DOMAIN FUNCTION AND REGULATION OF Ste12p
by
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B.Sc.(Hons), Queen's University, 1994

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Date July 11, 2001

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Abstract

Ste12p is a transcriptional activator and an effector of two MAP kinase cascades in the yeast *Saccharomyces cerevisiae*. Ste12p activates transcription required for both pheromone response and filamentous growth. The goal of this work was to further define the domain structure of Ste12p and the mechanisms that govern Ste12p activity.

In response to mating pheromone, Ste12p activates transcription of *FAR1*. Farlp interacts with the cell cycle machinery in order to cause G1 growth arrest. This work showed that overexpression of Ste12p in the absence of pheromone induced G1 growth arrest that was independent of *FAR1* and of transcription from pheromone responsive elements. The growth arrest did not reflect cell death and could be eliminated by overexpression of Rst1p and Rst2p, two negative regulators of Ste12p. These data indicated a novel role for Ste12p in pheromone-responsive growth arrest.

Rst1p and Rst2p, two negative regulators of Ste12p, are genetically redundant and, as such, are proposed to regulate Ste12p function by a common mechanism. This study demonstrated that overexpression of amino acids 262 to 594 of Ste12p activated transcription and that concurrent overexpression of either *RST1* or *RST2* abrogated that activation. The region 262 to 594 was able to interact with Rst1p, but not with Rst2p, in yeast extracts. Rst2p, in contrast, interacted with the DNA binding domain of Ste12p. Further, Rst2p, but not Rst1p, was shown to inhibit interaction of the Ste12p DNA binding domain with

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DNA *in vitro* and to inhibit the function of a Ste12pDBD-VP16 fusion *in vivo*. In addition, recombinant Rst1p and Rst2p were shown to interact directly with these distinct domains of Ste12p *in vitro*. These results showed that Rst1p and Rst2p function by non-identical mechanisms, as they interact with separate domains of Ste12p in order to inhibit its function.
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List of Abbreviations

% w/v  percent weight per volume
% v/v  percent volume per volume
α  alpha
β  beta
Δ  delta
γ  gamma
α-Gal4p  anti-Gal4p
α-histidine  anti-histidine
α-Ste12p  anti-Ste12p
A  (deoxy)adenosine
aa  amino acid
bp  base pairs
C  (deoxy)cytidine
CAK  CDK activating kinase
CDK  cyclin dependent kinase
CTD  C-terminal domain
dATP  deoxyadenosine triphosphate
DBD  DNA binding domain
DNA  deoxyribonucleic acid
DTT  dithiothreitol
EDTA  disodium ethylenediamine tetraacetate
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<table>
<thead>
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<th>Abbreviation</th>
<th>Description</th>
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<tbody>
<tr>
<td>EMSA</td>
<td>electrophoretic mobility shift assay</td>
</tr>
<tr>
<td>ERK</td>
<td>extracellular signal regulated kinase</td>
</tr>
<tr>
<td>E. coli</td>
<td><em>Escherichia coli</em></td>
</tr>
<tr>
<td>FIP</td>
<td>filamentation-invasion pathway</td>
</tr>
<tr>
<td>FRE</td>
<td>filamentous response element</td>
</tr>
<tr>
<td>G</td>
<td>(deoxy)guanosine</td>
</tr>
<tr>
<td>GAP</td>
<td>GTPase activating protein</td>
</tr>
<tr>
<td>GDP</td>
<td>guanosine diphosphate</td>
</tr>
<tr>
<td>GST</td>
<td>glutathione-s-transferase</td>
</tr>
<tr>
<td>GTP</td>
<td>guanosine triphosphate</td>
</tr>
<tr>
<td>his'</td>
<td>yeast media containing no histidine</td>
</tr>
<tr>
<td>HOG</td>
<td>high osmolarity glycerol</td>
</tr>
<tr>
<td>Inr</td>
<td>initiator</td>
</tr>
<tr>
<td>K. lactis</td>
<td><em>Kluyveromyces lactis</em></td>
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<tr>
<td>MAP kinase</td>
<td>mitogen activated protein kinase</td>
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<td>MAP/ERK kinase</td>
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<tr>
<td>mRNA</td>
<td>messenger ribonucleic acid</td>
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<tr>
<td>MW</td>
<td>molecular weight</td>
</tr>
<tr>
<td>Ni</td>
<td>nickel</td>
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<td>Abbreviation</td>
<td>Full Form</td>
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<tr>
<td>--------------</td>
<td>-----------</td>
</tr>
<tr>
<td>NR</td>
<td>negative regulation</td>
</tr>
<tr>
<td>OD&lt;sub&gt;600&lt;/sub&gt;</td>
<td>optical density at 600 nanometers</td>
</tr>
<tr>
<td>PIC</td>
<td>pre-initiation complex</td>
</tr>
<tr>
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<td>pheromone induction domain</td>
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<tr>
<td>PMSF</td>
<td>phenylmethylsulfonyl fluoride</td>
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<td>pheromone response pathway</td>
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<td>Saccharomyces cerevisiae</td>
</tr>
<tr>
<td>SDS</td>
<td>sodium dodecyl sulphate</td>
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<tr>
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<td>serine</td>
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<tr>
<td>SRE</td>
<td>stress responsive element</td>
</tr>
<tr>
<td>SRF</td>
<td>serum response factor</td>
</tr>
<tr>
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<tr>
<td>TCS</td>
<td>TEF-1, Tec1 and Aba DNA consensus sequence</td>
</tr>
<tr>
<td>TEY</td>
<td>threonine-glutamic acid-tyrosine</td>
</tr>
<tr>
<td>Thr</td>
<td>threonine</td>
</tr>
<tr>
<td>TPCK</td>
<td>N-tosyl-L-phenylalanine chloromethylketone</td>
</tr>
<tr>
<td>TRIS</td>
<td>tris(hydroxymethyl)aminomethane</td>
</tr>
<tr>
<td>Abbreviation</td>
<td>Description</td>
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<tr>
<td>--------------</td>
<td>----------------------------------</td>
</tr>
<tr>
<td>TXY</td>
<td>threonine-unspecified amino acid-tyrosine</td>
</tr>
<tr>
<td>Tyr</td>
<td>tyrosine</td>
</tr>
<tr>
<td>UAS</td>
<td>upstream activating sequence</td>
</tr>
<tr>
<td>URS</td>
<td>upstream repressing sequence</td>
</tr>
<tr>
<td>Wt</td>
<td>wild type</td>
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Acknowledgements

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1 Introduction

1.1 MAP kinase cascades affect gene expression in eukaryotes

1.1.1 Function and regulation of MAP kinase cascade components

In order to survive and flourish in a constantly changing environment, all cells must be able to detect and respond to environmental signals. In many cases, the signal to which a given cell responds is detected at the cell surface and must be transmitted to intracellular or intranuclear effector molecules, which include transcription factors like Ste12p. One archetypal signal transduction pathway is the MAP kinase cascade, a three component signaling module that is conserved throughout eukarya (Gustin et al., 1998; Widmann et al., 1999). The three conserved components of a MAP kinase cascade are a MAP kinase (MAPK), a MAPK/extracellular signal-regulated kinase kinase (MAPKK or MEK) and a MAPK/extracellular regulated kinase kinase kinase (MAPKKK or MEKK). One example of a MAPK cascade is the pheromone response pathway, which is illustrated in Figure 1 (Bardwell et al., 1994; Gustin et al., 1998). The following sections will review MAP kinase cascades with particular focus on the Saccharomyces cerevisiae (yeast) pheromone response pathway and its effector, Ste12p.

The activation of a MAP kinase cascade begins with an input signal, such as the activation of cell surface receptors by mating pheromone in yeast. The change in receptor activity activates an associated kinase (or kinases) or a GTP-
The pheromone response pathway in yeast is activated by pheromone binding to receptors at the cell surface. A signal transduction pathway, that includes a MAP kinase cascade, transmits the extracellular signal, culminating in the activation of Ste12p and Far1p, effectors of the pheromone response pathway. The tripartite pheromone response includes activation of transcription, G1 growth arrest and formation of a shmoo mating projection.
associated protein, which, in turn, activates the MAPK cascade. The cascade of phosphorylations, from MEKK to MEK to MAPK, results in an active MAPK, which can phosphorylate its substrate proteins and stimulate changes in gene expression or protein activity within the cell. Most MAP kinase substrates are transcription factors, like Ste12p, although MAP kinases also phosphorylate kinases, phosphatases and other effector molecules.

1.1.2 MAP kinase cascades in *Saccharomyces cerevisiae*

1.1.3 The five MAP kinase cascades of yeast

MAP kinase cascades have been studied in a wide range of eukaryotes, including *Saccharomyces cerevisiae*, *Dictyostelium discoideum*, *Drosophila melanogaster* and cultured mammalian cells (Widmann et al., 1999). Of these systems, the MAP kinases of yeast are the best characterized. With the advantages of genetic accessibility and a relatively small number of MAP kinases (6 yeast MAPKs, versus more than 12 mammalian MAPKs), *Saccharomyces cerevisiae* is an excellent model for deciphering the mechanics of MAP kinase signaling (Madhani and Fink, 1998) (Garrington and Johnson, 1999; Widmann et al., 1999).

The yeast *Saccharomyces cerevisiae* has five MAP kinase cascades that are required for pheromone response, invasive/filamentous growth, high osmolarity growth (HOG), cell integrity and spore wall assembly (Gustin et al., 1998; Madhani and Fink, 1998). The components of the pathways are described
in Figure 2, with predicted, but as yet undefined, components indicated by question marks.

Each MAPK cascade in yeast has a unique MAP kinase, but other components of the signaling machinery are shared between the pathways. All of the pathways are believed to require an upstream kinase, such as Ste20p, which activates the MEKK of the pheromone response (PRP), filamentation-invasion (FIP) and high osmolarity glycerol (HOG) pathways (Leberer et al., 1992; Liu et al., 1993; Mosch et al., 1996; O'Rourke and Herskowitz, 1998; Ramer and Davis, 1993; Roberts and Fink, 1994). The MEK Ste7p is shared between invasive growth and pheromone response (Roberts and Fink, 1994) and Ste11p, a MEKK, is shared by the HOG, pheromone response and filamentation-invasion pathways (Posas and Saito, 1997; Roberts and Fink, 1994). Although the pathways share components, their input signals are distinct and their outputs are unique. For example, the PRP and FIP share an upstream kinase, MEKK and MEK, and activate a shared transcription factor, Ste12p, yet they control two independent developmental pathways in response to two different signals (Gustin et al., 1998).

