spatial requirements for mating, one interpretation for this behavior is that the *far1-c* mutants can mate only with a wild-type cell that are located in the axial position and are not distracted by other cells, either wild-type or sterile cells, located elsewhere on the cell.

The Importance of Spatial Orientation in Cell-Cell Communication.

Examination of the mating process between individual cells established the importance of spatial localization in mating. Mating of *far1-c* mutants to a wild-type cells was found to be dependent on the orientation of the cells. The spatial requirements for mating of *far1-c* cells to wild-type cells explain the reduced mating efficiency to wild-type cells: the wild-type cell needs to be the proper orientation for mating. *far1-c* cells have much stronger mating defects to another *far1-c* cell. This bilateral mating defect suggests that the spatial requirements for the position of the two *far1-c* cells for a successful mating may be impossibly stringent, and that when mating to a wild-type cell, the wild-type cell can compensate for the defects in the *far1-c* cell.

Why should the orientation of polarity important for mating? In the mating cell, products necessary for mating, such as the FUS1 product, as well products necessary for morphogenesis, such as chitin, are localized in the region in the cell adjacent to the mating partner (Trueheart et al., 1987; Schekman and Brawley, 1979). The mislocalization of products such as FUS1 may explain why *far1-c* cells mate very poorly to cell fusion mutants. Like *far1-c* mutants , *fus1 fus2* mutants mate well to wild-type cells but mate poorly to other *fus1 fus2* mutants. *far1-c* mutants may express fusion products but may be defective in presenting the fusion products at the proper location and thus fail to mate.

The spatial localization of the mating factors and the receptors may also be important. When the a *far1-c* mutant is placed in the **trans** position to a wild-type cell, neither cell exhibits any sign of responding to the other cell. One explanation is that the

receptors and the secretion of mating factors may be primarily localized at the bud or the bud neck, away from the site where the two cells are touching. In the mating between two *FAR+* cells, there must be some communication, perhaps by low concentrations of stray receptors, which are not localized at the bud. This communication is then strengthened as the two cells sense each other and begin to direct receptors and factor towards each other by polarizing toward each other. An indication of this relocalization of the receptors is that the α-factor receptor STE2 has been found to be rapidly endocytosed in response to α-factor, and new receptor is deposited soon after (Jenness and Spatrick, 1986). In the matings with *far1 -c* mutants (in the **trans** position), neither cell directs receptors or factors towards each other, and although initial communication may occur, the communication is not strengthened, and neither cell commits to a mating program. Since one of the partners is wild-type, the lack of response illustrates how this process is cooperative: both cells need to commit to the mating process.

Role of the budding machinery in mating.

The behavior of *far1-c* reveals that products involved in bud site selection may also be involved in shmoo tip formation and mating. First, shmoo tip formation and mating in the *far1-c* mutant was found largely to be restricted to a site adjacent to the site of the last bud. This is where a haploid cell would bud in the absence of mating partners, and suggests that the same machinery which usually directs budding at that site also directs the site of polarization in mating in this mutant. Since FAR+ cells can mate or shmoo at any site on the cell surface, this machinery directing the axial polarity must be somehow inhibited or relocated in FAR1+ cells.

A genetic interaction between *FAR1* and *BUD5*, a gene involved in bud site selection, further supports the role of the budding machinery and *FAR1* in polarity in mating. *far1-c bud5* double mutants exhibit a stronger defect in shmoo formation than either

mutant alone and form depolarized balls in response to a-factor (which very slowly form a shmoo tip). The behavior of this mutant is analogous to the behavior of a *bem1 bud5* double mutant, which is defective in budding and exhibits the depolarized balls at restrictive temperature (Chant et al., 1991). The behavior of the *bem1 bud5* double mutant indicates a possible functional interaction between *BEM1* and *BUD5.* Although it is unlikely that *FAR1* physically associates with *BUD5*, it is possible that in response to a-factor,*FAR1* regulates a gene involved in polarity which interacts with the BUD5 product (see below).

These observations indicate that the gene products directing budding also direct the location and formation of shmoos. I predict that some products will play roles in both budding and shmooing (such as CDC24 and BUD5), other products (such as FAR1) will only be specific only to one of the processes.

A model for shmoo formation and the role of *FAR1*.

This section describes a model which encompasses many aspects of reorientation discussed so far. In the absence of α-factor, a complex of proteins, which might include *CDC24* and *BUD5*, dubbed "the polarity determinant", is localized near the last bud site and directs budding at that site. When an **a** cell contacts an α cell, it encounters a strong, local gradient of α-factor from the α cell. The α-factor binds to the α-factor receptor, STE2, and triggers a signal-transduction cascade inside the cell. The signal from the α-factor might act on polarity in two ways: first, it may inactivate the polarity determinant at the axial position; second, it may establish a new polarity determinant at the site where the signal is strongest, where the two cells touch. This new polarity determinant directs components of the cytoskeleton, growth, and secretion toward the mating partner. As a consequence of the localization of growth and secretion, new receptors and mating factor are presented at the junction between the two cells, resulting in intensified signalling between the two cells. The increased signalling

causes expression and activation of cell fusion products, and ultimately, cell fusion is achieved.

A defect in inactivating the polarity determinant at the axial position can explain some the properties of the *far1-c* mutant. Thus, some *far1-c* cells polarize using this axial determinant instead at a new site. One simple model is that *FAR1* inactivates one or more gene products of the polarity determinant in response to α-factor, which allows cells to efficiently polarize at other sites. This model is attractive given the fact the *FAR1* is already thought to act as an inhibitor -- of CLN2. By this model, a target for *FAR1* in regulating polarity may be identified by an extragenic suppressor mutation which allows *far1-c* mutants to mate more efficiently. One possibility is that FAR1 could act to inhibit a *BUD* gene. However, since BUD5 is required for shmoo formation in a *far1* strain, *BUD5* does not appear to be a target of FAR1.

Another possible defect in *far1-c* is that the formation of the new polarity determinant could be weak. Perhaps in the wildtype cell, the axial polarity determinant is abolished, but a new polarity determinant is formed which is stronger and takes precedence over the weaker axial determinant. If in the *far1-c* mutant, the new signal is weak, then the old axial signal might be used. *FAR1* thus could play a role in the amplification or spatial localization of the signal cascade or regulate more directly the formation of the new polarity determinant. This model most easily explains the delay in shmoo formation in *far1-c* mutants..

Chemotaxis and cell-cell communication

Mating between two yeast cells allows us to examine in molecular detail of how cells communicate with each other and how they polarize towards extracellular signals. These issues are of central importance in development and in the immune system. Our studies in yeast has shown the importance of the spatial orientation of cells in cell-cell

communication and describes the beginnings of pathways which lead from the receptor to the cytoskeleton.

EXPERIMENTAL PROCEDURES

Media and genetic methods: All media and yeast genetic techniques have been previously described. Bacto-Agar (Difco) was used.

Strains: FC224 (**a** *bud5::URA3*) was constructed by gene replacement using a *bud5::URA3* plasmid, kindly provided by John Chant, in FC140 (wt). The strain was confirmed to have a stable *URA3* marker integrated in the chromosome linked to the *BUD5* locus and to exhibit a random budding pattern; however, a Southern demonstrating proper replacement of the gene has not been performed. FC225 (**a** *far1-c bud5::URA3*) was constructed by a cross between FC224 and an isogenic strain, FC212 (α *far1-c*)

Assays for mating and shmoo formation: Assays for cell cycle arrest, mating, and mating factor production have been previously described (Chapter 1). Competition assays were carried out as described (Jackson and Hartwell, 1989), using $6x10^5$ test cells of each mating type and $3x10^7$ (50x excess) competitor cells. Filters were incubated on YEPD plates for 3 hours. Assays for shmoo tip direction were performed using the confrontation assay (Chapter 1). In Figure X , only cells close (one field or <25 cell widths) to the streak of a cells were assayed. Cell-to-cell matings were performed by placing **a** and α cells, which had been growing in exponential phase in YEPD, onto separate slabs of YEPD 4% agar. Small numbers of **a** and α cells to be tested were then transferred by micromanipulation onto a third slab of agar. Individual **a** and α cells with small buds were positioned together by persistent micromanipulation until

they fell in one the two configurations diagrammed in Table . The slab was than incubated in a moist chamber for 2.5 hours at 30°. The pairs of cells were then examined for the formation of zygotes by visual inspection of the cells. I found that agar obtained from Difco yielded optimal mating, whereas agar from BRL gave very poor mating in the cell-to-cell mating assays.

CONCLUSION

CONCLUSION

PERSPECTUS AND *PROSPECTUS*: RESPONSE TO NEGATIVE GROWTH FACTORS IN YEAST AND MAMMALIAN CELLS.

FAR1 and the α-factor Response Pathway in Yeast

This thesis has described a sequence of events that begins with the binding of α-factor on the cell surface and culminates with arrest of the cell cycle. The most significant aspect of this work has been to link events in the signal transduction pathway with the cell cycle, giving an overview of the pathway from beginning to end. A model incorporating much of our current knowledge is diagrammed in Figure 26.

The pathway begins with the α-factor receptor, *STE2*, a member of the integral membrane protein family with seven membrane-spanning regions (Burkholder and Hartwell, 1985; Nakayama et al., 1985). This receptor appears to function by communicating with a heterotrimeric G protein, composed of Gα, Gβ, and Gγ subunits coded by the *SCG1* (*GPA1*), *STE4*, and *STE18* genes respectively (Dietzel and Kurjan, 1987; Miyajima et al., 1987; Whiteway et al., 1989). Downstream of the G protein (Nakayama et al., 1988; Blinder et al., 1989) lies *STE5*, whose function is unknown, and FUS3, STE7, and STE11, which appear to be protein kinases (Elion et al., 1990; Teague et al., 1986; Rhodes et al., 1990). The STE12 protein appears to be a transcription factor that functions further downstream in the pathway: STE12 protein binds at a nucleotide sequence (termed the PRE, "pheromone response element"), located in the upstream regions of many α–factor-inducible genes, that confers inducibility by α-factor (Dolan et al., 1989; Errede and Ammerer, 1989). Induction of gene expression by α-factor is hypothesized to result from activation of the STE12

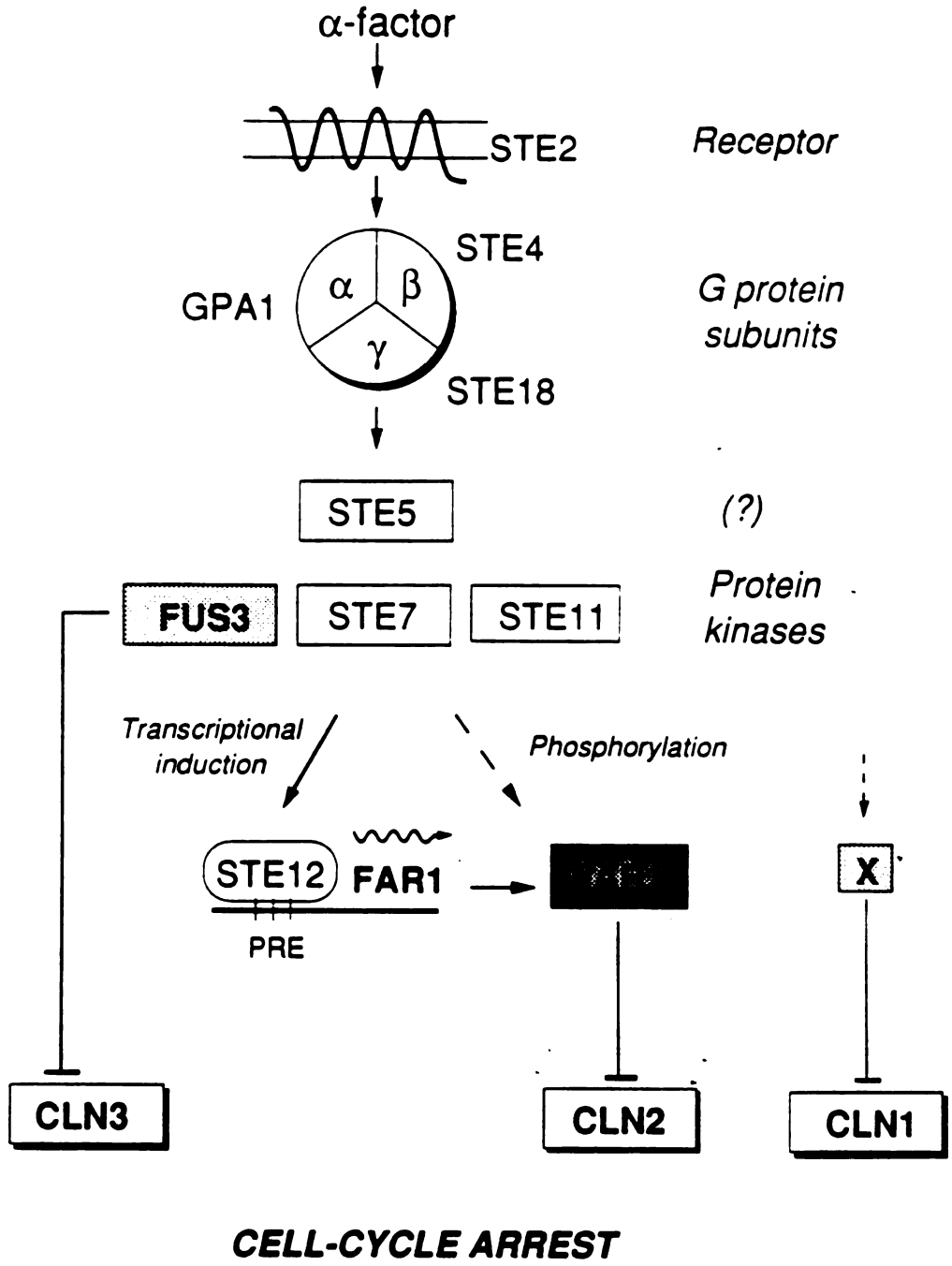


Figure 26: A model of *FAR1* and the α-factor response pathway in yeast. FAR1 is regulated by the signal transduction pathway both by transcription through STE12 and by phosphorylation. The specific kinases which might phosphorylate STE12 and FAR1 are not known, although FUS3, STE7, and STE11 are good candidates. See text for more details.

protein in some way, perhaps due to action of the protein kinases earlier in the pathway (see Dolan and Fields, 1990). STE12 activates transcription of a diverse set of genes which play different roles in the mating process.

One of these genes induced by α-factor is *FAR1*, which serves as a link between the signal transduction pathway described above and the cell-cycle. *FAR1* does not appear to function in the signal transduction pathway between the receptor and STE12, but has a specific role in cell-cycle arrest by α-factor. A genetic interaction with the G1 cyclin *CLN2* indicates that *FAR1* inhibits *CLN2* in cell-cycle arrest. This observation not only indicates a target for *FAR1*, but provides strong argument that α-factor causes cell-arrest by the inhibition of all three of G1 cyclins, which are functionally redundant. Since *FAR1* regulates only *CLN2*, we hypothesize that other factors of the mating factor response pathway must regulate *CLN1* and *CLN3*. One candidate for the inhibitor of *CLN3* is the protein kinase FUS3 (Elion et al., 1990).

One important question that remains is determining the mechanism by which the FAR1 product inhibits CLN2. Experiments with an *ADH-CLN2* construct demonstrate that FAR1 probably inhibits some aspect of the synthesis, activity, or degradation of the CLN2 protein. Characterization of CLN2 protein in cells carrying *ADH-CLN2* treated with α-factor will further illuminate how CLN2 might be inhibited. For instance, if CLN2 protein remains at high levels in response to a-factor, then FAR1 may inhibit the activity of the CLN2 protein. If CLN2 protein decreases in response to α-factor when CLN2 mRNA remains abundant, then FAR1 may either inhibit translation or stimulate the degradation of CLN2. Assaying the effect of α-factor on CLN2 in cycloheximide-treated cells might distinguish whether FAR1 affects CLN2 translation or degradation.

. The next question will then be to ask if the inhibition of CLN2 by FAR1 is direct or indirect. For instance, FAR1 could inhibit an activator of CLN2. The availability of

αFAR1 and αCLN2 antibodies will be useful for biochemical characterization of the process.

How does α-factor regulate the activity of FAR1? FAR1 has been found to be regulated in three ways. First, the transcription of *FAR1* is induced five-fold in response to α-factor by STE12 and the signal transduction pathway. Second, the FAR1 protein is phosphorylated in response to α-factor. Three protein kinases in the signal transduction pathway, STE7, STE11, and FUS3, are candidates for the protein kinase responsible for the FAR1 phosphorylation. The effect of phosphorylation on FAR1 activity however is unknown. Third, the expression of the FAR1 protein is restricted to a window in G1 (see Appendix A). This cell-cycle regulation may involve the regulation of FAR1 protein stability by the ubiquitination pathway. This cell-cycle regulation of FAR1 may insure that FAR1 does not inhibit G2 cyclins and arrest the cell in G2. Whether the activity of FAR1 itself is regulated by α-factor or if FAR1 is constitutively active is unknown. It is possible that FAR1 works in conjunction with other proteins, which are activated by α-factor.

Characterization of different alleles of *FAR1* indicate that it may have three functions. The first function, as described above, is the inhibition of CLN2 for cell-cycle arrest. A second function of *FAR1* is in directing polarity of the cell towards its mating partner in the mating process. Insertion mutations demonstrate that the C-terminal 400 amino acids of FAR1 are necessary for this mating function but are not necessary for its function in cell-cycle arrest. The structure of *FAR1* thus may be divided into domains with specific functions. This additional function in mating suggests that *FAR1* may act on a second (as yet unidentified) target, which may be involved in cell polarity. *FAR1* may also have a third function, which was revealed by the fact that the *far1 cln2* double mutant does not arrest as well as a *FAR*$^+$ strain at intermediate concentrations of α-factor (Chapter 2). This third function may be to inhibit *CLN3* at

intermediate concentrations of α-factor or may be to affect the signal transduction pathway. It is possible that this third function is the same as the mating function (the second function) of *FAR1*.

Why should there be three *CLN* genes and why is the regulation of these genes so complex? Having multiple *CLN* products may facilitate fine adjustments in the length of G1 needed in the regulation of cell size. Also, specific CLN products may have different roles in recovery from cell cycle arrest after starvation or mating. For instance, following the formation of the zygote, the diploid cell resumes division. One model is that CLN3 may the first CLN product that stimulates the first cell division. In the initial steps in recovery from arrest, the CLN3 protein may be reactivated simply by dephosphorylation. This activated CLN3 then might act, probably via the CDC28 kinase, to stimulate the synthesis of CLN1 and CLN2 (F. Cross, personal communication).

Why are the G1 cyclins the targets of α-factor regulation instead of CDC28 itself? One obvious reason is that the CLN products are required only in G1, whereas CDC28 is required both in G1 and G2. Thus, regulation of the CLN products may insure that the cell arrests only in G1 and not in G2. Since the G1 cyclins are unstable proteins, their activity may be easily regulated by controlling their synthesis.

Finally, I would to comment on how fortunate I was to find the genetic interactions described in this thesis. First, in the initial screens, it was not obvious that *far* mutants could be easily obtained. For instance, it was possible that the only genes which were specifically involved in cell-cycle arrest were essential for cell-cycle progression and thus would be difficult to identify in a mutant screen. Second, the identification of the genetic interaction between *FAR1* and *CLN2* rested on the unique nature of three functionally redundant products that are independently regulated (as well as on the generosity of the Reed lab in supplying me with the *cln* mutants.) My

original hypothesis was that *FAR1* might inhibit a single essential cell-cycle gene. In that case, a null allele of this essential gene would result in a dead cell and would not be useful in studying α-factor arrest. Furthermore, if the pathway leading to cell-cycle arrest were linear, a mutation in *FAR1* would always block cell-cycle arrest by α-factor, no matter what genes were disrupted downstream. Thus, I did not anticipate to be able to identify the target of *FAR1* by suppression analysis. Third, given the multiple modes of regulation of CLN2, I was also fortunate to identify the *FAR1* gene. α-factor inhibits *CLN2* in at least two ways, by inhibition of its transcription and by inhibition of the protein. *FAR1* is only directly responsible for one of these ways, the inhibition of the CLN2 protein. If these two ways of turning off CLN2 were entirely independent, then a single mutation in *FAR1* would not produce a defect in cell-cycle arrest, since CLN2 could still be inhibited transcriptionally. In reality, *CLN2* transcription is inhibited only when cells arrest, so that the single mutation in FAR1 affects both modes of inhibition and thus causes an α-factor resistance phenotype..

This scenario of "functionally redundant" modes of inhibition by α-factor might explain why the inhibitor of *CLN1* has not yet been identified. Like *CLN2*, *CLN1* may also be under multiple modes of inhibition by α-factor (F. Cross, personal communication). If these modes of inhibition are independent, then as discussed above, a single mutation would not be sufficient to block inhibition of *CLN1*. To circumvent this problem, an inhibitor of *CLN1* could perhaps be identified in a screen for α-factor resistant (*far*-like) mutants in a strain carrying *GAL-CLN1*, which exhibits cell-cycle arrest in response to α-factor (F. Cross, personal communication). In this strain, α-factor cannot inhibit CLN1 by transcription, and thus mutations which affect the second mode of *CLN1* regulation might be able to exhibit an α-factor resistance phenotype.

Cancer and the Response to Negative Growth Factors in Mammalian Cells.

One of the hopes of this work was that it might provide insights into the pathogenesis of cancer. It is increasingly apparent that cancer is a genetic disease, in which multiple mutations in cells lead to the loss of the control of proliferation (for review, see Bishop, 1991). The mutations which contribute to the formation of the cancer cell can be divided into two classes: gain of function (or new function) mutations and loss-of-function mutations. The gain-of-function mutations generally occur in genes involved in response to positive growth factors. For instance, mutations in the EGF receptor that cause constitutive activation of a signal transduction pathway that promotes proliferation contribute to transformation (see Cantley et al., 1991).

The second class of mutations is loss-of-function mutations in genes termed "tumor suppressor" genes. Recently, mutations in tumor suppressor genes such as RB and p53 have been widely implicated in many kinds of cancer. People who carry a deletion in either RB or p53 on one of their chromosomes have a very high risk of developing a variety of cancers, which arise when the other copy of the tumor suppressor gene is also mutated (see Vogelstein, 1990; Hansen and Cavenee, 1988). The lack of a functional RB gene was shown to contribute directly to the development of these cancers by demonstrating that reintroducing a functional RB gene into transformed cells lacking RB suppresses tumorgenicity (Bookstein et al., 1990; Huang et al., 1988). A further indication that the loss of RB function can lead to cancer is that transforming viruses such as SV40 encode for products like T antigen which bind RB and presumably inhibits it (DeCaprio et al., 1989). Specific mutations in T antigen which prevent binding to RB inactivate the transforming function of T antigen, suggesting that T antigen may transform cells in part by inactivating RB. Since the absence of RB or p53 function

contributes to tumor formation, these tumor suppressor gene products are hypothesized be involved in regulatory pathways which act to inhibit proliferation in normal cells. Thus, a key goal now in understanding the pathogenesis of cancer is to understand the pathways which negatively regulate the cell-cycle.

The control of proliferation of cells in the body is controlled by extracellular factors such as cell-cell contact and negative growth factors. The best characterized negative growth factor is TGF-β. Like α-factor, TGF-β is a potent differentiation factor and negative growth factor, which causes cells to exhibit changes in morphology, express specialized sets of genes, and arrest the cell cycle in G1. TGF-β has been shown to be important in development and wound healing, and its widespread expression and effects on many kinds of embryonic and adult tissues suggest that it may be important in the differentiation and growth inhibition of many different types of cells (see Massague, 1990 for review).

Our work on yeast provides a framework for thinking how factors such as TGF-β might regulate the cell-cycle in mammalian cells. I propose that the response pathway in mammalian cells to negative growth factors, such as TGF-β, might be similar to the α-factor response pathway in yeast (Figure 27). One possibility is that TGF–β may cause arrest in G1 by inhibiting the G1 cyclins and p34/CDC28. Individual components of the pathways, such as *FAR1*, may be conserved and play similar roles in both yeast and mammalian cells. In yeast, loss-of-function mutations in most genes of the pathway, such as the *STE* genes and *FAR1*, cause the cell to be insensitive to growth inhibition to α-factor. We can imagine that mutations in components of the TGF-β response pathway would make cells insensitive to growth inhibition and might contribute to the development of the cancer cell. Thus, these components of the TGF-β response pathway might be tumor suppressor genes.

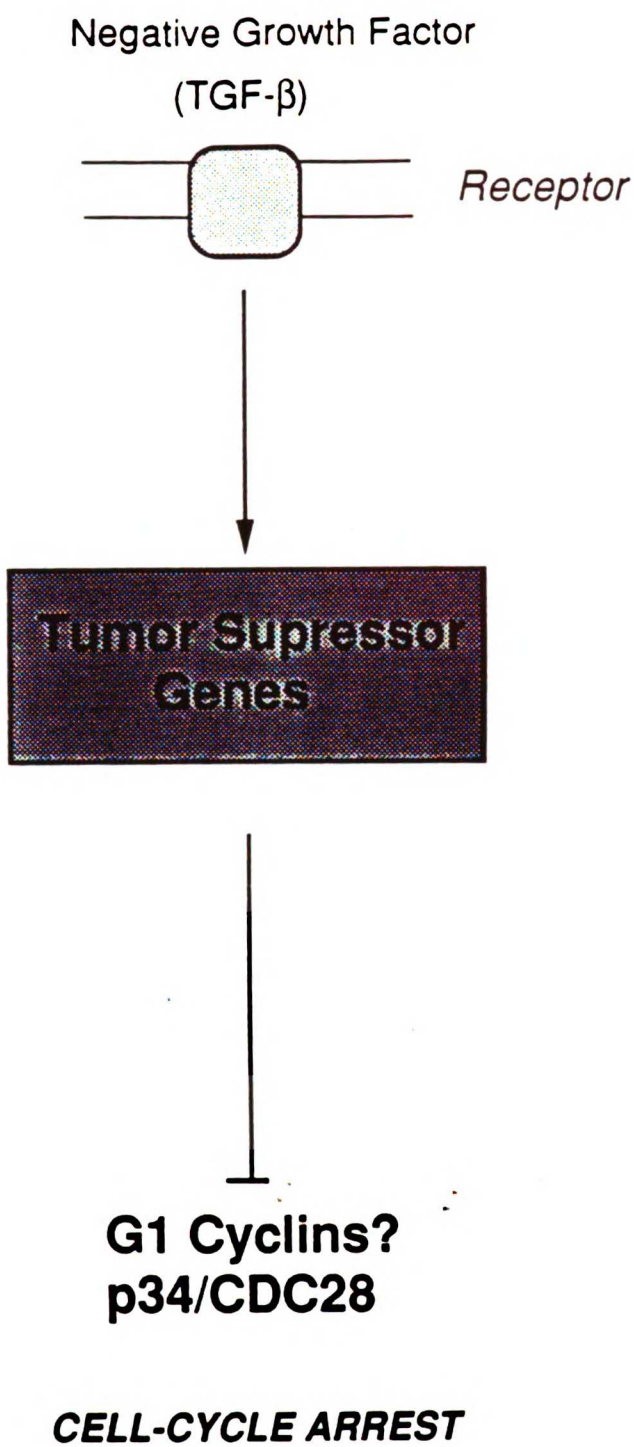


Figure 27: A model of a response pathway to negative growth factors in mammalian cells.
See text for details.