The different pathways may also have multiple functions under different growth conditions. The components of the FIP, for example, have been implicated in the maintenance of cell wall integrity during vegetative growth (Lee and Elion, 1999). In addition, the pathways may be activated sequentially. Global expression profiling shows that following PRP activation, the cell integrity
Figure 2. MAP kinase cascades in yeast
There are five known MAP kinase cascades in yeast. The cascades share some components but have different outputs. Figure adapted from Gustin et al. (1998).
Introduction

pathway is activated, which may stabilize the cell during the formation of the shmoo mating projection (Roberts et al., 2000).

There are six MAP kinases encoded by the yeast genome, five of which have been defined as members of the signaling cascades in Figure 2. The sixth kinase is Mlp1p, which is most similar to Mpk1p, has been implicated as being a second MAP kinase in the cell integrity pathway signals (Gustin et al., 1998).

Although the MAP kinases control several important life processes in *Saccharomyces cerevisiae*, they are not essential genes. A yeast strain with all six MAP kinase genes deleted remains viable, although it cannot mate, sporulate or regulate filamentation (Madhani et al., 1997).

1.2 The pheromone response pathway – A model MAP kinase cascade

The yeast pheromone response pathway (PRP), shown in Figure 1, is an excellent model for studying MAP kinase regulation, and is one of the best characterized protein kinase cascades.

When haploid yeast are exposed to pheromone from yeast of the opposite mating type, they rapidly (within one doubling time), differentiate into mating competent cells. The pheromone response includes G1 growth arrest, shmoo formation (a projection of the cell surface toward the nearest mating partner) and activation of transcription of pheromone responsive genes (Bardwell et al., 1994; Gustin et al., 1998). Pheromone responsive transcription is mediated through the transcription factor Ste12p, which is activated by the PRP. The pheromone response also includes activation of the genes and gene products that are
Introduction

required for signal attenuation to allow reentry into the cell cycle in the absence of a mating partner (Dietzel and Kurjan, 1987a; Gustin et al., 1998; Kronstad et al., 1987). Regulation of Ste12p activity within the context of the pheromone response has been the major focus of this work.

1.2.1 Components of the PRP which function upstream of Ste12p

1.2.1.1 The cell surface receptors

With the exception of cell-type specific pheromones and pheromone receptors, the PRP components are identical in \( \alpha \) and \( \alpha \) cells (Bardwell et al., 1994). The PRP is stimulated when pheromone from yeast of one mating type binds to a receptor on the cell surface of a yeast of the opposite mating type. \( \alpha \) cells produce \( \alpha \) factor, which is a 12-amino-acid farnesylated peptide exported from \( \alpha \) cells by Ste6p, a transporter protein (Kuchler et al., 1989; McGrath and Varshavsky, 1989). \( \alpha \) factor binds to Ste3p, the \( \alpha \) factor receptor, at the cell surface of \( \alpha \) cells and activates the PRP (Hagen et al., 1986). The PRP in \( \alpha \) cells is stimulated by 13-amino-acid peptide \( \alpha \) factor, produced and secreted from \( \alpha \) cells, binding to Ste2p at the cell surface of \( \alpha \) cells (Figure 1)(Dmochowska et al., 1987; Jenness et al., 1983; Julius et al., 1983; Julius et al., 1984a; Julius et al., 1984b).

Ste2p and Ste3p are members of the serpentine family of cell surface receptors, which have seven membrane-spanning hydrophobic \( \alpha \)-helical domains (Dohlman et al., 1991). Intracellular domains of both Ste2p and Ste3p are
associated with a heterotrimeric G protein complex consisting of Gpa1p (Gα), Ste4p (Gβ) and Ste18p (Gγ) (Boone et al., 1993; Clark et al., 1994; Stefan and Blumer, 1994). The association of Ste2p or Ste3p with pheromone results in a change in receptor conformation and a consequent dissociation of the Gα from the Gβ/γ complex (Blinder et al., 1989; Dietzel and Kurjan, 1987b; Jahng et al., 1988; Kurjan and Dietzel, 1988; Miyajima et al., 1988; Whiteway et al., 1989).

1.2.1.2 The complex of Ste4p-associated PRP proteins

The free Gβ/γ complex interacts with multiple proteins, including Ste5p, Ste20p, Ste50p, Far1p, Bem1p and Cdc42p (Cairns et al., 1992; Leberer et al., 1993; Stevenson et al., 1992; Whiteway et al., 1995). The protein complex is anchored to the plasma membrane through the prenylation and palmitoylation of Ste18p (Gγ) (Hirschman et al., 1997; Hirschman and Jenness, 1999; Manahan et al., 2000; Pryciak and Huntress, 1998; Whiteway and Thomas, 1994). This complex of proteins at the cell surface propagates the pheromone signal, ultimately activating PRP effectors including Ste12p.

Ste5p interacts with Ste4p

Following pheromone stimulation, Ste5p is recruited to the plasma membrane by interaction with Gβ (Mahanty et al., 1999; Pryciak and Huntress, 1998; Whiteway et al., 1995). At the cell membrane, Ste5p acts as a scaffold for the kinases of the PRP MAPK cascade, associating with Ste11p (MEKK), Ste7p (MEK) and Fus3p (MAPK) (Choi et al., 1999; Printen and Sprague, 1994).
Disruption of these interactions interrupts PRP signaling (Choi et al., 1994; Inouye et al., 1997a; Kranz et al., 1994; Marcus et al., 1994; Printen and Sprague, 1994).

The interaction of the PRP kinases with Ste5p has two results. First, interaction with Ste5p increases the efficiency of PRP signaling by co-localizing the kinases of MAPK cascade and Ste20p, the kinase that phosphorylates and activates Ste11p. (Leberer et al., 1992; Ramer and Davis, 1993; van Drogen et al., 2000). Second, by co-localizing and sequestering active PRP components, Ste5p prevents inappropriate crosstalk between MAP kinase cascades (Choi et al., 2000; Yashar et al., 1995).

In addition to recruitment of Ste5p to the plasma membrane, the interaction of Ste5p with Ste4p is postulated to result in conformational changes to Ste5p. According to this model, the conformation changes rearrange the scaffolded PRP kinases, making them more accessible to Ste20p and/or increasing their ability to cross-phosphorylate one another (Inouye et al., 1997b; Sette et al., 2000; Yablonski et al., 1996).

The interaction of Ste5p and Ste4p, and subsequent activation of the PRP, requires the shuttling of Ste5p through the nucleus (Mahanty et al., 1999). Ste5p shuttles through the nucleus during vegetative growth, and pheromone treatment results in enhanced nuclear export of Ste5p (Mahanty et al., 1999). The requirement for nuclear shuttling may prevent cytoplasmic Ste5p from activating the downstream kinases in the absence of pheromone.
Ste20p interacts with Ste4p

Another PRP component that interacts with Ste4p is Ste20p, the founding member of the p21-activated kinase (PAK) family. Ste20p activates Ste11p (MEKK) through phosphorylation of Ser302 and/or Ser306 and Thr307 in yeast (Leberer et al., 1992; Ramer and Davis, 1993; van Drogen et al., 2000). Phosphorylation of Ste11p by Ste20p is required to transmit the PRP signal from the Ste4p/Ste18p complex to Ste12p (Leberer et al., 1992; van Drogen et al., 2000; Wu et al., 1995).

Ste20p activation requires the interaction of Ste20p and Cdc42p, another membrane-associated member of the PRP (Moskow et al., 2000; Simon et al., 1995; Zhao et al., 1995). In addition, the Cdc42p-Ste20p interaction is required to localize Ste20 to regions of polarized growth, like the shmoo tip (Leberer et al., 1997a; Leberer et al., 1997b; Moskow et al., 2000; Peter et al., 1996; Ziman et al., 1993).

Ste50p associates with membrane-localized proteins

Another member of the complex of PRP proteins at the cell membrane is Ste50p (Rad et al., 1998; Rad et al., 1992). Ste50p acts in the PRP between Ste4p/Ste18p and Ste11p. Ste50p associates with the N-terminal domain of Ste11p and disrupts the interaction between the regulatory (N-terminal) and catalytic domains (C-terminal) of Ste11p in vitro (Wu et al., 1999). The same study demonstrates that the interaction of Ste50p with Ste11p is important but not essential for Ste11p activation in pheromone response. Since Ste50p and
Ste5p interact with adjacent regions of Ste11p, it may be that these two interactions, in concert with the activity of Ste20p, work to activate Ste11p during pheromone response.

**Ste4p-associated proteins localize to the shmoo tip**

Cdc42p, a Rho-like small GTP binding protein, in conjunction with its guanine nucleotide exchange factor, Cdc24p, regulates morphological changes in yeast, including the development of the shmoo mating projections that yeast develop prior to conjugation (Adams et al., 1990; Drubin and Nelson, 1996; Li et al., 1995a; Zheng et al., 1994; Ziman et al., 1993). The mating projections form in the direction of the nearest mating partner in a manner that is dependent on Cdc42p (Dorer et al., 1995; Nern and Arkowitz, 1998; Nern and Arkowitz, 1999; Schrick et al., 1997; Segall, 1993). The activation of Cdc42p in response to pheromone requires the nuclear export of Cdc24p-Far1p complexes, mediated by the exportin Msn5p (Blondel et al., 1999; Nern and Arkowitz, 2000; Shimada et al., 2000).

Multiple protein-protein interactions coordinate shmoo formation. The Ste4p-Ste20p-Cdc24p-Ste5p complex is proposed to limit pheromone signaling and shmoo formation to the regions of the cell surface immediately adjacent to the activated pheromone receptors (Nern and Arkowitz, 1998; Shimada et al., 2000; Zhao et al., 1995). Simultaneously, the Bem1p-Ste20p-Ste5p-actin-Far1p complex is postulated to tether the signaling pathway to the cytoskeleton (Butty et al., 1998; Leeuw et al., 1995; Lyons et al., 1996) and polarize the cytoskeleton
along the pheromone gradient (Valtz et al., 1995). Although there is genetic evidence for the function of these complexes, evidence of their physical existence is still required.

1.2.2 The MAP kinase cascade - Ste11p, Ste7p and Fus3p

Following interaction of Ste5p with activated Ste4p, the PRP MAP kinase cascade is activated. The MEKK of the PRP, Ste11p, is required for the activation of Ste7p (MEK) and consists of two domains, a kinase domain and a regulatory domain. Deletion of the N-terminal regulatory domain converts Ste11p to a constitutively active form, which can activate the pheromone response in the absence of upstream components of the PRP (Cairns et al., 1992; Ramer et al., 1992; Stevenson et al., 1992). The mechanism for pheromone responsive activation of Ste11p has not been determined, but Ste20p can activate Ste11p as a kinase and Ste50 and/or Ste5p may interact with the regulatory domain of Ste11p, resulting in an active MEKK (Leberer et al., 1992; Ramer and Davis, 1993; van Drogen et al., 2000; Wu et al., 1999; Wu et al., 1995).

Next, Ste11p (MEKK) phosphorylates Ste7p (MEK) and stimulates its ability to phosphorylate Fus3p (MAPK) in vitro and in vivo (Cairns et al., 1992; Zhou et al., 1993). In vitro, Ste11p has been demonstrated to phosphorylate Ste7p at residue Thr363, which is analogous to the activating phosphorylation of mammalian MEK1 (MEK) by MEKK (MEKK) (Neiman and Herskowitz, 1994; Zheng and Guan, 1994). Mutagenesis studies have indicated that phosphorylation of Ste7p at residue Ser359 is also required for pheromone
response, but serine phosphorylation of Ste7p by Ste11p has not been
demonstrated \textit{in vitro} (Neiman and Herskowitz, 1994; Pages et al., 1994; Zheng
and Guan, 1994). Ste7p, like other MEKs, is a dual specificity kinase and
phosphorylates Fus3p and Kss1p on both threonine and tyrosine in a -TEY-
sequence in response to pheromone (Errede et al., 1993; Gartner et al., 1992;
Ma et al., 1995). Ste7p is associated with Fus3p and Kss1p in tight complexes,
which may contribute to signaling specificity (Bardwell et al., 1996).

1.2.2.1 \textbf{Fus3p activates Ste12p and other effectors of the PRP}

Fus3p is the MAP kinase of the PRP. Upon activation by Ste7p in
response to pheromone, Fus3p phosphorylates the effector proteins of the PRP.
Fus3p substrates include Ste12p, a transcriptional activator that mediates
pheromone responsive transcription, and Far1p, an effector of both cell cycle
arrest and chemotropism (Chang and Herskowitz, 1990; Chang and Herskowitz,
1992; Elion et al., 1993; Hung et al., 1997; Song et al., 1991). Fus3p also
phosphorylates Rst1p (Dig1p) and Rst2p (Dig2p), negative regulators of Ste12p
(Cook et al., 1996; Tedford et al., 1997). Activation of Ste12p and relief of
repression leads to increased transcription of pheromone-responsive genes,
which allows the yeast to proceed through the mating reaction.

Characterization of a series of partial function mutants of Fus3p
demonstrated that mutations in the residues of Fus3p that are conserved
between MAP kinases can selectively affect multiple aspects of pheromone
response including Ste12p activation, G1 arrest, shmoo formation and recovery
Introduction

(Farley et al., 1999). Further, the work showed that not all of these Fus3p functions are dependent upon activation of transcription by Ste12p (Farley et al., 1999).

1.2.2.2 Fus3p and Kss1p function in different pathways

Fus3p is closely related to the MAPK Kss1p, a gene whose overexpression promotes recovery from pheromone response (Courchesne et al., 1989; Elion et al., 1990). Early work indicated that the functions of Kss1p and Fus3p were redundant, as the mating competence of a fus3Δ kss1Δ strain is lower than that of a fus3Δ strain, but further work has revealed differences in both function and regulation that point to Fus3p as the PRP MAP kinase (Elion et al., 1991). First, Fus3p expression is limited to haploids and FUS3 gene expression is pheromone-induced, while Kss1p is expressed in all cell types and KSS1 gene expression is not induced by pheromone (Elion et al., 1990; Liu et al., 1993). Second, overexpression of KSS1 promotes recovery from pheromone induced arrest, whereas FUS3 overexpression increases pheromone sensitivity in yeast (Courchesne et al., 1989; Elion et al., 1990). Finally, Fus3p facilitates growth arrest in response to pheromone, through the phosphorylation of Far1p. Kss1p does not have an apparent growth arrest function (Elion et al., 1991; Elion et al., 1990; Farley et al., 1999; Tyers and Futcher, 1993).

Kss1p can function in pheromone response, but does not normally function as a major positive regulator of pheromone response and is only able to do so when FUS3 is deleted from the genome. In other words, the absence of
Introduction

*FUS3* from the genome results in inappropriate crosstalk between MAPK pathways (Madhani et al., 1997). Other results show that, although Kss1p may function in the PRP, it is only in a manner that is much more limited than Fus3p and does not affect the same range of substrate proteins (Farley et al., 1999). These results demonstrate that, in cells with intact *FUS3* and *KSS1* genes, Fus3p is the principal MAP kinase of the PRP, while Kss1p functions primarily in filamentation (Figure 3).

Global gene expression profiling confirms that Fus3p is the dominant MAP kinase in pheromone response. In *kss1Δ* yeast, Fus3p directs transcription in response to mating pheromone that is very similar to the response seen in wild type cells. In contrast, the transcription profiles in *fus3Δ* cells exposed to pheromone feature preferential induction of genes that have been implicated in filamentation (Roberts et al., 2000).

1.2.3 Recovery from Pheromone Response

1.2.3.1 PRP signal attenuation at the cell surface

After a period of exposure to pheromone, yeast that have not found a mating partner will recover from pheromone-induced cell cycle arrest and reenter the cell cycle. Attenuation of the pheromone signal is mediated by several mechanisms which affect the PRP at multiple levels, ultimately reducing the activity of PRP effectors including Ste12p.
Figure 3. Fus3p and Kss1p function in separate pathways
Current models suggest that Fus3p and Kss1p function in separate developmental pathways in yeast. Fus3p is required to activate Ste12p for mating in response to pheromone. Kss1p, which can both repress and activate Ste12p function, controls the activity of Ste12p-Tec1p dimers at filamentous response elements. Figure adapted from (Madhani and Fink, 1998).
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There are several proteins which down-regulate pheromone response upstream of the MAP kinase cascade. Bar1p is a protease that is secreted by a cells that cleaves \(\alpha\) factor, inhibiting propagation of the pheromone signal. When a cells are exposed to \(\alpha\) factor, the expression of \(BAR1\) is induced (Kronstad et al., 1987; MacKay et al., 1988). Ste2p and Ste3p undergo feedback regulation, as well. Upon activation of the PRP, the cytoplasmic tails of the pheromone receptors become hyperphosphorylated, and Ste2p ubiquitinated. These modifications target Ste2p for endocytosis, which eventually leads to downregulation of pheromone response (Feng and Davis, 2000; Hicke et al., 1998; Schandel and Jenness, 1994). Another mediator of pheromone response attenuation is Sst2p. SST2, a pheromone-inducible gene, encodes a putative GTPase activating protein (GAP) for \(Gpa1p\) (\(G\alpha\)) (Apanovitch et al., 1998; Dohlman et al., 1995; Dohlman et al., 1996). In order for \(G\alpha\) to dissociate from \(G\beta/\gamma\), it must exchange a bound GDP for a GTP. Hydrolysis of GTP to GDP results in the re-association of \(G\alpha\) with \(G\beta/\gamma\) and inhibition of pheromone signaling. A second negative regulator of G protein-mediated signaling is \(SIG1\), a gene that suppresses dominant negative \(G\beta\) by an unknown mechanism. Deletion of \(SIG1\) results in pheromone hypersensitivity (Leberer et al., 1994).

1.2.3.2 Attenuation of MAP kinase activity

Fus3p (MAPK), the terminal kinase of the PRP, is the target of at least three phosphatases, that down regulate Fus3p activity and promote recovery from pheromone response. \(MSG5\), a gene whose expression is pheromone-
induced, encodes a dual specificity phosphatase that is able to dephosphorylate both phosphothreonine and phosphotyrosine residues (Doi et al., 1994). Ptp2p and Ptp3p work in concert as protein tyrosine phosphatases (Zhan et al., 1997; Zhan and Guan, 1999).

As well as phosphorylating effectors of the PRP, Fus3p also phosphorylates both Ste7p and Ste5p, two upstream members of the PRP cascade (Elion et al., 1993; Errede et al., 1993; Kranz et al., 1994). The significance of these phosphorylations is unknown, but it has been postulated that they may act as attenuation signals. Kss1p, which was cloned as a gene that promotes recovery from pheromone, inhibits the activated PRP MAP kinase cascade at or below the level of Ste11p (Cherkasova et al., 1999). Fus3p may also promote pheromone recovery through modulation of Mcm1p activity. Overexpression of the transcription factor Mcm1p can promote proliferation in the presence of pheromone in a Fus3p-dependent manner (Cherkasova et al., 1999).

Inactivation of Fus3p may also promote recovery. Catalytically inactive Fus3p has been shown to repress activation of FUS1 transcription by active Fus3p and catalytically inactive Fus3p accumulates in yeast after extended exposure to mating pheromone (Choi et al., 1999; Farley et al., 1999).

1.2.3.3 PRP signal attenuation and transcription

Signal attenuation in the pheromone response may also be achieved by altering the activity of transcription factors. Rst1p (Dig1p) and Rst2p (Dig2p) are
known to be negative regulators of Ste12p and substrates of Fus3p and Kss1p (Bardwell et al., 1998b; Cook et al., 1996; Pi et al., 1997; Tedford et al., 1997) and RST2 expression is pheromone induced (Cook et al., 1996). Although the Rstps have not been linked to recovery, the genes were cloned in two-hybrid screens as proteins that interact with Cln1p, Cln2p and Kss1p, all three of which are implicated in recovery (Cherkasova et al., 1999; Cook et al., 1996; Courchesne et al., 1989; Oehlen and Cross, 1994; Oehlen and Cross, 1998b; Tedford et al., 1997; Wu et al., 1998) (see The pheromone response and the cell cycle, below).