The characterization of the TGF-β response pathway is still in a very preliminary stage. However, recent work has provided some hints that the product of a tumor suppressor gene, RB, indeed might play a role in TGF-β response. Introduction of the transforming protein, large T antigen, which binds and inactivates RB, makes cells resistant to growth inhibition by TGF-β (Laiho et al., 1990; Pietenpol et al., 1990). Introduction of T antigen mutants which do not inactivate RB do not affect cell-cycle arrest by TGF-β, suggesting that the defect in the cell-cycle arrest caused by T Antigen is due to the inactivation of RB. T antigen appears to affect specifically cell-cycle arrest by TGF-β and does not affect the transcriptional responses to TGF-β (Lailo et al., 1991); this phenotype is analogous to a *far* phenotype. Thus, RB may be like FAR1 in that it may have a specific role in arresting the cell cycle in G1 in response to a negative growth factor. However, this hypothesis needs to tested more directly with cells carrying deletions of the RB locus.

RB and FAR1 are also regulated in similar ways: both may active only during the G1 phase of the cell cycle, and both may be regulated by phosphorylation in response to a negative growth factor. RB has been found to be dephosphorylated during a window in G1 (Buchkovich et al., 1989; Chen et al., 1989; DeCaprio et al., 1989; Mihara et al., 1989). This dephosphorylated form of RB is thought to be the active form of the protein (Ludlow et al., 1989). Thus like FAR1, RB may be active only during G1, which may insure that cells do not arrest in inappropriate phases of the cell cycle. TGF-β has been found to prevent the phosphorylation of RB and thus may keep RB in an active form. This effect of TGF-β on the phosphorylation state of RB is not dependent on cell-cycle arrest and thus may represent a primary effect of TGF-β on RB (Laiho et al., 1990). Despite these possible similarities of RB with FAR1, FAR1 has no significant sequence similarity to any of the known tumor suppressor genes.

One current burning question is whether there is a mammalian homologue of *FAR1*. Such a homologue might be another tumor suppressor gene like RB. In collaboration with Vivian Chan, we have found that affinity-purified αFAR1 antibody recognizes one major band of approximately 70 kD and two minor bands of approximately 105 kD and 150kD. These proteins are good candidates for proteins with homology to *FAR1*. The genes encoding these proteins may be cloned by screening expression libraries using αFAR1 antibody. Other approaches to finding a *FAR1* homologue include finding genes with nucleotide homology using PCR or hybridization methods and possible complementation methods.

APPENDIXES

APPENDIX A: Cell cycle regulation of FAR1.

APPENDIX B: Regulation of *HO* expression by α-factor and CDC28.

APPENDIX C: Strain List

APPENDIX D: References

APPENDIX A

CELL CYCLE REGULATION OF FAR1

INTRODUCTION

In previous sections of the thesis, I have shown that *FAR1* is regulated in two ways: in response to α-factor, FAR1 transcription is induced five-fold , and FAR1 protein is phosphorylated. Here I present evidence that the presence of the FAR1 protein is restricted to a window in G1. This regulation may involve controlling protein stability. The presence of FAR1 mirrors the presence of the G1 cyclins, and may insure that FAR1 inhibits only a G1 cyclin and not cyclins in other phases of the cell cycle.

RESULTS

The cell cycle expression of FAR1 was determined by assaying FAR1 protein by Western blotting in *cdc* mutants which arrest at 37° at different phases of the cell cycle (Figure 1; panel A). FAR1 protein was overexpressed in cells arrested in G1: in a *cln1 cln2 cln3* mutant (lane 4), in a *cdc28-4* mutant (lane 6), and in a *cdc34* mutant (lane 8). FAR1 was not expressed in cells which are arrested in S phase, in a *cdc8* mutant (lane 10) and in hydroxyurea-treated cells (panel B; lane 3). FAR1 was also not expressed in cells arrested at G2 or M phase: in *cdc13* (lane 12), *cdc20* (lane 14), and *cdc15* mutants(lane 16). The *cdc* mutants at the permissive temperature (22°)

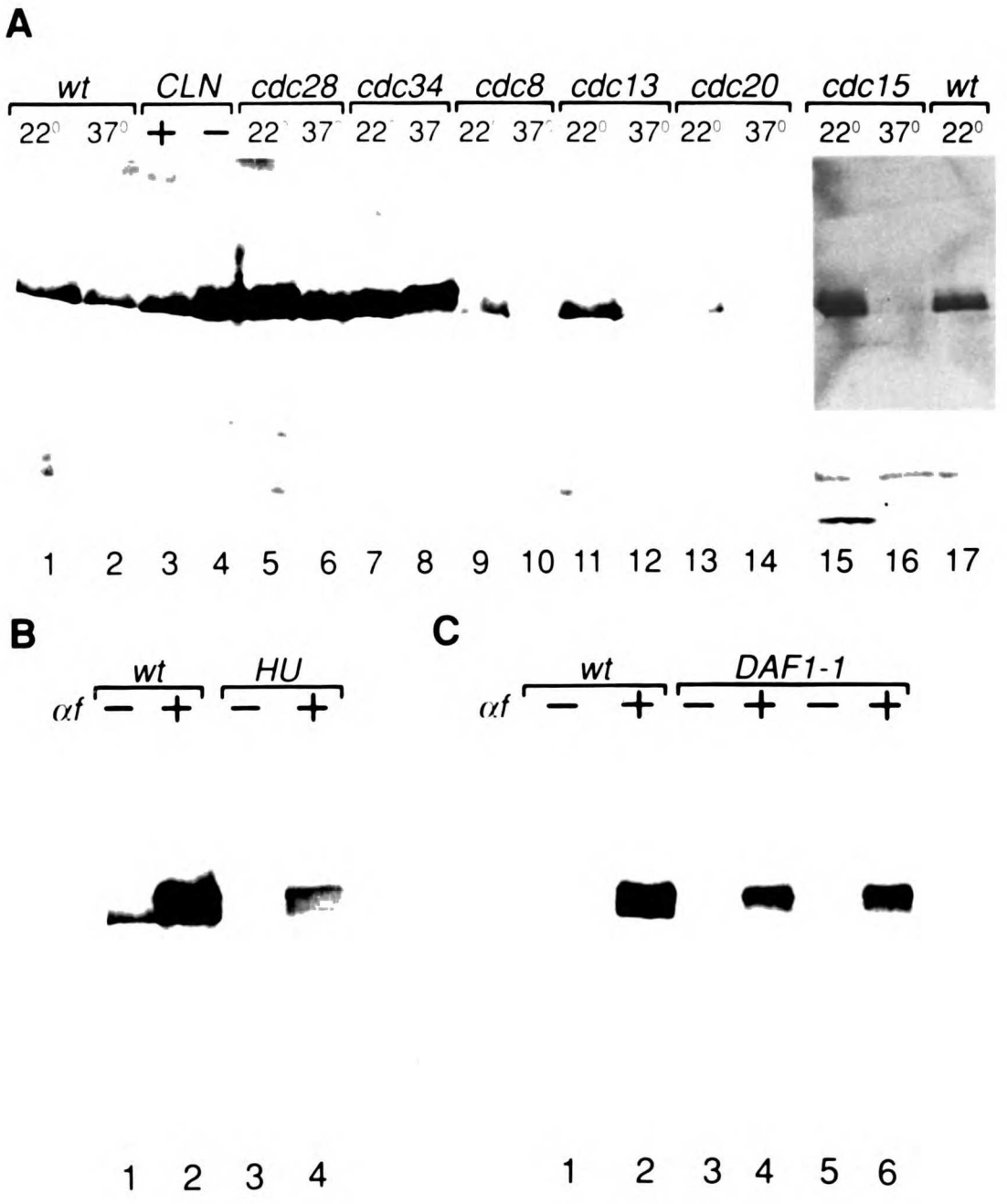
Figure 1: Cell cycle regulation of FAR1 protein.

Cells were grown in conditions described below, and then were harvested, examined for morphology by phase microscopy, extracted with SDS sample buffer, electrophoresed on SDS-PAGE, and Western blotted with αFAR1 antibody.

Panel A. In lanes 1,2, 5-17, cells of the indicated genotype were grown in exponential phase cultures in YEPD at 22°C and were shifted to 37° or kept at 22° for 3 hrs. Cells in lanes 3 and 4 were grown in YEP+galactose and then washed and resuspended in YEPD (glucose)media and grown for 3 hrs at 30°. All the *cdc* mutants arrested with uniform (> 95% cells) morphologies at restrictive conditions. The strains used were: *wt*: FC279; *cdc28-4* :FC391; *wt:* FC279; *cln1 cln2 cln3 GAL-cln2*: PLY1; *cdc34*: FC 377; *cdc8-1*: FC379; *cdc13-1*: FC380; *cdc20-1*: FC382; *cdc15-1*: IH2372.

Panel B: Wildtype **a** cells (FC279) growing in YEPD were treated with 5mg/ml hydroxyurea (HU)(lanes 3-4) or mock treated (lanes 1,2) for 90 min, and then treated with 1μM α-factor (lanes 2 and 4) or mock treated (lanes 1 and 3) for 60 minutes. Cells treated with HU and α-factor exhibited 7% budded cells.

Panel C: **a** cells of the indicated genotype were grown in exponential phase cultures in YEPD and treated with α-factor (lanes 2, 4 ,6) or mock treated (lanes 1,3,5) for 60 min at 30°. Strains used were: lanes 1,2 FC344 (wt); lanes 3,4: FC342 (DAF1-1x8); lanes 5,6: FC342 (DAF1-1x8).


A
wt
CLN
cdc28
cdc34
cdc8
cdc13
cdc20
cdc15
wt
22° 37° + − 22° 37° 22° 37° 22° 37° 22° 37° 22° 37° 22° 37° 22° 37° 22°
1 2 3 4 5 6 7 8 9 10 11 12 13 14 15 16 17
B
wt
HU
αf − + − +
1 2 3 4
C
wt
DAF1-1
αf − + − + − +
1 2 3 4 5 6

expressed FAR1, indicating that these strains do not carry a *far1* mutation. This experiment indicates that FAR1 is expressed only in G1 of the cell cycle.

FAR1 appeared to be overexpressed in a *cdc28-4* mutant even at permissive temperature (lane 5). This strain has low histone H1 kinase activity at permissive temperatures even though the cell grows well. This observations may indicate a specific role of CDC28 in inhibiting levels of FAR1 protein.

FAR1 exhibited a striking shift in mobility in the *cdc34* strain arrested at late G1 after START. This form is normally not detected in the absence of α-factor. This shift in mobility is similar to the shift seen in FAR1 in response to α-factor, which has been shown to be due to phosphorylation (Chapter 3). Thus, this shift in FAR1 in a *cdc34* block is likely to caused by phosphorylation, although it is not known whether this phosphorylation is on the same sites as the α-factor-induced phosphorylations. Possible implications for this finding as discussed below.

I next determined if α-factor could induce expression of FAR1 in phase of the cell cycle where FAR1 is not expressed. Figure 1 panel B shows that cells arrested in S phase with hydroxyurea did not express FAR1 protein (lane 3). After addition of α-factor, low levels of FAR1, which exhibited the band characteristic of the α-factor-induced phosphorylation, was detected. This low level of expression in lane 4 could be due to the fact that the cells were not completely arrested in S-phase: 7% of the cells were unbudded and arrested by α-factor in G1. This subpopulation in G1 could account for the low level of expression of FAR1. However, the possibility that α-factor induces low levels of FAR1 expression in S phase cannot be ruled out.

FAR1 was also found to be reduced in cells which lack a G1 phase. *DAF1-1x8* cells carry eight copies of a truncated *CLN3* gene which cause cells to become smaller than wildtype cells and to have a reduced or no G1 phase of the cell cycle (Cross, 1988; Nash et al., 1988). In response in α-factor, *DAF1-1* cells do not exhibit cell cycle arrest or morphological changes, although they do exhibit induction of genes such as *FUS1*,

showing that the signal transduction pathway is functional in these cells (Cross, 1988). Figure 1 panel C demonstrates that FAR1 expression is greatly reduced in *DAF1-1* cells in the absence of α-factor (lanes 3 and 5). After treatment with α-factor, FAR1 is expressed at low levels and is phosphorylated (lanes 4 and 6). As observed for the HU-treated cells, this low level of FAR1 expression could represent a subpopulation of *DAF1-1* cells which arrest with α-factor and accumulate in G1. It is likely that the reduction of FAR1 in *DAF1-1* cells is a consequence of the reduced G1 phase in these cells, although other effects by *DAF1-1* may be possible.

DISCUSSION

These experiments demonstrate that FAR1 protein is expressed only in a window of the cell cycle, in G1. FAR1 was found to be overexpressed in mutants which are arrested in G1 and is not expressed in mutants which are arrested in other phases of the cell cycle. Also, FAR1 is not expressed in *DAF1-1* cells, where the G1 phase is greatly reduced. The cell cycle regulation of FAR1 in the absence of α-factor is clear. However, these experiments do not conclusively show whether α-factor can induce FAR1 expression in cells not in G1.