POG1 is a gene which, when overexpressed, inhibits both pheromone-induced growth arrest and repression of CLN1 and CLN2 expression (Leza and Elion, 1999). Deletion of POG1 results in enhanced pheromone sensitivity. Initial studies indicate that Pog1p may enhance pheromone recovery through an increase in CLN2 expression (Leza and Elion, 1999). As described below, Cln2p-Cdc28p complexes may inhibit the function of Ste20p in order to promote recovery from pheromone-induced arrest (Leza and Elion, 1999; Oehlen and Cross, 1994; Oehlen and Cross, 1998b; Wu et al., 1998).
1.2.4 Filamentous development: the pseudohyphal development and invasive growth pathways share components of the PRP

1.2.4.1 The phenotype of filamentous development

Under certain conditions, including nutrient limitation, *Saccharomyces cerevisiae* will exhibit filamentous growth (Gustin et al., 1998). When diploid yeast undergo the dimorphic transition from the yeast form to the filamentous pseudohyphal form, they switch from a bipolar to an apical budding pattern, their buds cease to detach and they grow as filaments of elongated cells that project outward from a colony in solid media (Gimeno et al., 1992; Mosch and Fink, 1997). Haploid yeast grow in a similar filamentous form, but their invasive growth is limited to projections beneath the colony, because their budding pattern is bipolar (Roberts and Fink, 1994).

The environmental signals that control filamentous growth are nutritional (Cullen and Sprague, 2000; Dickinson, 1994; Gimeno et al., 1992; Lo et al., 1997). Nitrogen starvation in the presence of abundant carbon can induce pseudohyphal development, as can the presence of metabolic byproducts (Gimeno et al., 1992; Lorenz et al., 2000a). In contrast, haploid yeast grow invasively in response to glucose depletion and their morphology is unaffected by nitrogen depletion (Cullen and Sprague, 2000).

The list of genes induced by the filamentation-invasion pathway remains incomplete, although it is known to include *TEC1*, *FLO11* and *PHD1* (Gimeno and Fink, 1994; Lo and Dranginis, 1998; Madhani and Fink, 1997; Oehlen and
Cross, 1998a; Rupp et al., 1999). Recent analysis has also shown that different genes may be induced in the filamentation-invasion response in haploids and diploid, although *FLO11* expression is induced in both (Madhani et al., 1999).

### 1.2.4.2 Signal transduction and filamentous development

At least two distinct signal transduction pathways stimulate filamentous development in yeast. Ras2p can stimulate both pathways, possibly in response to the different signals (Gimeno et al., 1992; Kubler et al., 1997; Lorenz and Heitman, 1997; Mosch et al., 1999; Mosch et al., 1996). One pathway is controlled by Gpr1p (G-protein coupled receptor) stimulation of cAMP-PKA and modulates the activity of the transcription factor Flo8p (Liu et al., 1996; Lorenz et al., 2000b; Pan and Heitman, 1999; Rupp et al., 1999; Tamaki et al., 2000). The second pathway that regulates filamentous development in yeast is the filamentation-invasion MAP kinase cascade (Figures 2 and 3), which shares several components with the PRP including Ste20p, Ste50p, Ste11p, Ste7p, Rst1p, Rst2p and Ste12p (Liu et al., 1993; Roberts and Fink, 1994). The filamentation-invasion pathway (FIP) does not respond to pheromone, however, and the FIP MAP kinase is Kss1p, and not Fus3p (Madhani et al., 1997; Roberts et al., 2000).

### 1.2.4.3 The filamentation-invasion MAP kinase pathway

In the filamentation-invasion pathway, Ras2p signals through Bmh1p and Bmh2p, two 14-3-3 proteins, to activate Cdc42p (Gimeno et al., 1992; Mosch et
al., 1996; Roberts et al., 1997). Active Cdc42p stimulates Ste20p, which in turn activates Ste11p to initiate the MAPK cascade (Leberer et al., 1997b; Mosch et al., 1996; Peter et al., 1996). Ste50p has also been implicated in the activation of Ste11p for filamentation-invasion, possibly by a mechanism analogous to its role in pheromone response (see Ste50p associates with membrane-localized proteins, above) (Rad et al., 1998). Upon activation of Kss1p, negative regulation of Ste12p by Rst1p and Rst2p is relieved and transcription of filamentation genes is induced (Bardwell et al., 1998a; Bardwell et al., 1998b; Cook et al., 1996; Cook et al., 1997; Madhani et al., 1997; Tedford et al., 1997).

Activation of transcription in the filamentation-invasion pathway requires two transcription factors, Ste12p and Tec1p. Ste12p and Tec1p bind to filamentous response elements (FREs) (Figures 3 and 4) and direct transcription of genes such as FLO11 (Baur et al., 1997; Gavrias et al., 1996; Laloux et al., 1994; Lo and Dranginis, 1998; Madhani and Fink, 1997; Mosch and Fink, 1997). FRE elements consist of a Tec1p binding site adjacent to a Ste12p binding site (Madhani and Fink, 1997).

1.2.4.4 Maintaining MAP kinase cascade signaling specificity

The FIP and the PRP control two separate developmental pathways, yet they share most of their signaling components, including the transcription factor, Ste12p (Gustin et al., 1998; Madhani and Fink, 1998). Several mechanisms have been proposed as models for how the two signal pathways may be kept
Several categories of promoters have been defined that respond to Ste12p. PRE sequences \([\text{(A)TGAAACA}]\) are found in pheromone responsive genes. PQ elements, which consist of a P box \([\text{CC(NNNN)GG}]\) adjacent to a Q box \([\text{CTGTCATTGT}]\), are found in \(\alpha\)-specific genes. P/PRE sites, which combine a P box with a PRE, are found in a-specific genes. FRE elements, composed of a TCS \([\text{CATTCT}]\) and a PRE, are found upstream of genes induced during filamentation. See Table 1 for examples of genes containing these elements. All of these elements are recognition sites for transcription factors. As depicted, Ste12p binds to PRE elements, Mcm1p binds to P boxes, \(\alpha1p\) binds to Q boxes and Tec1p binds to TCS elements.
<table>
<thead>
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<th>Gene Name</th>
<th>Promoter Type</th>
<th>Gene Product Function</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Ty1 LTR</td>
<td>Filamentous</td>
<td>Yeast transposon</td>
<td>(Company et al., 1988; Laloux et al., 1994; Mosch and Fink, 1997; Mosch et al., 1996)</td>
</tr>
<tr>
<td>FLO11</td>
<td>Filamentous</td>
<td>Cell surface flocculin</td>
<td>(Lo and Dranginis, 1998; Rupp et al., 1999)</td>
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<tr>
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<td>RST2/DIG2</td>
<td>Haploid specific</td>
<td>Negative regulator of pheromone response</td>
<td>(Cook et al., 1996)</td>
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<td>FUS1</td>
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<td>(Hagen et al., 1991; McCaffrey et al., 1987; Trueheart et al., 1987)</td>
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<tr>
<td>FAR1</td>
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<td>(Chang and Herskowitz, 1990)</td>
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<td>Recovery from pheromone arrest</td>
<td>(Dietzel and Kurjan, 1987a)</td>
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<td>α-factor protease, degrades α-pheromone</td>
<td>(Kronstad et al., 1987)</td>
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<table>
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<th>Function</th>
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</table>
Introduction

distinct. First, combinatorial control of transcription distinguishes filamentation-invasion from pheromone response. The cAMP pathway and the FIP converge on the *FLO11* promoter, simultaneously modulating gene expression from independent promoter elements (Rupp et al., 1999). A second mechanism that likely maintains PRP/FIP specificity is the interaction of Ste12p with different transcription factor partners. Ste12p interacts with different partners at FREs and PREs, although it is unknown how the partners are selected (Baur et al., 1997; Hwang-Shum et al., 1991; Laloux et al., 1994; Madhani and Fink, 1997; Oehlen et al., 1996; Yuan et al., 1993). Cell-type-specific gene expression distinguishes the FIP from the PRP in diploids, as diploids do not express Ste5p, Fus3p, pheromone receptors, Gpa1p, Ste18p or Ste4p (Elion et al., 1990; Hartig et al., 1986; Leberer et al., 1993; Mukai et al., 1993; Perlman et al., 1993; Sprague et al., 1983; Whiteway et al., 1989). Scaffold proteins, such as Ste5p, may also contribute to specificity, although no scaffold has been identified for the FIP (Widmann et al., 1999).

The MAP kinases of the PRP and FIP are different, which may be an important source of signal specificity. Originally, Kss1p and Fus3p were postulated to have overlapping functions in pheromone response and invasive growth, as deletion of one gene was insufficient to abolish either response. The two kinases do have different substrate proteins and different activities (see The MAP kinase cascade, above) and recent observations have demonstrated that the two MAP kinase have independent functions (Cook et al., 1997; Madhani et al., 1997; Roberts et al., 2000). Fus3p is the major MAP kinase of the PRP and
may exclude Kss1p from the Ste5p complex, effectively eliminating Kss1p from the pheromone response (Madhani et al., 1997). Kss1p acts as both a negative and a positive regulator of filamentous development (Bardwell et al., 1998a; Bardwell et al., 1998b). Deletion of KSS1 from the genome does not eliminate invasive growth, but it is likely that the cAMP signal and the elimination of the negative regulation by Kss1p combine to permit invasion (Madhani et al., 1997; Rupp et al., 1999). Collectively, these results indicate that the FIP and the PRP employ different and specific MAP kinases, which may prevent unwanted crosstalk.

The role of Kss1p as a negative regulator may also be essential to signaling specificity. Kss1p binds to Ste12p and potentiates Rst1p- and Rst2p-dependent inhibition of Ste12p function at filamentous response elements (FREs) (Bardwell et al., 1998a; Bardwell et al., 1998b). Inactive Kss1p is postulated to stabilize the interaction between Rst1p and Ste12p in a complex containing all three proteins (Bardwell et al., 1998a; Bardwell et al., 1998b). Activation of Kss1p by Ste7p in the FIP results in dissociation of Kss1p from Ste12p, relieving repression at FREs (Cook et al., 1997). A similar effect is observed at pheromone responsive elements (PREs), however, it is much weaker. In a kss1Δ strain, under conditions of vegetative growth, FRE reporters show sixty percent of FIP-induced activity, whereas PRE reporters show seven percent of PRP-induced function (Cook et al., 1997). These results suggest that an inactivated MAP kinase may act as a pathway-specific inhibitor of activator function.
1.2.5 The pheromone response and the cell cycle

*Saccharomyces cerevisiae* proliferate by budding to produce two progeny cells with identical genetic material. Newly budded yeast, in the presence of sufficient nutrients, proceed through the four ordered phases of the cell cycle (G1, S, G2 and M) in order to replicate their DNA and distribute their genetic material to their progeny (Herskowitz, 1988). Pheromone response halts the ordered progression of the cell cycle in haploid yeast before the process of DNA replication begins, which ensures that yeast mate with a 1N complement of DNA. As described below, the interaction of the cell cycle machinery and the PRP is dynamic. The PRP can halt cell cycle progression by several mechanisms, while the activity of the cell cycle machinery limits the growth arrest function of the PRP to the G1 phase of the cell cycle. The following sections will review the events surrounding the G1 to S transition in yeast and the effects of pheromone and Ste12p-dependent transcription on those events.

1.2.5.1 Cdc28p - the cell cycle kinase

Progression through the cell cycle in yeast is dependent upon the activity of Cdc28p, the cyclin dependent kinase (CDK) that coordinates the events of the *Saccharomyces cerevisiae* cell cycle. CDK activity is both positively and negatively regulated. Activation of CDKs requires cyclin binding and phosphorylation by a CAK (CDK activating kinase). Even in the presence of these activating stimuli, CKIs (cyclin dependent kinase inhibitors) can limit CDK
function by binding to CDK-cyclin complexes and directly inhibiting their activity (Mendenhall and Hodge, 1998).

Cdc28p is a constitutively expressed protein that is regulated at the post-transcriptional level (Mendenhall and Hodge, 1998; Mendenhall et al., 1987). The activity of Cdc28p reflects the balance of cyclins and CKIs in a cell, which varies in response to proliferation and arrest signals. At several checkpoints in the cell cycle, influences such as nutrient availability, mating pheromone and DNA damage can halt or enhance cell cycle progression through an influence on Cdc28p activity (Mendenhall and Hodge, 1998). The first checkpoint of the cell cycle is Start, which occurs at the G1 to S transition. Cells that have passed the Start checkpoint begin to replicate their DNA, form buds and duplicate their spindle pole bodies. Passage through Start reflects a commitment to completing the cell cycle and once past Start, yeast are unable to respond to pheromone until they complete one cell cycle (Mendenhall and Hodge, 1998). Response to pheromone, however, arrests yeast at the Start checkpoint and inhibits further cell cycle progression (Gustin et al., 1998; Mendenhall and Hodge, 1998).

1.2.5.2 The cyclins and CKIs of Start

There are five cyclins and two CKIs associated with Start in *Saccharomyces cerevisiae*. The three G1 cyclins are Cln1p, Cln2p and Cln3p (Andrews and Measday, 1998), the two B-type cyclins are Clb5p and Clb6p (Andrews and Measday, 1998), and Sic1p and Far1p are the two CKIs (Mendenhall and Hodge, 1998). The relative amount of these cyclins and CKIs
in a cell is regulated by a combination of transcription and proteolysis and determines when the cells will progress through Start.

Cln1p and Cln2p, which are 57% identical, are expressed maximally at Start (Hadwiger et al., 1989; Tyers et al., 1993; Wittenberg et al., 1990). Cln1p- and Cln2p-Cdc28p complexes initiate bud formation, spindle pole body duplication and proteolysis of the Sic1p CKI (Deshaies and Kirschner, 1995; Dirick et al., 1995; Lew and Reed, 1993; Schneider et al., 1996; Schwob et al., 1994; Tyers, 1996; Verma et al., 1997a; Verma et al., 1997b). \( CLN1 \) and \( CLN2 \) expression is activated in G1 by the SBF transcription factor in response to Cln3p-Cdc28p (Dirick et al., 1995; Nasmyth and Dirick, 1991; Ogas et al., 1991; Stuart and Wittenberg, 1995; Tyers et al., 1993; Wittenberg et al., 1990). Cln1p and Cln2p are ubiquitinated and degraded following Start (Blondel and Mann, 1996; Deshaies et al., 1995). Cln3p, the third G1 cyclin, is expressed throughout the cell cycle, with its levels increasing during the M/G1 phase under the control of the Mcm1p transcription factor (Kuo and Grayhack, 1994; McInerny et al., 1997). \( CLN1 \), \( CLN2 \) and \( CLN3 \) are genetically redundant and yeast require at least one G1 cyclin for viability (Cross, 1990; Richardson et al., 1989).

A \( SIC1 \) deletion will restore viability to a \( cln1\Delta cln2\Delta cln3\Delta \) strain (Tyers, 1996). Sic1p is a CKI that inhibits the function of Clb5p- and Clb6p-Cdc28p complexes via exclusion of substrate (Mendenhall, 1993; Schwob et al., 1994). Sic1p is only present in yeast during G1 (Donovan et al., 1994; Mendenhall et al., 1987; Schwob et al., 1994). Upon accumulation of Cln1p and Cln2p at Start,
Sic1p is subject to ubiquitin-mediated proteolysis (Dirick et al., 1995; Verma et al., 1997a; Verma et al., 1997b). The absence of Sic1p enables Clb5p- and Clb6p-Cdc28p complexes to initiate DNA synthesis and spindle pole body separation (Schwob et al., 1994), two events which represent passage though Start. CLB5 and CLB6 are 50% identical and are maximally expressed at Start under the control of the MBF transcription factor (Epstein and Cross, 1992; Koch et al., 1993; Kuhne and Linder, 1993; Schwob et al., 1994; Schwob and Nasmyth, 1993).

Overall, the passage through the Start checkpoint from G1 to S phase requires the accumulation of sufficient Clnp-Cdc28p activity to initiate Sic1p destruction and allow Clb5/6p-Cdc28p complexes to function (Dirick et al., 1995; Mendenhall and Hodge, 1998) (see Figure 5, NO pheromone).

1.2.5.3 Pheromone induced G1 arrest

When haploid yeast respond to pheromone they arrest in G1 as unbudded cells with a 1N DNA content. Pheromone-induced G1 arrest is a result of inhibition of Clnp-Cdc28p activity. Several mechanisms have been proposed which may combine to inhibit Clnp-Cdc28p, including direct inhibition of kinase activity, degradation of G1 cyclin proteins and repression of the expression of G1 cyclin genes (Gustin et al., 1998; Mendenhall and Hodge, 1998).
Figure 5. Pheromone response and the G1 to S transition
Cln1p, Cln2p and Cln3p control the activity of Cdc28p at the G1 to S transition. Activation of Cdc28p by the Clnps results in budding and degradation of Sic1p. When Sic1p is deleted from the cell, Clb5p and Clb6p are able to activate Cdc28p and, thereby, stimulate DNA synthesis. The activity of the G1 cyclins can be inhibited in yeast in the presence of pheromone by the combined activities of Fus3p and Far1p. The direct inhibition of Cln1/2p-Cdc28p by Far1p remains in dispute. See text for details.
Farlp is a pheromone-induced CKI

Farlp is a CKI and an effector of the pheromone response pathway that is required for pheromone-induced G1 arrest (Chang and Herskowitz, 1990). $FAR1$ is required for pheromone-induced repression of transcription. Analysis of global gene expression shows that pheromone exposure causes repression of multiple genes in a $FAR1$-dependent manner. The repressed genes are generally cell cycle-regulated genes whose expression peaks outside of the G1 phase (Roberts et al., 2000). Repression is likely an indirect result of Farlp activation by Fus3p, since Farlp inhibits the function of Cln-Cdc28p complexes in a pheromone-dependent manner, facilitating pheromone-induced G1 arrest (Gustin et al., 1998; Mendenhall and Hodge, 1998).

The mechanism of Farlp action remains in dispute; for completeness, all proposed mechanisms of Farlp function are included in Figure 5. Farlp is known to inhibit all three Cln-Cdc28p complexes in a PRP-dependent manner and has been detected in Cln-Cdc28p immunoprecipitates from pheromone-treated yeast (Jeoung et al., 1998; Peter et al., 1993; Tyers and Futcher, 1993). Early evidence indicated that Farlp inhibits Cln-Cdc28p by excluding other substrates. Cln-Cdc28p complexes are able to phosphorylate Farlp, which implies that the kinase remains active (Tyers and Futcher, 1993). In some experiments, however, immunoprecipitated Cln2p-Cdc28p complexes from pheromone treated, $FAR1$ cells demonstrate reduced specific activity, compared to their counterparts from far1 yeast (Peter et al., 1993). Importantly, when
Farlp is removed from Cln2p-Cdc28p-Far1p complexes by washing, Cln2p-Cdc28p kinase activity towards substrates other than Far1p is restored (Peter et al., 1993). In direct conflict with these observations, more recent work has demonstrated that Cln2p-Cdc28p complexes immunoprecipitated from pheromone-treated cells do not exhibit a Far1p-dependent reduction in specific activity (Gartner et al., 1998). These authors agree that Far1p is present in Cln2p-Cdc28p complexes in pheromone treated cells, but they propose that Far1p does not act through substrate exclusion (Gartner et al., 1998). The authors suggest that previous results were biased by CLN2 overexpression (Gartner et al., 1998). In contrast, all evidence to date supports direct inhibition of Cln3p-Cdc28p by Far1p in response to pheromone (Jeoung, et al., 1998). Further work will be required to determine the complete biochemical mechanism for Far1p action.

Far1p CKI activity is modulated by regulation of its expression, protein degradation and pheromone-induced phosphorylation (Blondel et al., 1999; Blondel et al., 2000; Gustin et al., 1998; Mendenhall and Hodge, 1998). Maximal FAR1 gene expression is detected in G2/M and pre-Start G1 yeast (Oehlen et al., 1996). The G2/M expression is dependent upon Mcm1p and the G1 expression requires Ste12p (Oehlen et al., 1996). Far1p levels are highest during G1, not G2/M, due to increased protein stability as, outside of G1, Far1p is subject to ubiquitin-mediated proteolysis (McKinney et al., 1993; McKinney and Cross, 1995). At the G1-S transition, nuclear localized Far1p is ubiquitinated by
Introducing the SCF<sub>Cdc4p</sub> complex and directed to the 26S proteasome (Blondel et al., 1999; Blondel et al., 2000).

The presence of pheromone increases <i>FAR1</i> expression four-fold in a Ste12p-dependent manner (Chang and Herskowitz, 1990). While increased Far1p levels are required for pheromone-induced growth arrest, PRP-dependent Far1p phosphorylation is also required for Far1p to function as a CKI (Chang and Herskowitz, 1992; McKinney et al., 1993; Oehlen et al., 1996; Peter et al., 1993; Peter and Herskowitz, 1994; Tyers and Futcher, 1993). Pheromone-induced phosphorylation allows Far1p to associate with Clnp-Cdc28p complexes in the nucleus (Chang and Herskowitz, 1992; Peter et al., 1993; Peter and Herskowitz, 1994; Tyers and Futcher, 1993). The association of Far1p with Clnp-Cdc28p complexes results in further phosphorylation and Far1p ubiquitin-mediated degradation, which may be a mechanism for recovery from pheromone response (Blondel et al., 1999; Blondel et al., 2000; Henchoz et al., 1997; Peter et al., 1993; Tyers and Futcher, 1993).