Preliminary evidence suggests that FAR1 may be regulated by protein stability. Northern blots show that *FAR1* mRNA is present in parts of the cell cycle where FAR1 protein is not expressed (F. Cross, unpublished). Thus, the cell cycle regulation is likely to occur by the regulation of either the synthesis or degradation of the FAR1 protein. Cycloheximide treatment of cells inhibits protein synthesis and halts the cell cycle. The FAR1 protein (data not shown) was shown to be fairly stable in cycloheximide-treated cells. Thus FAR1 is stable when the cell cycle is stopped, and

unstable when the cell cycle progresses from G1 to S, suggesting that its stability may regulated at the G1/S boundary..

One protein responsible for the degradation of FAR1 may be CDC34. CDC34 is a ubiquitin-conjugating enzyme, which may be involved in targeting proteins for degradation (Goebel ref). The *cdc34* mutant arrests in a point in the cell cycle in late G1 after START, suggesting that *CDC34* may be required for the degradation of protein(s) in late G1 for cell cycle progression. FAR1 is overexpressed in the *cdc34* mutant and may be phosphorylated in a form that is not seen in asynchronous cultures. This behavior of FAR1 suggests a model where FAR1 is phosphorylated at START and is degraded by CDC34 and the ubiquitin system. The phosphorylated form of FAR1 seen in the *cdc34* mutant thus may an intermediate form of FAR1 which is rapidly degraded in wildtype cells, but accumulates in cells defective in the degradation pathway.

The protein kinase CDC28 may be responsible for the putative phosphorylation of FAR1 at START. FAR1 is overexpressed in the *cdc28-4* mutant, both at permissive and restrictive temperatures, suggesting that CDC28 may regulate FAR1 independently of cell cycle position. However, this possibility needs to be tested more directly.

This cell cycle regulation of FAR1 mirrors the expression pattern of its target, CLN2 (Wittenberg et al., 1990). The G1 regulation of FAR1 may insure that FAR1 inhbits the G1 cyclin and not G2 cyclins, which would cause the cell to arrest in G2. The rapid degradation of FAR1 at G1/S may also be instrumental in allowing cells to recover from arrest following mating or removal of α-factor.

The coordinate regulation of FAR1 with the G1 cyclins suggests that similar mechanisms, possibly involving CDC28 and CDC34, may be responsible the cell cycle regulation of the G1 cyclins. The protein stability of cyclin B in Xenopus oocytes has been found to regulated by ubiquitination (Glotzer et al., 1991). Thus, CDC34, a ubiquitin conjugating enzyme, is a good candidate as the enzyme which targets the G1

cyclins and FAR1 for destruction. The study of the cell cycle regulation of FAR1 thus could aid the study of how the G1 cyclins are regulated.

The effect of the *DAF1-1* alleles on FAR1 demonstrates that a mutation of one G1 cyclin, *CLN3* (DAF1), can have pleiotropic effects which affect CLN2. We believe that *DAF1-1* affects FAR1 by reducing the length of G1, although this possibility remains untested. The *DAF1-1* mutant thus causes a defect in α-factor arrest not only because its CLN3 product may resistant to inactivation by α-factor, but also because FAR1 is not expressed and thus CLN2 is also not inhibited by α-factor.

EXPERIMENTAL PROCEDURES

Strains: Most of the *cdc* strains (except the *cdc34* strain) are from the Hartwell original collection and were obtained from Rong Li. The *cdc34* strain was a gift from Ray Deshaies. The *CDC+* strain was a gift from Peter Sorger. The *cln1 cln2 cln3* strain was a gift from Pierre Leopold (see Chapter 2). The *DAF1-1* strains were a gift from Fred Cross.

Western Blots: Westerns were performed with affinity purified αFAR1 antibodies (1/200 dilution) as described in Chapter 3.

APPENDIX B

REGULATION OF *HO* EXPRESSION BY α-FACTOR AND CDC28

INTRODUCTION

HO encodes an endonuclease responsible for mating type interconversion in *Saccharomyces cerevisiae*. Transcription of *HO* is regulated in at least three different ways: 1. *HO* is expressed in **a** and α cells, but not in **a**/α diploids; 2. *HO* is expressed only in a window in the cell cycle, in late G1; 3. *HO* is expressed in mother cells and not daughter cells. The promoter sequences in *HO* as well as many *trans*-acting factors involved in the regulation of *HO* have been identified and are being characterized (see Herskowitz, 1989 for review).

The cell cycle regulation of *HO* expression is dependent on the cell cycle regulator *CDC28*. *HO* transcription is off in some *cdc28* temperature sensitive strains at restrictive temperatures as well as in α-factor-arrested cells (Nasmyth, 1983). The CCB (cell cycle box) sequence is present in multiple copies dispersed in the HO promoter and is sufficient to confer cell cycle regulation (Nasmyth, 1985; Breeden and Nasmyth, 1987). Two trans-acting factors, SWI4 and SWI6, activate transcription through the CCB elements in a cell cycle dependent manner(Breeden and Nasmyth, 1987), and SWI4 has been found to be part of a complex (CCBF) which binds specifically to the CCB sequence (Andrews and Herskowitz, 1990). One very simple and attractive model is that the cell cycle regulation of *HO* might result from the activation of a component of CCBF by phosphorylation by the CDC28 protein kinase. Components involved in the activation of CCBF are potential substrates of CDC28 during the G1/S transition. Identification and

characterization of CDC28 substrates could lead to advances in the understanding of how CDC28 functions to drive a cell from G1 into S phase.

This section presents experiments concerning two aspects of *HO* regulation. First, I describe evidence that α-factor regulates *HO* expression, independent of cell cycle arrest. Second, I give some genetic evidence that *CDC28* and not just cell cycle position per se activates *HO* in G1 of the cell cycle.

RESULTS

The Inhibition of *HO* Expression by α-factor is Independent of Arrest.

Cells treated with α-factor do not express *HO* (Nasmyth, 1983). Since α-factor inhibits CDC28 activity, and *cdc28* mutants do not express *HO*, the effect of α-factor has been interpreted to be a consequence of α-factor turning off CDC28 activity and arresting the cell at a point in the cell cycle where *HO* is not expressed. I describe here two lines of evidence that indicate that α-factor may have negative effects on *HO* in addition to its cell cycle effect.

The assay for *HO* expression in these experiments was an *HO-lacZ* fusion, which contained the N-terminal 88 amino acids fused to *lacZ*, and which was integrated into the chromosome and thus contained the full promoter (Russell et al., 1986). Although the effects on lacZ activity were assumed to be transcriptional, RNA levels were not directly assayed in this experiments and thus effects on protein synthesis and stability of the lacZ fusion cannot be ruled out.

Figure 1 shows that *HO-lacZ* activity drops when cells are treated with α-factor, with a half life of approximately one hour. This fusion is thus sufficiently unstable to measure a reduction in *HO* transcription.

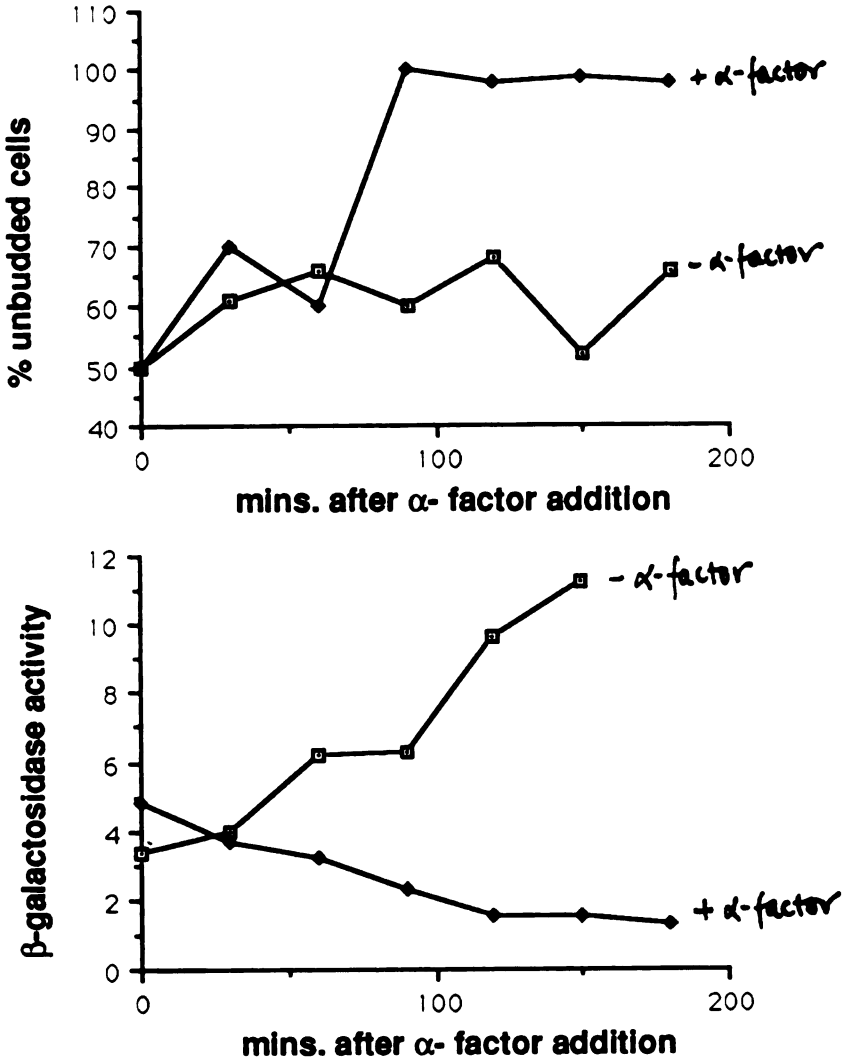


Figure 1: *HO-lacZ* activity decreases in response to α-factor.
α-factor ($6x10^{-8}$M) was added to a culture of wildtype cells (FC115-5A *bar1-1 HO-lacZ*) at time 0, and fractions were removed every 30 minutes and assayed for **A.** % unbudded cells, showing fraction of cells in G1. **B.** *HO-lacZ* activity as measured by the total β-galactosidase activity/ ml culture (not normalized to cell mass).

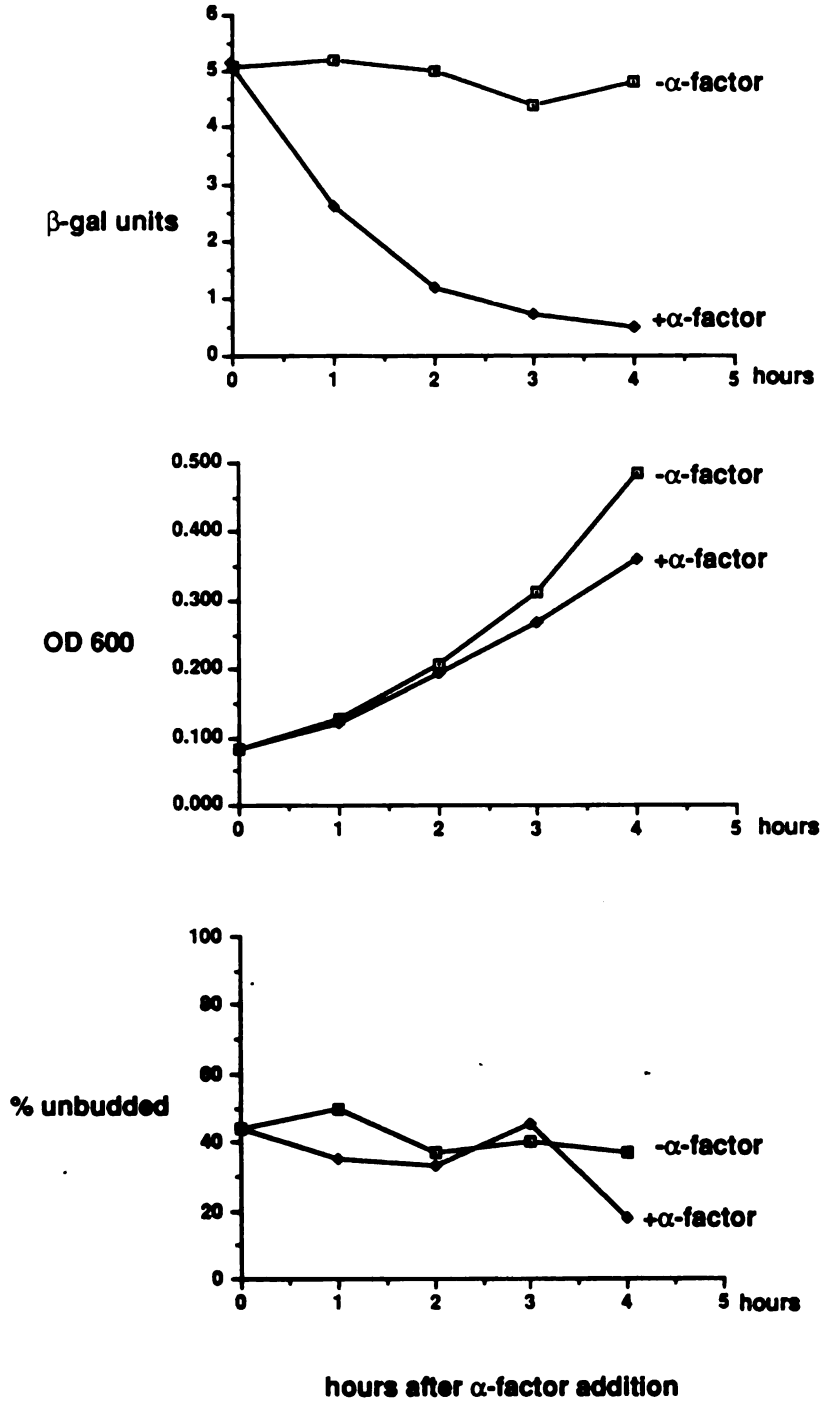


Figure 2: *HO-lacZ* activity decreases in response to α-factor in *far1* cells. α-factor (10^{-6}M) was added to a culture of *far1* cells (FC1022-8C *a bar1-1 far1-1 HO-lacZ*) at time 0. Fractions were removed at the indicated times and assayed for %unbudded cells, OD_{600}, and *HO-lacZ* activity. **A.** β-galactosidase activity as normalized to cell mass (OD_{600}). **B.** OD_{600} showing cell growth **C.** % unbudded cells, showing the fraction of cells in G1.