Fus3p is postulated to be the PRP-dependent Far1p kinase (Chang and Herskowitz, 1992; Elion et al., 1993; Peter et al., 1993; Tyers and Futcher, 1993). Fus3, but not Kss1p, has been implicated in pheromone-induced arrest, functions upstream of Far1p in the PRP and is required for Far1p activity (Elion et al., 1991; Elion et al., 1990; Elion et al., 1993). Biochemically, it has been demonstrated that Far1p is an <i>in vitro</i> substrate of Fus3p from pheromone.
treated cells and Far1p from fus3Δ cells does not associate with Clnp-Cdc28p complexes (Elion et al., 1993; Peter et al., 1993; Tyers and Futcher, 1993).

Together these results suggest a model for G1 growth arrest in which FAR1 expression and Far1p phosphorylation increase in response to pheromone and allow Far1p to bind to and inhibit Clnp-Cdc28p complexes (Gustin et al., 1998; Mendenhall and Hodge, 1998). In this model, Far1p directly inhibits Clnp-Cdc28p complexes, which inhibits Cln3p-Cdc28p-mediated induction of CLN1 and CLN2 expression and prevents Sic1p degradation (see Figure 5).

Additional mechanisms for growth arrest

Far1p inhibition of Clnp-Cdc28p complexes is the best understood mechanism for pheromone induced growth arrest in yeast, but other mediators have also been described.

Hyperactivated Fus3p and Kss1p have been demonstrated to induce pheromone-responsive G1 arrest in far1Δ cells. In these yeast, Fus3p and Kss1p inhibit the transcription of CLN1, CLN2, and CLB5, which results in decreased Clnp-Cdc28p activity. Normally, Cln3p-Cdc28p activates transcription in G1 through the SBF transcription factor, but Fus3p and Kss1p do not appear to inhibit SBF, so other factors must be involved (Cherkasova et al., 1999).

FAR3 was isolated in a screen for yeast mutants which maintain Ste12p-responsive transcription but resist growth arrest in yeast that overexpress STE4, which encodes the Gβ of the PRP (Horecka and Sprague, 1996). The screen also identified CLN3, FUS3 and FAR1 mutants. FAR3 is constitutively expressed
in all cell types and is not pheromone inducible. A far3Δ mutant increased the pheromone resistance of a far1Δ fus3Δ yeast strain, indicating that FAR3 inhibits growth by a separate pathway (Horecka and Sprague, 1996). In addition, FAR3 does not appear to alter Clnp-Cdc28p activity, CLN expression or Clnp proteolysis (Horecka and Sprague, 1996). A model for the action of the FAR3 gene product has not been proposed.

G1 growth arrest can be induced by overexpression of Ste12p in the absence of pheromone (Dolan, 1996; Dolan and Fields, 1990). The arrest is independent of Far1p, so it may involve induction of other unidentified genes that mediate growth arrest (Dolan, 1996).

1.2.5.4 Cdc28p activity impacts on the PRP

The basal activity of the PRP kinases Ste7p and Fus3p is increased in G1 but decreases, in a CLN1- and CLN2-dependent manner, along with the expression of pheromone-responsive genes, as the Start checkpoint approaches (McKinney et al., 1993; Oehlen and Cross, 1994; Oehlen et al., 1996; Wassmann and Ammerer, 1997; Zanolari and Riezman, 1991). The decreased activity of the PRP correlates with peak expression of the G1 cyclins, indicating that the cell cycle may regulate PRP activity (Oehlen et al., 1996; Wassmann and Ammerer, 1997).

Clnp-Cdc28p complexes may inhibit PRP function through the post-translational modification of Ste20p (Oehlen and Cross, 1998b; Wu et al., 1998). Overexpression of CLN2 represses PRP activation by pheromone, by deletion of
Gpa1 (Gα), and by Ste4p activation (Oehlen and Cross, 1998b). In contrast, CLN2 overexpression does not counteract PRP stimulation by activated Ste20p, Ste11p or Ste12p, indicating that Ste20p may be the target of Cln2p down-regulation (Oehlen and Cross, 1998b). In addition, Ste20p accumulates a post-translational modification (possibly phosphorylation) which correlates with both maximal CLN2 expression around Start and a decrease in PRP signaling (Oehlen and Cross, 1998b; Wu et al., 1998). The Ste20p modification correlates with a change in electrophoretic mobility that can be duplicated by Cln2p-Cdc28p complexes in vitro (Wu et al., 1998).

The effect of G1 cyclins on the PRP is specific, as overexpression of CLB5 and CLB6 does not alter the mobility of Ste20p (Wu et al., 1998). Cln2p-dependent reduction of PRP signaling may limit pheromone response to G1 and may have a role in recovery from pheromone response (see PRP signal attenuation and transcription).

1.3 Ste12p – A transcription factor for MAP kinase-induced development

1.3.1 Important features of Ste12p function

Ste12p is a transcription factor in the yeast Saccharomyces cerevisiae that responds to two separate MAP kinase signaling pathways; the pheromone response pathway (PRP) and the filamentation-invasion pathway (FIP) (Liu et al., 1993; Roberts and Fink, 1994) (see Filamentous development: the pseudohyphal development and invasive growth pathways share
components of the PRP, above). In response to both pathways, Ste12p binds to PREs (pheromone responsive elements) and directs transcription (Dolan et al., 1989; Errede and Ammerer, 1989; Gavrias et al., 1996; Laloux et al., 1994; Liu et al., 1993; Madhani and Fink, 1997; Roberts and Fink, 1994). Since Ste12p responds to two separate signal transduction pathways and activates the transcription of separate cohorts of genes in response to those signals, any model of Ste12p function must consider how specificity of Ste12p function is maintained.

A schematic of the known functional domains and protein-protein interactions of the 688 amino acid, 78 kDa Ste12p is shown in Figure 6 (Errede and Ammerer, 1989). The following sections will discuss the current model for Ste12p function and regulation, and address mechanisms for pathway-specific function of the transcription factor.

1.3.2 DNA binding and protein-protein interactions of Ste12p

1.3.2.1 Pheromone responsive elements

Most Ste12p responsive promoters share a common sequence known as the pheromone responsive element (PRE), whose sequence is defined as 5'-(A)TGAAACA-3' (Kronstad et al., 1987; Van Arsdell and Thorner, 1987). The PRE was originally described as the UAS (upstream activating sequence) which mediates α-factor stimulation of a-specific genes (Kronstad et al., 1987; Van Arsdell and Thorner, 1987). Subsequently, it has been demonstrated that PREs
Figure 6. Features of Ste12p
Three functional domains have been described for Ste12p. The DBD (amino acids 1 to 215) is the DNA binding domain, the NR (amino acids 305 to 669) is involved in negative regulation and the PID (amino acids 301 to 335) is the pheromone induction domain. Three transcription factor interaction domains have been identified in Ste12p. α1p interacts with amino acids 215 to 688 of Ste12p, Mcm1p interacts with amino acid 470 to 688 and Tec1p interacts with amino acids 1 to 215. For details, see text.
direct both basal and pheromone-induced transcription of haploid-specific genes (Hagen et al., 1991). In addition to its function in pheromone response, a PRE element can form one half of the FRE (filamentous response element), which consists of a PRE adjacent to a Tec1p binding site (Figure 4) (Laloux et al., 1994; Madhani and Fink, 1997).

Several experiments have demonstrated that Ste12p binds directly to DNA at pheromone responsive elements. First, Ste12p was detected in EMSA complexes on the transcriptional control elements of α-specific genes and TY1 promoters, both of which contain PRE sequences (Dolan et al., 1989; Errede and Ammerer, 1989). Next, recombinant Ste12p was shown to bind directly to PREs in both EMSA and footprint assays on both wild-type promoter fragments and oligonucleotides containing PREs (Yuan and Fields, 1991). Recently, Ste12p was shown to bind to pheromone-responsive promoters by chromatin immunoprecipitation and microarray analysis (Ren et al., 2000). The study also demonstrated that Ste12p binds to selected promoters prior to pheromone response and that further Ste12p accumulates after pheromone treatment (Ren et al., 2000).

The minimum Ste12p fragment required for binding to PREs is amino acids 41 to 204, although amino acids 1 to 215 are generally referred to as the DNA binding domain (Figure 6) (Yuan and Fields, 1991). Amino acids 1-19, in fact, may reduce the ability of Ste12p to interact with DNA in vivo (Crosby et al., 2000).
1.3.2.2 Categories of Ste12p responsive promoters

Examples of Ste12p-inducible genes are listed in Table 1. Some genes, such as *FLO11*, *PHD1* and *TEC1* are induced in yeast in the processes of filamentous development and invasive growth (Gimeno and Fink, 1994; Lo and Dranginis, 1998; Madhani and Fink, 1997). Other genes are induced by pheromone, and can be divided into three categories. *STE2*, the α-factor receptor, is expressed only in α cells and is a model α-specific gene (Hartig et al., 1986; Nakayama et al., 1985). Genes expressed only in α-cells, such as the α-factor receptor *STE3*, are called α-specific genes (Sprague et al., 1983). Some pheromone-inducible genes are expressed in both a and α yeast and are known as haploid-specific genes. *FUS1*, which is required for cell fusion, is a haploid-specific gene (McCaffrey et al., 1987; Trueheart et al., 1987).

At each type of promoter, Ste12p activates transcription through a different protein-DNA complex, which may contribute to pathway-specific signaling (Figure 4). Haploid-specific genes, such as *FUS1*, have multiple PREs in their promoters through which Ste12p directs both basal and pheromone-induced transcription, possibly as a homodimer (Hagen et al., 1991; McCaffrey et al., 1987; Trueheart et al., 1987; Yuan and Fields, 1991). Mcm1p-Ste12p complexes activate *STE2* and other α-specific genes through adjacent Ste12p and Mcm1p binding sites, respectively PREs and P boxes (Dolan et al., 1989). α-specific genes, like *STE3*, are regulated through PQ elements (Sengupta and Cochran, 1990). PQ elements consist of an Mcm1p binding site (P) adjacent to
an α1p binding site (Q), but do not have an associated PRE (Jarvis et al., 1988). Since Ste12p can interact with both α1p and Mcm1p, Ste12p activation of α-specific genes may be mediated by protein-protein interaction rather than protein-DNA interaction (Kirkman-Correia et al., 1993; Yuan et al., 1993). The fourth category of promoter to which Ste12p binds contains filamentous response elements (FREs), also known as SREs (stress response elements) (Laloux et al., 1994; Madhani and Fink, 1997). Genes involved in yeast filamentation, which include FLO11 and TEC1, are regulated by Tec1p and Ste12p bound to adjacent TCS and PRE elements in FREs (Lo and Dranginis, 1998; Madhani and Fink, 1997). TCS (TEA (TEF-1, Tec1, and AbA motif) DNA consensus sequence) elements are Tec1p binding sites (Baur et al., 1997).

1.3.3 The interaction of Ste12p with other transcriptional activators

In order for the STE12 gene product to activate transcription of α- and α-specific pheromone-responsive genes, it must interact with Mcm1p and α1p, two other transcription factors involved in pheromone response (Kirkman-Correia et al., 1993; Yuan et al., 1993). The carboxy-terminal 219 amino acids of Ste12p interact with Mcm1p to activate α-specific genes such as STE2 (Figure 6) (Errede and Ammerer, 1989; Kirkman-Correia et al., 1993). The Ste12p (aa 469 to 688) interaction with Mcm1p was demonstrated by EMSA using myc-tagged deletions of Ste12p (Errede and Ammerer, 1989). The C-terminus of Ste12p also interacts with α1p (Figure 6). A ste12 phenotype in S. cerevisiae can be complimented by co-expression of α1p and Ste12p from K. lactis. Expression of a series of
chimeric Ste12p constructs, combining Ste12p domains from either species, in S. cerevisiae revealed that the C-terminus of Ste12p interacts with α1p. Recombinant Ste12p (aa 215 to 688) can also interact with α1p in the absence of DNA (Yuan et al., 1993). In addition, some Ste12p-α1p interactions may fall outside the carboxy terminus of Ste12p. The T-50 allele of STE12, which converts alanine 50 of Ste12p to a threonine residue, reduces the interaction of Ste12p and α1p, indicating that the Ste12p DNA binding domain may also be involved in the Ste12p-α1p interaction (La Roche et al., 1995).

Another subset of pheromone-responsive genes that Ste12p controls is the karyogamy-specific genes, a set of genes required for nuclear fusion during mating. Both Ste12p and Kar4p modulate pheromone-responsive expression of these genes, which include KAR3, KAR5 and CIK1 (Gammie et al., 1999; Kurihara et al., 1996). Neither the DNA sequence elements nor the protein domains involved in the regulation of gene expression by Ste12p and Kar4p are known, although the KAR3 promoter does contain three potential PREs (Kurihara et al., 1996).

Activated transcription from FREs is regulated by Ste12p and its transcription factor partner, Tec1p (Baur et al., 1997; Gavrias et al., 1996; Laloux et al., 1994; Madhani and Fink, 1997). Although direct interaction of Ste12p and Tec1p in the absence of DNA has not been demonstrated, their binding to the FRE is believed to be cooperative and only requires amino acids 1 to 215 of Ste12p (Baur et al., 1997; Madhani and Fink, 1997).
Ste12p and Tec1p, genes required for filamentation-invasion may also require the input of other transcription factors, such as Flo8p, which regulates the expression of *FLO11*, through independent sequence elements (Rupp et al., 1999).

### 1.3.4 MAP kinase-dependent phosphorylation of Ste12p

Since Ste12p is known to function downstream of the MAP kinases Fus3p and Kss1p, one attractive model for Ste12p regulation is the accumulation of activating phosphorylations when the MAP kinases are stimulated. When Ste12p-Gal4p fusion proteins are expressed in yeast cells, it is possible to observe pheromone-dependent phosphorylation of the fusion proteins which correlates with transcriptional activation (Song et al., 1991). In addition, Ste12p has been demonstrated to be a substrate of pheromone-activated Fus3p (Elion et al., 1993).

Substantial effort has been expended to identify pheromone-induced phosphorylations of Ste12p. Ste12p has been shown by peptide mapping to have eight constitutive phosphorylated peptides, whose phosphorylation is independent of the protein kinases of the pheromone response pathway (Hung et al., 1997). Ste12p accumulates two transient minor phosphopeptides in the presence of pheromone which are dependent upon the pheromone response pathway and localization of Ste12p to the nucleus (Hung et al., 1997). The identity of the two phosphopeptides is not known. As a result, it is not possible to test their function or to demonstrate that the peptides are direct targets for Fus3p.
or Kss1p phosphorylation. It is possible, therefore, that the phosphorylation of other target molecules, such as Rst1p (Dig1p) and Rst2p (Dig2p) (described below) controls pheromone-responsive transcription (Cook et al., 1996; Tedford et al., 1997).

1.3.5 Regulatory domains of Ste12p

The regulatory domains of Ste12p were originally characterized by examining a series of Gal4p-Ste12p fusion proteins and by analysis of a set of linker and deletion mutants of Ste12p (Kirkman-Correia et al., 1993; Song et al., 1991). The analyses revealed an activation domain in amino acids 214 to 473 and demonstrated that amino acids 305 to 669 comprise a target of negative regulation in the absence of pheromone (Kirkman-Correia et al., 1993; Song et al., 1991).

One study showed that amino acids 214 to 473 of Ste12p can activate transcription in the absence of pheromone, when fused to Gal4p. Activation by amino acids 214 to 473 was one hundred and eighty fold more efficient than activation by amino acids 214 to 688, demonstrating that the C-terminal amino acids reduce the activity of Ste12p (Song et al., 1991). Confirming these results, the second study showed that three separate deletions in the region of STE12 encoding amino acids 305 to 669 of Ste12p resulted in substantially (six- to twenty-fold) increased levels of basal transcription from a FUS1-LacZ reporter (Kirkman-Correia et al., 1993).
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Subsequently, amino acids 301 to 335 of Ste12p have been identified as the pheromone inducible domain (PID) of Ste12p (Figure 6) (Pi et al., 1997). If this domain is absent from Ste12p, the activity of a $FUS1$-$LacZ$ reporter is no longer pheromone inducible. In addition, this domain confers pheromone inducibility on Ste12p-Gal4p fusions. It has been proposed that PID function may be regulated by phosphorylation. However, alanine substitution of the serine and threonine residues in the pheromone inducible domain of Ste12p has no apparent effect on Ste12p function, indicating that phosphorylation of Ser or Thr residues does not control PID function. Interestingly, the mutation of two tyrosines (residues 310 and 317) in the Ste12p "minimal pheromone induction domain" to alanines resulted in increased basal transcription and loss of pheromone induction of Gal4p-Ste12p fusions (Pi et al., 1997). Tyrosine phosphorylation of Ste12p has not been demonstrated (Hung et al., 1997). Based on these data, the PID may contribute to both the activation and inhibition of Ste12p function.

The PID is much smaller than either the activation domain (amino acid 214 to 473) or the regulatory domain (amino acids 305 to 669) defined by earlier studies. Future work will determine whether other amino acids within these domains have important regulatory functions.

1.3.6 Overexpression of Ste12p

When Ste12p is overexpressed in yeast cells, it is possible to observe some of the phenotypes of pheromone response in the absence of pheromone
(Dolan, 1996; Dolan and Fields, 1990). STE12 overexpression leads to an increase in transcription of pheromone-inducible genes and the arrest of the cells in the G1 stage of the cell cycle, independent of the FAR1 gene product. As well, the overexpression of STE12 can suppress the mating defect of ste mutants. Since STE12 overexpression can increase the activity of Ste12p in the absence of pheromone and deletions of regions of Ste12p can eliminate negative regulation (Dolan, 1996; Dolan and Fields, 1990; Kirkman-Correia et al., 1993), it has been postulated that Ste12p has an inherent activation function which is masked in the absence of pheromone. One possible explanation of these results is that there is a direct negative regulator (or regulators) of Ste12p, similar to the Gal80p regulator of Gal4p. Two candidate proteins for the role of direct negative regulator of Ste12p are Rst1p (Dig1p) and Rst2p (Dig2p) (Bardwell et al., 1998a; Bardwell et al., 1998b; Cook et al., 1996; Pi et al., 1997; Tedford et al., 1997).

1.3.7 Rst1p (Dig1p) and Rst2p (Dig2p) are MAP kinase substrates and negative regulators of Ste12p

Rst1p (Dig1p) and Rst2p (Dig2p) are negative regulators of Ste12p and regulate both its pheromone-responsive and filamentous growth functions (Bardwell et al., 1998a; Bardwell et al., 1998b; Cook et al., 1996; Pi et al., 1997; Tedford et al., 1997). Rst1p (Dig1p) and Rst2p (Dig2p) were cloned simultaneously in two separate two-hybrid screens (Cook et al., 1996; Tedford et al., 1997). One screen identified Rst1p (Dig1p) and Rst2p (Dig2p) as proteins that interact with Cln1 and Cln2p, two of the G1 cyclins (Tedford et al., 1997),
while the other screen cloned genes encoding proteins that interact with Kss1p (Cook et al., 1996). Further characterization has shown that Rst1p (Dig1p) and Rst2p (Dig2p) are nuclear proteins which co-immunoprecipitate with Kss1p, Fus3p and Ste12p, and that Rst1p (Dig1p) and Rst2p (Dig2p) are substrates for MAP kinase phosphorylation by both Kss1p and Fus3p (Cook et al., 1996; Tedford et al., 1997).

In addition to being substrates for the MAP kinases, Rst1p (Dig1p) and Rst2p (Dig2p) are known to be essential to the negative regulation function of Kss1p (Bardwell et al., 1998a; Bardwell et al., 1998b). Kss1p, the MAP kinase that regulates the invasive growth pathway, functions as both an activator and an inhibitor of Ste12p-dependent transcription (Madhani et al., 1997). Recent data has demonstrated that the negative regulation function of Kss1p is dependent upon Rst1p (Dig1p) and Rst2p (Dig2p) (Bardwell et al., 1998a; Bardwell et al., 1998b). Although Kss1p, Ste12p, Rst1p (Dig1p) and Rst2p (Dig2p) coexist in a complex, the mechanism for the negative regulation has yet to be described (Cook et al., 1996; Tedford et al., 1997).