The *far1* mutant does not arrest with α-factor but still expresses many responses associated with α-factor response, such as induction of mating genes and morphological changes (Chang and Herskowitz, 1990). I tested whether α-factor affects *HO* expression in the absence of arrest by assaying *HO-lacZ* expression in a *far1* mutant. *HO-lacZ* was found to drop in the *far1* mutant after addition of α-factor(Figures 2B and 2C). The *far1* mutant in this experiment did not exhibit cell cycle arrest, as shown by the low percentage of unbudded cells throughout the experiment (Figure 2A). These observations indicate that α-factor negatively regulates *HO* expression even in the absence of cell-cycle arrest.

I found a further indication that α-factor affects *HO* expression. Cells released from starvation express a burst of *HO* in G1 of the first cell cycle after release Nasmyth et al., (1987a). Figure 3 shows that cells arrested by α-factor behave differently. Following release from the α-factor block, cells, which synchronously progress through the cell cycle, do not express *HO-lacZ* in the first cell cycle, although they do in the second cell cycle. This observation was also made concurrently by Nasmyth et al.(1987a), measuring *HO* RNA levels. Thus α-factor inhibits *HO* expression in the subsequent cell cycle after the α-factor has been washed away.

cdc28-1 Produces Hyperexpression of *HO*

Two types of models exist for why *HO* is not expressed in a cell arrested a *cdc28-4* allele at restrictive temperature: first, *HO* is not expressed in that part of the cell cycle; second, *HO* is activated directly by CDC28. I have found some genetic evidence for the second model.

Previous observations showed that *HO* is not expressed in *cdc28-4* mutants at restrictive temperature (Nasmyth, 1983) I assayed *HO-lacZ* activity in a strain carrying the *cdc28-1* allele, which was the only *cdc28* allele available in the laboratory. The *cdc28-1* strain exhibited a striking induction of *HO-lacZ* (Figure 4) when the cells

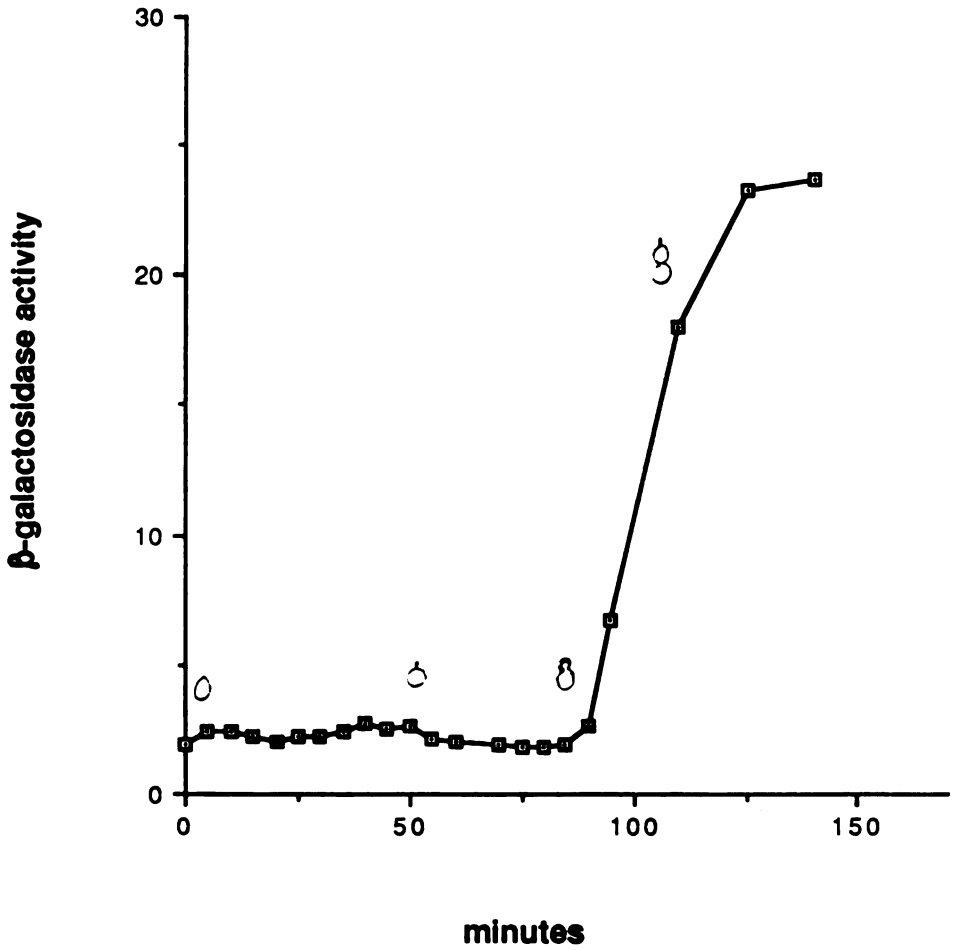


Figure 3: *HO-lacZ* is not expressed in the first cell cycle following release from arrest by α-factor.

FC115-5A (a *bar1-1 HO-lacZ*) cells were arrested with $6x10^{-8}$M α-factor in YEPD for 95 mins. The cells were then washed and resuspended in YEPD media without α-factor. Alloquats were removed at the indicated times (every 5 to 15 minutes) for *HO-lacZ* assays and every 20 minutes for examination of cell morphology to moniter cell cycle synchrony. In the first cell cycle, cells were >80% synchronous on the basis of bud size. Drawings of cells represent the morphology of cells present at that particular time point. β-galactosidase activity represents total activity per ml of cells (not normalized to cell mass).

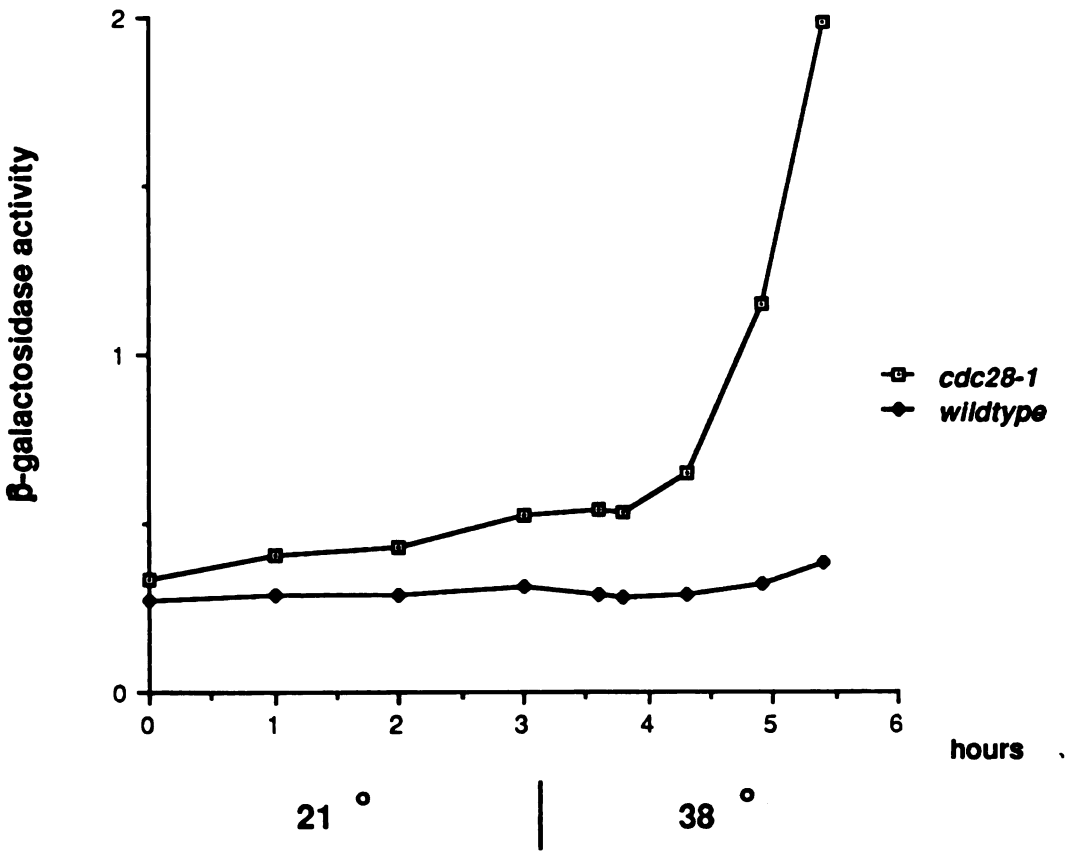


Figure 4: *HO-lacZ* is induced in a *cdc28-1* strain at restrictive temperature. FC121-1C (*cdc28-1 HO-lacZ*) and IH1107 (wildtype *HO-lacZ*) were resuspended from a fresh plate grown at 21° into a YEPD culture at time 0 and were grown in YEPD at 21° for 3.2 hrs. The culture was then shifted to 38°. Examination of cell morphology showed that most cells in both strains were budding before the shift to 38°, and after 4 hours at 38°, most of the FC121-1C cells were arrested and formed projections. (Similar results on *HO-lacZ* expression were also obtained in temperature shift experiments using logarithmic cultures of 121-1C)

were shifted to restrictive temperature. The temperature shift did not affect *HO-lacZ* levels in a wildtype strain (Figure 4). The *cdc28-1* allele thus exhibited an opposite phenotype to the *cdc28-4* allele: *HO* is expressed in the *cdc28-1* mutant and is not expressed in *cdc28-4* mutant. In both cases, the cells arrest as unbudded cells (data not shown).

One explanation for this difference in alleles is that *CDC28*, and not merely cell cycle position, activates *HO*. The cdc28-4 mutant product may be inactive for all *CDC28* functions, while cdc28-1 mutant product may be able to phsophorylate some substrates but not all. One of the substrates still activated in *cdc28-1* leads to the activation of *HO* expression. However, some substrates necessary for cell cycle progression are not activated in *cdc28-1*, and thus the cell arrests.

Effect of Individual *CLN* genes on *HO* Expression.

The *CDC28* protein kinase is thought to be activated during G1 by the three G1 cyclins, *CLN1*, *CLN2*, and *CLN3* (Richardson et al., 1989). These *CLN* genes appear to be functionally redundant, so that one or two *CLN* genes can be deleted without causing cells to arrest. However, some strains deleted for two *CLN* genes possess morphological abnormalities and slow growth, suggesting that, although the *CLN* genes can functionally substitute for each other, the *CLN* genes may not be entirely equivalent. The cyclins have been proposed to impart substrate specificity on the CDC28 protein kinase (see Richardson et al, 1989). Thus, it is possible that the different *CLN* products confer slightly different substrate specificity on *CDC28*. Since the observations above suggest that *HO* might be a nonessential substrate of CDC28, I tested whether different *CLN* genes might affect *HO* expression differently.

A plasmid containing a *lacZ* fusion driven by a fragment containing multiple CCB elements which confer cell-cycle regulation was transformed into strains in which two of the three *CLN* genes had been deleted. The *CCB-lacZ* fusion was expressed equally in a

wildtype, *cln1 cln2*, *cln1 cln3*, and *cln2 cln3* strains, as measured by filter assay. This result suggests that the different *CLN* genes at do not affect *HO* expression (CCB activity) differently.

DISCUSSION

α-factor inhibits *HO* expression.

Observations presented here show that α-factor negatively regulates *HO* expression independently of cell cycle arrest. The strongest piece of evidence is that synthesis of *HO* RNA is still repressed by α-factor in a *far1* mutant, which does not arrest. I propose that some product in the α-factor response pathway (such as a *STE* gene) acts to inhibit the activity or synthesis of one the activators (a *SWI* gene) necessary for *HO* expression. In a wildtype cell, α-factor might inhibit *HO* expression in two ways: by directly inhibiting an activator of *HO*, and by arresting the cell cycle, which inhibits CCBF activity.

Which *SWI* gene(s) might be targeted by the α–factor response pathway? α-factor turns off *HO* expression in the cell cycle after cells recover from α-factor arrest. Nasmyth et al. (1987b) have found that cells carrying a *sin3* mutation express *HO* in the first cell cycle after an α-factor arrest. The *sin3* mutation makes *HO* expression independent of *SWI5* (Sternberg et al., 1986; Nasmyth et al., 1987b) The suppression of the α-factor effect by *sin3* suggests that α-factor may inhibit *HO* expression by inhibiting *SWI5*. A direct test of this model would be to examine the effect of α-factor in *far1 sin3* double mutants; the model would predict that the *sin3* mutation would prevent the inhibition of *HO* transcription seen in *far1* cells in response to α-factor.

HO is one of several products whose activity or synthesis is repressed by α-factor, including the *CLN* genes (Wittenberg et al., 1990) and glycogen synthase (Francois et al., 1991). The negative regulation of *HO* is most similar to the inhibition of *CLN1*:

transcription of both genes is regulated in the cell cycle and are rapidly repressed in response in α-factor. The repression in both cases is largely independent of *FAR1* activity. One possibility might be that these two genes could share a common trans-acting factor, such as SWI4 or SWI5.

Why should α-factor regulate *HO* expression? One reason could be that it is advantageous for cells not to switch mating type when cells of the opposite mating type are in the vicinity.

CDC28 activates *HO* expression

One of the most exciting aspects of studying the cell cycle regulation of *HO* is that the trans-activators are potential substrates for central cell cycle regulators, such as *CDC28*. The *cdc28-1* allele uncouples two activities of *CDC28*: activation of *HO* and progression through G1. This uncoupling provides strong evidence that *CDC28* may have multiple substrates at G1. This observation also supports the idea that *HO* may represent a direct or indirect substrate of *CDC28*. Although the effect of *CDC28* on *HO* may be many steps removed, the amino acid sequence of *SWI4* contains *CDC28* phosphorylation consensus sequences (Andrews and Herskowitz, 1990) and raises the possibility that the CDC28 protein kinase may directly phosphorylate *SWI4*.

EXPERIMENTAL PROCEDURES

Strains and Materials: FC115-5A (**a** *ura3 leu2 his3 met⁻ bar1-1* HO^{88}*-lacZ*) was a segregant of cross between IH1402 and IH917. FC1022-8C (**a** *ura3 leu2 trp1 met-bar1-1 far1-1*) was a segregant in a cross between 115-5A and FC1002-2D (α *far1-1*) The *HO-lacZ* construct in these strains encodes 88 aa of HO fused to lacZ (Russell et al., 1987). FC121-1C (**a** *tyr1 met⁻ his⁻ lys2 leu2 cdc28-1* HO^{46}*-lacZ*) was a

segregant of a cross between IH1778 and FC117-2B (derived from IH1213 and IH1107). The *HO-lacZ* construct in FC121-1C and IH1107 was obtained from K. Nasmyth and encodes 46 aa of HO fused to lacZ. The CCB-lacZ fusion was a gift from B. Andrews. The *cln* strains were FC317 (**a** *cln3Δ cln2::LEU2*), FC320 (**a** *cln3Δ cln1::TRP1*), IH2370 (**a** *cln1::TRP1 cln2::LEU2*), which were gifts from H. Richardson.

β-galactosidase Assays: 1 ml aliquots of cultures were quick frozen in a dry ice bath. Cells were then thawed, and β-galactosidase assays were performed as described in Chapter 1.

APPENDIX C

STRAIN LIST

The FC140 isogenic set: all these strains are *HMRa HMLa ura3-52 met1* very leaky *lys bar1-1* This is the strain for the original *far* mutant hunt.

FC139 **a**

FC140 **a**

FC146 **a** pSB234 (2u *FUS1-lacZ*)

D1 **a** *far1-1*

E2 **a** *far1-2*

FF **a** *far1-3*

FC148 **a** *far1-1* pSB234

FC170 **a** *far1-a* (Tn3::URA3 insertion mutant)

FC171 **a** *far1-b* "

FC172 **a** *far1-c* "

FC173 **a** *far1-e* "

FC174 **a** *far1-g* "

FC175 **a** *far1-h* "

FC200 **a** *FUS1-lacZ::URA3* integrated

FC201 α *FUS1-lacZ::URA3* integrated

FC202 **a** *far1-1 FUS1-lacZ::URA3* integrated

FC203 **a** *far1-c FUS1-lacZ::URA3* integrated

FC204 **a** *far1::URA3* (pFC13 replacement)

FC209 **a**/α diploid

FC212 α *far1-c*

FC216 α *far1-1*

FC224 **a** *bud5::URA3*

FC225 **a** *bud5::URA3 far1-c*

FC271 **a** *far1-1 gpa1::URA3*

FC273 α *far1-1 gpa1::URA3*

FC296 α *far1::URA3*

FC311 **a** *FUS1-lacZ URA3* integrated

FC312 **a** *far1::URA3 FUS1-lacZ URA3* integrated

The 15D isogenic set:
all these strains are isogenic to BF264-15D (S. Reed strain) and contain ura3-del trp1 leu2 ade1 his2. The strain is good for gal inductions.

FC278 α *cln3::URA3*

FC279 **a** *bar1::LEU2*

FC280 **a** *far1::URA3 bar1::LEU2*

FC281 **a** *bar1::LEU2* pFC24 (*GAL-FAR1 TRP1* cen plasmid)

FC283 **a** *far1::URA3 bar1::LEU2 pFC24 (GAL-FAR1 TRP1* cen plasmid)

FC289 **a** *cln1::TRP1 far1::URA3 bar1::LEU2*

FC290 **a** *cln1::TRP1 cln2::LEU far1::URA3 bar1::LEU2*

FC291 **a** *cln2::LEU bar1::LEU2*

FC292 **a** *cln1::TRP1*

FC293 α *cln2::LEU2 cln3::URA3*

FC294 α *cln1::TRP1 cln3::URA3*

FC297 α *far1::URA3 (BAR+)*

FC308 **a**/α pFC24 (*GAL-FAR1 TRP1* cen plasmid)

FC310 **a** *far1::URA3 cln2::LEU2 bar1::LEU2*

FC313 **a** *ste4::LEU2 pFC24 (GAL-FAR1 TRP1* cen plasmid)

FC315 **a** *ste12::LEU2 pFC24 (GAL-FAR1 TRP1* cen plasmid)

FC317 α? *cln2::LEU2 cln3::del* (from F. Cross)

FC318 **a** *ste5::URA3 bar1::LEU2*

FC319 **a** *far1::URA3 cln3::URA3 cln1::TRP1 bar1::LEU2*

FC320 α? *cln1::TRP1 cln3::del*

FC321 **a** *far1::URA3 cln1::TRP1 cln3::URA3 bar1::LEU2*

FC322 **a** *far1::URA3 cln2::LEU2 cln3::URA3 bar1::LEU2*

FC329 **a** *far1::URA3 cln3::URA3 bar1::LEU2*

FC336 **a** *cln1::TRP1 cln3::URA3 bar1::LEU2*

FC339 **a** *cln3::URA3 bar1::LEU2*

FC375 **a** *cln1::TRP1 cln3::del cln2::GAL-CLN2::LEU2* from Pierre Leopold

<u>Other strains:</u>

FC1001-1C **a** *trp1 leu1 met1 ade5 bar1-1*

FC1002-1B **a** *ura3 leu2 his lys far1-1 bar1-1*

FC1002-2D **a** *ura3 leu trp1 met1 far1-1*

FC1011-2C *mata1::TRP1 ura3 leu2 his4 trp1 far1-1*

FC177 **a** *far1-c (Tn3::URA3) ura3-52 leu2 trp1 his4 can1* (EG123)

KMY381-1C α *ura3 trp1 his3 sgv1-1* (K. Matsumoto)

KMY381-1D **a** *ura3 trp1 his3 leu2 lys2 ade8 sgv1-1*

I-24 **a** *bar1 DAF1-1x8* (Fred Cross)

I-8 **a** *bar1* isogenic to IH2315

FC345 **a** *FUS1-lacZ::LEU2* pDB20 (*ADH* promoter vector *URA3*)

FC347 **a** *FUS1-lacZ::LEU2* pDB20-CLN2 (*ADH-CLN2* plasmid *URA3*)

406 **a** *trp1-289 ura3-52 his7 can1 cdc4-1*

a *his7 ura1 cdc8-1* (A. Murray)

a *his7 ura1 cdc13-1*

a *his7 ura1 cdc14-1*

a *his7 ura1 cdc20-1*

a *his7 ura1 cdc28-4* (P. Sorger)

APPENDIX D: REFERENCES

Anderegg, R.J., R. Betz, S.A. Carr, J.W. Crabb, W. Duntze. (1988). Structure of *Saccharomyces cerevisiae* mating hormone a-factor. J. Biol. Chem. 263: 18236-18240.

Andrews, B. J. and I. Herskowitz. (1989). The yeast SWI4 protein contains a motif present in developmental regulators and is part of a complex involved in cell-cycle-dependent transcription. Nature. 342: 830-833.

Ausubel, F. M., R. Brent, R. E. Kingston, D. D. Moore, J. G. Seidman, J. A. Smith and K. Struhl. (1989). Current protocols in molecular biology.

Beach, D., B. Curkacz and P. Nurse. (1982). Functionally homologous cell cycle control genes in budding and fission yeast. Nature. 300: 706-709.

Bender, A. and G. F. J. Sprague. (1986). Yeast peptide pheromones, α-factor and a factor, activate a common response mechanism in their target cells. Cell. 47: 929.

Bennetzen J.L. and B.D. Hall. (1982). The primary structure of the *Saccharomyces cerevisiae* gene for alcohol dehydrogenase I. J. Biol Chem. 257: 3018-3025.

Berlin, V., C.A. Styles, and G.R. Fink. (1990). BIK1, a protein required for microtubule function during mating and mitosis in *Saccharomyces cerevisiae*, colocalizes with tubulin. J. Cell Biol. 111: 2573-2586.

Bishop, M. (1991). Molecular themes in oncogenesis. Cell 64: 235-248.

Blumer, K. J., J. E. Renke and J. Thorner. (1988). The STE2 gene product is the ligand-binding component of the α factor receptor of *Saccharomyces cerevisiae*. J. Biol. Chem. 263: 10836.

Blumer, K. J. and J. Thorner. (1990). Beta and gamma subunits of a yeast guanine nucleotide-binding protein are not essential for membrance àssociation of the alpha subunit but are required for receptor coupling. Proc. Nat. Acad. Sci. 87: 4363-4367.

Boeke, J. D., H. Xu and G. R. Fink. (1988). A general method for the chromosomal amplification of genes in yeast. Science. 239: 280-282.

Booher, R.N. C.E. Alfa, J.S. Hyams, and D.H. Beach. (1989). The fission yeast cdc2/cdc13/suc1 protein kinase: regualtion of catalytic activity and nuclear localization. Cell 58: 485-497.

Buchkovich, K., L.A. Duffy and E. Harlow. (1989). The retinoblastoma protein is phosphorylated during specific phases of the cell cycle. Cell 58: 1097-1105.

Bucking-Throm, E., W. Duntze, L. H. Hartwell and T. R. Manney. (1973). Reversible arrest of haploid cells at the initiation of DNA synthesis by a diffusible sex factor. Exp. Cell Res. 76: 99-110.

Burkholder, A. C. and L. H. Hartwell. (1985). The yeast α factor receptor: Structural properties deduced from the sequence of the *STE2* gene. Nucleic Acids Res. 13: 8463-8475.

Byers, B and L. Goetxch. (1974). Behaviors of spindles and spindle plaques in the cell cycle and conjugation in *Saccharomyces cerevisiae.* J. Bacteriol. 124: 511-523.

Cantley, L.C., K.R. Auger, C. Carpenter, B. Duckworth, A. Granziani, R. Kapeller, and S. Soltoff. (1991). Oncogenes and signal transduction. Cell 64: 281-302.

Chaleff, D. T. and K. Tatchell. (1985). Molecular cloning and characterization of the *STE7* and *STE11* genes of *Saccharomyces cerevisiae.* Mol Cell. Biol. 5: 1878-1886.

Chan, R. K. and C. A. Otte. (1982). Isolation and genetic analysis of *Saccharomyces cerevisiae* mutants supersensitive to G1 arrest by a-factor and α-factor pheromones. Mol. Cell. Biol. 2: 11-20.

Chen, P.L., R. Scully, J.Y. Shew, J.Y.J. Wang, and W.H. Lee. (1989). Phosphorylation of the retinoblastoma gene product is modulated during the cell cycle and cellular differentiation. Cell 58: 1193-1198.

Conde, J. and G.R. Fink. (1976). A mutant of *Saccharomyes cerevisiae* defective for nuclear fusion. Proc. Nat. Acad. Sci. USA. 73: 3651-3655.

Cole, G.M., D.E. Stone, and S.I. Reed. (1990). Stoichiometry of G protein subunites affects the *Saccharomyces cerevisiae* mating pheromone signal transduction pathway. Mol. and Cell. Biol. 10: 510-517.

Cross, F., L. H. Hartwell, C. Jackson and J. B. Konopka. (1988). Conjugation in *Saccharomyes cerevisiae.* Annu. Rev. Cell Biol. 4: 429-457.

Cross, F. R. (1988). DAF1, a mutant gene affecting size control, pheromone arrest, and cell cycle kinetics of *Sadccharomyces cerevisiae.* Mol. Cell. Biol. 8(11): 4675-4684.

Cross, F., J. Roberts, and H. Weintraub. (1989). Simple and complex cell cycles. Annu.Rev. Cell Biol. 5: 341-395.

de Barros Lopes, M., J.Y. Ho, S.I. Reed. (1990). Mutations in cell division cycle genes *CDC36* and *CDC39* activate the *Saccharomyces cerevisiae* mating pheromone response pathway. Mol. Cell Biol. 10: 2966-2972.

DeCaprio, J.A., J.W. Ludlow, J. Figge, J.W. Shew, C.M. Huang, W.H. Lee, E. Marsilio, E. Paucha, and D.M. Liveingston. 1988. SV40 large tumor antigen forms a specific complex with the product of the retinoblastoma susceptibility gene. Cell 54: 275-283.

DeCaprio, J.A., J.W. Ludlow, D. Lynch, Y. Lurukawa, J. Griffin, H. Piwnica-Worms, C.M. Huang, and D.M. Livingston. (1989). The product of the retinoblastoma susceptibility gene has properties of a cell cycle regulatory element. Cell 58: 1085-1095.

Dietzel, C. and J. Kurjan. (1987). The yeast *SCG1* gene: a Gα-like protein implicated in the a- and α– factor response pathway. Cell. 50: 1001-1010.

Dietzel, C. and J. Kurjan. (1987). Pheromonal regulation and sequence of the *Saccharomyces cerevisiae SST2* gene: A model for desensitization to pheromone. Mol. Cell. Biol. 7: 416-427.

Dolan, J. W., C. Kirkman and S. Fields. (1989). The yeast STE12 protein binds to the DNA sequence mediating pheromone induction. Proc. Nat. Acad. Sci. USA. 86: 5703-5707.

Dunphy, W.G., L. Brizuela, D. Beach, and J. Newport. (1988). The Xenopus cdc2 protein kinase is a component of MPF, a cytoplasmic regulator of mitosis. Cell 54: 423-431.

Draetta, G., F. Luka, J. Westendorf, L. Brizuela, J. Ruderman and D. Beach. (1989). CDC2 protein kinase is complexed with both cyclin A and B: Evidence for proteolytic inactivation of MPF. Cell. 56(829-838):

Dutcher, S. K. and L. H. Hartwell. (1982). The role of *Saccharomyces cerevisiae* cell division cycle genes in nuclear fusion. Genetics. 100: 175-184.

Errede, B. and G. Ammerer. (1989). STE12, a protein involved in cell-type specific transcription and signal transduction in yeast is part of protein-DNA complexes. Genes and Dev. 3: 1349-1361.

Evans, T., E. T. Rosenthal, J. Youngblom, D. Distel and T. Hunt. (1983). Cyclin: A protein specified by materanl mRNA in seq urchin eggs that is destroyed at each cleavage division. Cell. 33: 389-396.

Field, C., and R. Schekman. (1980). Localized secretion of acid phophatase reflects the pattern of cell surface growth in Saccharomyces cerevisiae. J. Cell Biol. 86: 123-128.

Fields, S. and I. Herskowitz. (1985). The yeast *STE12* is required for two sets of cell-type specific expression. Cell. 42(923):

Fields, S., D. T. Chaleff and G. F. J. Sprague. (1988). Yeast *STE7*, *STE11*, and *STE12* genes are required for expression of cell-type-specific genes. Mol. Cell. Biol. 8: 551.

Francois, J., D.L. Higgens, F. Chang, and K. Tatchell. (1991). Inhibition of glycogen synthesis in Saccharomyces cerevisiae by the mating pheromone α-factor. J. Biol. Chem. in press.

Freifelder, D. (1960). Bud formation in *Saccharomyces cerevisiae*. J. Bacteriol. 80: 567-568.

Gautier, J., C. Norbury, M. Lohka, P. Nurse, and J. Maller. (1988). Purified maturation-promoting factor contains the product of a Xenopus homolog of the fission yeast cell cycle control gene *cdc2+*. Cell 54: 433-439.

Gautier, J., J. Minshull, M. Lohka, M. Glotzer, T. Hunt, and J.L. Maller. (1990). Cyclin is a component of maturation-promoting factor from Xenopus. Cell 60: 487-494.

Gould, K.L. and P. Nurse. (1989). Tyrosine phosphorylation of the fission yeast cdc2+ protein kinase regulates entry into mitosis. Nature 342: 39-45.

Gould, K.L., S. Moreno, N.K. Tonks, and P. Nurse. (1990). Complementation of the mitotic activator, p80cdc25, by a human protein-tyrosine phosphatase. Science 250; 1573-1576.

Glotzer, M., A.W. Murray, and M.W. Kirschner. (1991). Cyclin is degraded by the ubiquitin pathway. Nature, in press.

Hadwiger, J. A., C. Wittenberg, H. E. Richardson, M. D. B. Lopes and S. I. Reed. (1989). A family of cyclin homologues that control the G1 phase in yeast. Proc. Natl. Acad. Sci. USA. 86:

Hagen, D. C. and G. F. J. Sprague. (1984). Induction of the yeast a-specific STE3 gene by the peptide pheromone a factor. J. Mol. Biol. 178: 835.

Hagen, D. C., G. McCaffrey and G. F. J. Sprague. (1986). Evidence the yeast STE3 gene encodes a receptor for the peptide pheromone, a factor: Gene sequence and implications for the structure of the presumed receptor. Proc. Natl. Acad. Sci. USA. 83: 1418.

Hansen M.F. and W.K. Cavenee. (1988). Retinoblastoma and the progression of tumor genetics. Trends Genet. 4: 125-128.

Hartig, A., G. Holly, G. Saari and V. L. MacKay. (1986). Multiple regulation of *STE2*, a mating-type-specific gene of *Saccharomyces cerevisiae*. Mol. Cell. Biol. 6: 2106.

Hartwell, L.H. and M.W. Unger. (1977). Unequal division in *Saccharomyces cerevisiae* and its implications for the control of cell division. J. Cell Biol. 75: 422-435.

Hartwell, L. H., J. Culotti, J. R. Pringle and B. J. Reid. (1974). Genetic control of the cell division cycle in yeast. Science. 183: 46-51.

Hartwell, L. H. (1980). Mutants of *Saccharomyes cerevisiae* unreponsive to cell division control by polypeptide mating hormones. J. Cell Biol. 85: 811-822.

Hartwell, L. H. and T. A. Weinert. (1989). Checkpoints: controls that ensure the order of cell cycle events. Science. 246(629-634):

Hasek J., I. Rupes, J. Svobodova, and E. Streiblova. (1987). Tubulin and actin topology during zygote formation of *Saccharomyces cerevisiae*. J. Gen. Microbiol. 133: 3355-3363.

Herskowitz, I. and L. Marsh. (1987). Conservation of a recptor/signal transduction system. Cell. 50: 995-996.

Herskowitz, I. (1989). A regulatory hierachy for cell specialization in yeast. Nature. 342: 749-757.

Hicks, J. B. and I. Herskowitz. (1976). Interconversion of yeast mating types I. Direct observation fo the action of the homothallism (HO) gene. Genetics. 83: 245-258.

Hicks, J. B. and I. Herskowitz. (1976). Evidence for a new diffusible element of mating pheromones in yeast. Nature. 260: 246-248.

Hicks, J. B. and I. Herskowitz. (1976). Interconversion of yeast mating types. 1. Direct observation of the action of the homothallism (HO) gene. Genetics. 83: 245-258.

Hoffman, C. S. and F. Winston. (1987). A ten-minute DNA preparation from yeast efficiently releases autonomous plasmids for tranformation of *Escherichia coli*. Gene. 57: 267-272.

Iida H, Y.Yagawa, Y. Anraku. (1990). Essential role for induced $Ca2^+$ influx followed by $[Ca2^+]i$ rise in maintaining viability of yeast cells late in the mating pheromone

response pathway. A study of [Ca2+]i in single *Saccharomyces cerevisiae* cells with imaging of fura-2. J. Biol. Chem.265 :13391-9.

Ito, H., Y. Fukuda, K. Murata and A. Kimura. (1983). Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. 153: 163-168.

Jackson, C. L. and L. H. Harwell. (1989). Courtship in yeast: an early cell-cell interation during mating. Mol. Cell. Biol. :

Jackson, C. L. and L. H. Harwell. (1990). Courtship in yeast: Both cell types choose mating partners by responding to the strongest pheromone signal. Cell 63: 1039-1051.

Jahng, K. Y., J. Ferguson and S. I. Reed. (1988). Mutations in a gene encoding the a subunit of a *Saccharomyces cerevisiae* G protein indicate a role in mating pheromone signalling. Mol. Cell. Biol. 8(2484): 2484-2493.

Jenness, D. D. and P. Spatrick. (1986). Down regulation of the α-factor pheromone receptor in *S. cerevisiae.* Cell. 46: 345.

Jenness, D.D., B.S. Goldman, L.H. Hartwell. (1987). *Saccharomyces cerevisiae* mutants unresponsive to alpha-factor pheromone: alpha-factor binding and extragenic suppression. Mol. Cell. Biol. 7: 1311-1319.

Jensen, R., G. F. J. Sprague and I. Herskowitz. (1983). Regulation of yeast mating-type interconversion: Feedback control of HO gene expression by the mating-type locus. Proc. Natl. Acad. Sci. USA. 80: 3035-3039.

Johnson, D.I. and J. Pringle. Molecular characterization of CDC42, a *Saccharomyces cerevisiae* gene involved in the development of cell polarity. J. Cell Biol. 111: 143-152.

Johnston, G.C., J.A. Predergast, and R.A. Singer. (1991). The S. cerevisiae *MYO2* gene encodes an essential mysoin for vectorial transport of vesicles. Submitted.

Kang Y.S., J. Kane, J. Kurjan, J.M. Stadel, D.J. Tipper. (1990). Effects of expression of mammalian G alpha and hybrid mammalian-yeast Galpha proteins on the yeast pheromone response signal transduction pathway. Mol. Cell. Biol. 10 :2582-90.

Keller, E.F. (1983). A Feeling for the Organism: the Life and Work of Barbara McClintock. New York: W. H. Freeman and Company.

Konopka, J. B., D. D. Jenness and L. H. Hartwell. (1988). The C-terminus of the *S. cerevisiae* α–pheromone receptor mediates an adaptive response to pheromone. Cell. 54: 609-620.

Kronstad, J. W., J. A. Holly and V. L. MacKay. (1987). A yeast operator overlaps an upstream activation site. Cell. 50: 369-377.

Laiho, M., J.A. DeCaprio, J.W. Ludlow, D.W. Livingston, and J. Massague. (1990). Growth inhibition of TGF-β linked to suppression of retinoblastoma protein phosphorylation. Cell 62: 175-185.

Laiho, M., L. Roonstrand, J. Heino, J.A. DeCaprio, J.W. Ludlow, D.W. Livingston, and J. Massague. (1991). Control of JunB and extracellular matrix protein expression by

transforming growth factor-b1 is independent of Simian Virus 40 T Antigen-sensitive growth-inhibitory events. Mol. Cell. Biol. 11: 972-978.

Lee, M. G. and P. Nurse. (1987). Complementation used to clone a human homologue of the fission yeast cell cycle control gene cdc2. Nature. 327: 31-35.

Levi, J. D. (1956). Mating reaction in yeast. Nature. 177: 753-753.

Lipke, P. N., D. Wojciechowicz and J. Kurjan. (1989). *AGα1* is the structural gene for the *Saccharomyces cerevisiae* a-agglunin, a cell surface glycoprotein involved in cell-cell interactions during mating. Mol. Cell. Biol. 9: 3155-3165.

Lohka, M. J., J. L. Kyes and J. L. Maller. (1988). Purification of maturation-promoting factor, an intracellular regulator of eacly mitotic events. Proc. Natl. Acad. Sci. USA. 85: 3009-3013.

Lord, P. G. and A. E. Wheals. (1984). Size control in a small size mutant of *Saccharomyces cerevisiae.* J. Gen. Microbiology. 130: 280;3-2808.

Lorincz, A. T. and S. I. Reed. (1984). Primary structure homology between the product of the yast cell civision control gene *CDC28* and vertebrate oncogenes. Nature. 307(183-185):

Ludlow, J.W., J.A. DeCaprio, C.M. Huang, W.H. Lee, E. Paucha, and D.M. Livingston. (1989). SV40 large T antigen binds preferentially to an underphosphorylated member of the retinoblastoma susceptibility gene product family. CdII 56: 57-65.

MacKay, V. L. and T. R. Manney. (1974a). Mutations affacting sexual conjugation and related processes in *Saccharomyes cerevisiae.* I. Isolation and phenotypic characterization of non-mating mutants. Genetics. 76: 255-271.

MacKay, V. and T. R. Manney. (1974b). Mutations affecting sexual conjugation and related processes in *Saccharomyes cerevisiae.* II. Genetic analysis of nonmating mutants. Genetics. 76: 273-288.

Maniatis, T., E. F. Fritsch and J. Sambrook. (1982). Molecular Cloning: A Laboratory Manual. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory.

Manney, T. R. and V. Woods. (1976). Mutants of *Saccharomyes cerevisiae* resistant to the α mating-type factor. Genetics. 82: 639-644.

Marsh, L. and I. Herskowitz. (1988). From membrane to nucleus: the pathway of siganl transduction in yeast and its genetic control. Cold Spring Harbor Symposia on Quantitative Biology. 53: 557-565.

Marsh, L. and I. Herskowitz. (1988). STE2 protein of *Saccharomyces kluyveri* is a member of the rhodopsin/β-adrenergic receptor family and is responsible for recognition of the peptide ligand a factor. Proc. Natl. Acad. Sci. 85: 3855-3859.

Massague, J. (1989). The transforming growth factor-β family. Annu. Rev. Cell Biol. 6: 597-641.

McCaffrey, G., F. Clay, K. Kelsay and G. F. Sprague Jr. (1987). Identification and regulation of a gene required for cell fusion during mating of the yeast *Saccharomyes cerevisiae.* Mol. Cell. Biol. 7: 2680-2690.

McKnight S.L. and R. Kingsbury. (1982). Transcriptional control signals of a eukaryotic protein coding gene. Science 217: 2316-2324.

McIntosh, J. R. and M. P. Koonce. (1989). Mitosis. Science. 246: 622-628.

Mendenhall, M. D., C. A. Jones and S. I. Reed. (1987). Dual regulation of the yeast CDC28-p40 protein kinase complex: cell cycle, pheromone, and nutrient limitation effects. Cell. 50: 927-935.

Meluh, P.B. and M.D. Rose. (1990). *KAR3*, a kinesin-related gene required for yeast nuclear fusion. Cell 60: 1029.

Michaelis, S. and I. Herskowitz. (1988). The a-factor pheromone of *Saccharomyces cerevisiae* is essential for mating. Mol. Cell. Biol. 8: 1309-1318.

Minshull, J., J. J. Blow and T. Hunt. (1989). Translation of cyclin mRNA is necessary for extracts of activated Xenopus eggs to enter mitosis. Cell. 56: 947-956.

Miyajima, I., M. Nakafuku, N. Nakayama, C. Brenner, A. Miyajima, K. Kaibuchi, K. Arai, Y. Kaziro and K. Matsumoto . (1987). *GPA1*, a haploid-specific essential gene, encodes a yeast homolog of mammalian G protein which may be involved in mating factor siganl transduction. Cell. 50: 1011-1019.

Miyajima, I., K. Arai and K. Matsumoto. (1989). *GPA1Val-50* mutation in the mating-factor signaling pathway in *Saccharomyces cerevisiae*. Mol. Cell. Biol. 9: 2289-2297.

Moreno, S., and P. Nurse. (1990). Substrates of p34cdc2. *In vivo veritas*? Cell 61: 549-551.

Murray, A. W. and M. W. Kirschner. (1989a). Dominoes and clocks: The union of two views of the cell cycle. Science. 246: 614-621.

Murray, A. W. and M. W. Kirschner. (198b). Cyclin synthesis drives the early embryonic cell cycle. Nature 339: 275-280.

Murray, A. W., M.J. Solomon, and M. W. Kirschner. (1989). The role of cyclin synthesis and degradation in the control of MPF activity. Nature 339: 280-286.

Nakayama, N., Y. Kaziro, K. I. Arai and K. Matsumoto. (1987). Common signal transduction system shared by *STE2* and *STE3* in haploid cells of *Saccharomyces cerevisiae*: Autocrine cell-cycle arrest results from forced expresssion of STE2. EMBO J. 6: 249.

Nakayama, N., Y. Kaziro, K. I. Arai and K. Matsumoto. (1988). Role of *STE* genes in the mating factor signaling pathway mediated by *GPA1* in *Saccharomyes cerevisiae*. Mol. Cell. Biol. 8(9): 3777-3783.

Nakayama, N., A. Miyajima and K. Arai. (1985). Nucleotide sequence of *STE2* and *STE3*, cell type-specific sterile genes from *Saccharomyces cerevisiae*. EMBO. 4: 2643-2648.

Nash, R., G. Tokiwa, S. Anand, K. Erickson and A. B. Futcher. (1988). The *WHI1+* gene of *Saccharomyces cereviesiae* tethers cell division to cell size and is a cyclin homolog. EMBO. 7(13): 4335-4346.

Nasmyth, K. (1983). Molecular analysis of a cell lineage. Nature 302: 670-676.

Nasmyth, K. and D. Shore. (1987). Transcriptional regulation in the yeast life cycle. Nature. 237: 1162.

Nurse, P. (1981). Genetic control of the yeast cell cycle: a reappraisal of 'start.'. The fungal nucleus. Cambridge, British Mycological Society.

Nomoto S, N. Nakayama, K. Arai K, K. Matsumoto. (1990). Regulation of the yeast pheromone response pathway by G protein subunits. EMBO J. 9:691-6.

Nurse, P. and Y. Bisset. (1981). Gene required in G1 for commitment to cell cycle and in G2 for control of mitosis in fission yeast. Nature. 292: 558-560.

O'Farrell, P. H., B. A. Edgar, D. Lakich and C. F. Lehner. (1989). Directing cell division during development. Science. 246: 635-640.

Pardee, A. B. (1989). G1 events and regulation of cell proliferation. Science. 246(603-608):

Peter, M, J. Nakgawa, M. Doree, J.C. Labbe, and E.A. Nigg. (1990). *In vitro* disassembly of the nuclear lamina and M phase-specific phsphorylation of lamins by cdc2 kinase. Cell 61: 591-602.

Pendergast, J., L. Murray, A. Rowley, D. Carruthers, R. Singer and G. Johnston. (1990). Size selection identifies new genes that regulate *Saccharomyces cerviesiae* cell proliferation. Cell. 124: 81-90.

Pientenpol, J.A., R.W. Stein, E. Moran, P. Yaciuk, R. Schlegel, R.M. Lyons, R.M. Pittelkow, K. Mugner, P.M. Howley, and H.L. Moses. (1990). TGF-β1 inhibition of c-myc transcription and growth in keratinocytes is abrogated by viral transforming protein with pRB binding domains. Cell 61: 777-785.

Pines, J. and T. Hunt. (1987). Molecular cloning and characterization of the mRNA for cyclin from sea urchin eggs. EMBO J. 6(10): 2987-2995.

Pines, J. and T. Hunter. (1989). Isolation of a human cyclin cDNA: evidence for cyclin mRNA and protein regulation in the cell cycle and for interaction with p34cdc2. Cell. 58: 833-846.

Polaina, J. and J. Conde. (1982). Genes involved in the control of nuclear fusion during the sexual cycle of *Saccharamyces cerevisiae*. Mol. Gen. Genet. 186: 253-258.

Pringle, J. R. and L. H. Hartwell. (1981). The *Saccharomyces cerevisiae* cell cycle. The Molecular Biology of the Yeast Saccharomyces. Life Cycle and Inheritance. Cold Spring Harbor, NY, Cold Spring Harbor Laboratory.

Pringle, J. R., R. A. Preston, A. E. M. Adams, T. Stearns, D. G. Drubin, B. K. Haarer and E. W. Jones. (1989). Fluorescence microscopy methods for yeast. Methods in Cell Biol. 3: 922-930.

Reed, S. I. (1980). The selection of *S. cerevisiae* mutants defective in the START event of cell division. Genetics. 95: 561-577.

Reed, S. I., J. A. Hadwiger and A. T. Lorincz. (1985). Protein kinase activity associated with the product of the yeast cell division cycle gene CDC28. Proc. Natl. Acad. Sci. USA. 82: 4055-4959.

Reid, B. J. and L. H. Hartwell. (1977). Regulation of mating in the cell cycle of *Saccharomyces cerevisiae*. J. Cell Biol. 75: 355-365.

Reneke, J. E., K. J. Blumer, W. E. Courchesne and J. Thorner . (1988). The carboxy-terminal segment of the yeast α-factor receptor is a regulatory domain. Cell. 55: 221-234.

Rhodes, N., L. Connell, and B. Errede. (1990). STE11 is a protein kianse required for cell-type-specific transcription and signal transduction in yeast. Genes and Devel. 4: 1862-1874.

Richardson, H. E., C. Wittenberg, F.Cross and S. I. Reed. (1989). An essential G1 function for cyclin-like proteins in yeast. Cell. 59: 1127-1133.

Roncero, C., M. H. Valdivieso, J. C. Ribas and A. Duran . (1988). Effect of calcufluor white on chitin synthases from Saccharomyes cerevisiae. J. Bact. 170(4): 1945-1949.

Rose, M. D., B. R. Price and G. R. Fink. (1986). *Saccharomyes cerevisiae* nuclear fusion requires prior activation by a-factor. Mol. Cell. Biol. 6: 3490-3497.

Rothstein, R. (1983). One-step gene disruption in yeast. Methods of Enzymol. 101: 202-209.

Russell, D.W., R. Jensen, M.J. Zoller, J. Burke, B. Errede, M. Smith, and I. Herskowitz. Structure of the *Saccharomyes cerevisiae HO* gene and analysis of its upstream regulatory region. Mol. Cell. Biol. 6: 4281-4294.

Russell, P. and P. Nurse. (1986). *cdc25+* functions as an inducer in the mitotic control of fission yeast. Cell 45: 145-153.

Russell, P. and P. Nurse. (1987). Negative regulation of mitosis by *wee1+*, a gene encoding a protein kinase homolog. Cell. 49: 559-567.

Russell, P. and P. Nurse. (1987). The mitotic inducer *nim1+* functions in a regulatory netword of protein kinase homologs controlling the initiation of mitosis. Cell. 49: 569-576.

Schekman, R. and V. Brawley. (1979). Localized deposition of chitin on the yeast cell surface in response to mating pheromone. Proc. Natl. Acad. Sci. USA. 76: 645-649.

Seifert, H. S., E. Y. Chen, M. So and F. Heffron. (1986). Shuttle mutagenesis: a method of transpososon mutagenesis for Saccharomyces cerevisiae. Proc. Natl. Acad. Sci. USA. 83: 735-739.

Sherman, F., G. R. Fink and J. B. Hicks. (1982). Methods in yeast genetics. Cold Spring Harbor, New York, Cold Spring Harbor Laboratory.

Simanis, V. and P. Nurse. (1986). The cell cycle control gene *cdc2+* of fission yeast encodes a proein kinase potenetially regulated by phosphorylation. Cell. 45: 261-268.

Sloat, B.F., A. Alison, and J.R. Pringle. (1981). Roles of the CDC24 gene product in cellular morphogenesis during the Sacchromyces cerevisiae cell cycle. J. Cell Biol. 89: 395-405.

Soloman, M., R. Booher, M. Kirschner and D. Beach. (1988). Cyclin in fission yeast. Cell. 54: 738-740.

Soloman, M, M. Blotzer, T.H. Lee,and M. Kirschner. (1990). Cyclin activation of p34cdc2. 63: 1013-1024.

Sorger P.K., M.J. Lewis, H.P. Pelham. (1987). Heat shock factor is regulated differently in yeast and HeLa cells. Nature 329: 81-84.

Sprague, G. F. J. and I. Herskowitz. (1981). Control of yeast cell type by the mating type locus. I. Identification and control of expression of the a-specific gene, *BAR1*. J. Mol. Biol. 153: 305-321.

Sprague, G.F.Jr., R.Jensen, and I. Herskowitz. (1983). Control of yeast cell type by the mating type locus: positive regulation of the a-speicifc *STE3* gene by the MAT a1 product. Cell 32: 409- .

Starnart, N., J. Minshull, J. Pnes and T. Hunt. (1987). Cyclin sysnthesis, modification and dstruction during meiotic maturation of the starfish ooctye. Devel. Biol. 124: 248-258.

Stern, M., R. Jensen and I. Herskowitz. (1984). Five *SWI* genes are required for expression of the *HO* gene in yeast. J. Mol. Biol. 178: 853-868.

Sternberg, P. W., M. J. Stern, I.Clark and I. Herskowitz. (1987). Activation of the yeast *HO* gene by release from multiple negative controls. Cell. 48: 567-577.

Stoltzer, D. and W. Duntze. (1976). Isolation and characterization of four related peptides exhibiting α-factor activity from *Saccharomyces cerevisiae*. Eur. J. Biodhem. 65: 257.

Streiblova, E. (1970). Study of scar fomation in the life cycle of heterothallic Can. J. Microbiol. 16: 827-831.

Sudbery, P. E. and A. R. Goodey. (1980). Genes which control cell proliferation in the yeast *Saccharomyces cerevisiae*.. Nature. 288: 401-404.

Teague, M. A., D. T. Chaleff and B. Errede. (1986). Nucleotide sequence of the yeast regulatory gene *STE7* predicts a protein homologous to protein kinases. Proc. Natl. Acad. Sci. USA. 83: 7371-7375.

Thomas, L. (1974). The Medusa and the Snail. New York: Bantum Books.

Trueheart, J., J. D. Boeke and G. R. Fink. (1987). Two genes required for cell fusion during yeast conjugation: evidence for a pheromone induced surface protein. Mol. Cell. Biol. 7: 2316-2328.

Van Arsdell, S. A., G. L. Stetler and J. Thorner. (1987). The yeast repeated element sigma contains a hormone-inducible gene. Mol. Cell. Biol. 7: 749.

Vogelstein, B. (1990). A deadly inheritance. Nature 348: 681-682.

Ward, G.E. and M.W. Kirschner (1990). Identification of cell-cycle regulated phosphorylation sites on nuclear lamin C. Cell 51: 561-577.

Weinert, T. and L.H. Hartwell. (1990). The *RAD9* gene controls the cell cycle reponse to DNA damage in Saccharomyces cerevisiae. Science 241: 317-322.

Whiteway, M., R. Freedman, S. V. Arsdell, J. W. Szostak and J. Thorner. (1987). The yeast *ARD1* gene product is required for repression of cryptic mating-type information at the HML Locus. Mol. Cell. Biol. 7(10): 3713-3722.

Whiteway, M., L. Hougan, D. Dignard, D. Y. Thomas, L. Bell, G. C. Saari, F. J. Grant, P. O'Hara and V. L. MacKay. (1989). The *STE4* and *STE18* genes of yeast encode potential β and γ subunits of the mating factor receptor-coupled G protein. Cell. 56: 467-477.

Whiteway, M., L. Hougan and D.Y. Thomas. (1990). Overexpression of the *STE4* gene leads to mating response in haploid *Saccharomyces cerevisiae.* Mol Cell. Biol. 10: 217-222.

Wittenberg, C., S. L. Richardson and S. I. Reed. (1987). Subcellular localization of a protein kinase required for cell cycle initiation in *Saccharomyces cerevisiae*: evidence for an association between the CDC28 gene product and the insoluble cytoplasmic matrix. J. Cell Biol. 105: 1527-1538.

Wittenberg, C. and S. I. Reed. (1988). Control of the yeast cell cycle is associated with assembly/ disassembly of the Cdc28 protein kinase complex. Cell. 54: 1061-1072.

Wittenberg, C. and S. I. Reed. (1989). Conservation of function and regulation within the Cdc28/cdc2 protein kinase family: Characterization of the human Cdc2Hs protein kinase in *Saccharomyces cerevisiae.* Mol. Cell. Biol. 9: 4064-4068.

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