1.3.7.1 RST1 and RST2 are redundant genes with non-identical functions

Rst1p (Dig1p) and Rst2p (Dig2p) are novel genes with substantial sequence homology to one another (27% amino acid identity, 42% amino acid similarity) but no significant primary sequence similarity to other known proteins (Cook et al., 1996; Tedford et al., 1997). Disruption of either RST1 or RST2 has no detectable effect on S. cerevisiae; however, disruption of both genes results in
constitutive invasive growth and elevated transcription of pheromone-responsive
genes (Cook et al., 1996; Tedford et al., 1997). In addition, \textit{rst1 rst2} cells grow
slowly, but all three \textit{rst1 rst2}-associated phenotypes are commonly lost after the
cells have been grown for several generations. The loss of the \textit{rst1 rst2}
phenotype can be attributed to accumulation of loss of function mutations in the
\textit{STE12} gene (M. Tyers, pers. comm.). Taken together, these results indicate that
Rst1p (Dig1p) and Rst2p (Dig2p) may be the direct negative regulators of Ste12p
and that they are redundant.

Two significant differences between Rst1p (Dig1p) and Rst2p (Dig2p)
have been observed. First, Rst1p (Dig1p) is constitutively expressed, while
Rst2p (Dig2p) is pheromone induced (Cook et al., 1996). Second, the two
proteins are not the same size. Rst1p (Dig1p) is 452 amino acids, while Rst2p
(Dig2p) is 323 amino acids, and the Rst1p (Dig1p) sequence contains several
stretches of amino acid sequence that have no homology to Rst2p (Dig2p) (Cook
et al., 1996; Tedford et al., 1997). So, although the two genes \textit{RST1} and \textit{RST2}
have been assumed to be redundant, the genes and the mechanisms by which
they are regulated are not identical.

\section{1.4 Transcriptional activators cause increased transcription by RNA
polymerase II}

Transcriptional activators, including Ste12p, regulate transcription in
response to the physiological needs of the cell (Kornberg, 1999). In order to
mate effectively, yeast cells that are exposed to mating pheromone must
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differentiate into mating competent cells (Herskowitz, 1988). The pheromone
response stimulates obligate transcriptional activation of gene expression,
including a one-hundred fold increase in the cellular level of FUS1 transcripts
(Hagen et al., 1991; McCaffrey et al., 1987; Trueheart et al., 1987). The increase
in FUS1 expression is a result of activation by Ste12p, which, upon stimulation by
the pheromone response pathway, causes increased RNA polymerase II (Pol II)
transcription of FUS1 and other pheromone responsive genes (Dolan et al.,
1989; Errede and Ammerer, 1989; Fields and Herskowitz, 1985; Fields and
Herskowitz, 1987; Song et al., 1991).

Activators are essential to regulated gene expression, since the amount of
Pol II in a cell is limited and activators increase the amount of Pol II transcription
from specific promoters (Hampsey and Reinberg, 1999; Kornberg, 1999).

Activation may act through multiple protein-protein interactions, since Pol
II is an enzyme which functions in conjunction with a large number of other
A schematic of the protein complexes required for activated transcription of a
eukaryotic promoter is presented in Figure 7. The following sections will outline
the function of these Pol II-associated factors, including TFIIA, the general
transcription factors, the Pol II core components, the Mediator and the
nucleosome remodeling complexes. The review will describe the steps which
precede transcription initiation and the mechanisms by which transcriptional
activators may affect the formation of the Pol II complex at promoters.
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Figure 7. A model for transcriptional activation
An RNA polymerase II pre-initiation complex including TFIIA, TFIID, the general transcription factors and the Mediator, is assembled at a hypothetical eukaryotic promoter. In this model, the activator enhances complex formation by interaction with Mediator components. Prior to the formation of the PIC, nucleosomal rearrangement by either SWI/SNF or SAGA complexes may have exposed the DNA elements to which the transcription factors are bound. Adapted from (Malik and Roeder, 2000).
1.4.1 TFIID and TFIIA

In order to initiate transcription of a structural gene, a pre-initiation complex (PIC), consisting of Pol II and the general transcription factors, must be assembled at the promoter (Hampsey, 1998). The first step in PIC assembly is promoter recognition by TFIID (Buratowski, 2000; Green, 2000; Kornberg, 1999). TFIID, a general transcription factor which contains TATA binding protein (TBP) and the TAF\(_{110}\)s, associates with the promoter in the absence of other factors and nucleates the formation of the PIC. The binding of TBP to the promoter is correlated with transcriptional efficiency (Kuras and Struhl, 1999; Li et al., 1999).

The TBP subunit of TFIID binds directly to the TATA box, straddling the DNA and bending the DNA in order to form a context for the subsequent TFIIB interaction (see The general transcription factors, below) (Kim et al., 1993; Nikolov et al., 1992). The TATA box is a core cis regulatory element found in typical class II eukaryotic promoters and is located 40 to 120 bp upstream of the transcription start site. Together with other core promoter elements, the TATA box defines the site where Pol II and the GTFs will bind and form the pre-initiation complex (PIC) (Hampsey, 1998; Leuther et al., 1996; Li et al., 1994).

Binding of TBP to the core promoter is stabilized by the factor TFIIA, which interacts with both TBP and the DNA flanking the TATA box (Buratowski et al., 1989; Imbalzano et al., 1994; Kang et al., 1995; Lee et al., 1992). TFIIA also inhibits the function of Mot1p, a protein that can dissociate TBP from the TATA element, and yTAF130, which inhibits TBP binding to the TATA sequence (Auble
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et al., 1994; Kokubo et al., 1998). In addition to its core promoter functions, TFIIA has activator-dependent functions (Stargell et al., 2000). TFIIA can interact directly with both activation domains and coactivators and TFIIA is necessary for the activator-dependent stabilization of the TFIID-TATA complex (Clemens et al., 1996; Damania et al., 1998; Kobayashi et al., 1995; Lieberman and Berk, 1994; Ozer et al., 1994; Ranish et al., 1999).

The other components of TFIID are the TAFs. In Saccharomyces cerevisiae, twelve TAFs have been identified, all of which have homologues in higher eukaryotes (Green, 2000). Unlike TBP, which is generally required for Pol II transcription, and whose occupancy of promoters is correlated to transcriptional activity, TAFs are promoter-selective (Kuras et al., 2000; Lee et al., 2000; Li et al., 2000). Genome-wide analysis has demonstrated that each TAF affects the expression of a characteristic subset of genes, ranging from three to sixty-seven percent of the genome, which indicates that TAFs may act as promoter selectivity factors (Lee et al., 2000).

TAFs have multiple functions, including contacting the DNA in the core promoter region, and histone acetyltransferase function (Green, 2000). Several observations indicate that certain TAFs may interact with core promoter sequences that flank the TATA box and confer binding specificity on TFIID (Green, 2000; Verrijzer et al., 1995; Verrijzer and Tjian, 1996). For example, yTAF145 recognizes and selects core promoters in a UAS-independent manner (Shen and Green, 1997). TAFs may also provide a catalytic activity that is essential for transcription; yTAF145 and its mammalian homologue, TAFI250,
have intrinsic histone acetyltransferase (HAT) activity, and could acetylate other proteins in the transcription complex (Mizzen et al., 1996). TAFII250 has also been demonstrated to act as a ubiquitin activating and conjugating enzyme for histone H1 (Pham and Sauer, 2000). Both of these modifications are postulated to affect nucleosomal architecture and transcription activity (Mizzen et al., 1996; Pham and Sauer, 2000).

TAFII55 may also interact transcriptional activators and increase TBP binding to promoters (Klebanow et al., 1997; Li et al., 1999; Moqtaderi et al., 1996; Poon et al., 1995; Reese et al., 1994). Activators have been shown to interact with isolated TAFII55, although TAFII55 are found in complexes, so the protein-protein interactions of isolated TAFII55 may not be physiologically relevant (Chen et al., 1994; Goodrich et al., 1993; Hoey et al., 1993; Reese et al., 1994; Thut et al., 1995). In addition, it has been shown that yTAF17-dependence of transcription is conferred by the UAS sequence, indicating a yTAF17-activator interaction (Michel et al., 1998; Moqtaderi et al., 1998). A recent study has also demonstrated that, at TAFII-dependent promoters, TAFII55 are co-recruited with TBP in a manner consistent with direct interactions between TAFII55 and activators (Li et al., 2000).

In addition to their role in TFIID, the TAFII55 are found in SAGA, another multi-subunit complex that affects transcription (see The SAGA complex contains a histone acetyltransferase below) (Brown et al., 2000; Green, 2000).
**1.4.2 The RNA polymerase II holoenzyme**

Following the association of TFIID and TFIIA with the core promoter elements, the RNA polymerase II holoenzyme is recruited to the promoter (Ranish et al., 1999). The Pol II holoenzyme is a DNA-independent complex of proteins composed of the RNA polymerase II core enzyme, the Mediator complex and, possibly, the general transcription factors (Kim et al., 1994; Koleske and Young, 1994; Thompson et al., 1993).

The scope of holoenzyme function *in vivo* was highlighted when DNA microarray analysis was used to monitor the effects of temperature sensitive holoenzyme components on transcription in yeast (Holstege et al., 1998). Srb4p, a member of the Mediator complex, was demonstrated to be required for transcription of most yeast genes, whereas mutation of Srb10p, a cyclin-dependent kinase from the holoenzyme, only affects selected genes (Holstege et al., 1998). While the limited effect of Srb10p indicates that some holoenzyme components are specific to particular genes or gene families, the global requirement of Srb4p for transcription indicates that the holoenzyme functions at most promoters.

**1.4.2.1 The core components of RNA polymerase II**

The twelve subunit RNA polymerase II complex, which is conserved in all eukaryotic organisms, is the enzyme that transcribes protein coding genes (Hampsey, 1998; Kornberg, 1999). Recent crystallographic analysis of yeast Pol II shows that the enzyme forms a clam shaped molecule around the DNA, with
binding sites for both DNA and RNA and a pore beneath the active site for entry of the NTPs and the exit of transcripts (Cramer et al., 2000). The two dominant components of the "clam" are Rpb1p (β') and Rpb2p (β), the two largest components of Pol II, which are responsible for binding DNA and synthesizing RNA, respectively (Hampsey, 1998). The smaller Pol II subunits are arranged around the periphery of the structure and help to form a set of "jaws" to grip the DNA downstream of the active center, and a clamp which holds the DNA in place at the active center (Cramer et al., 2000).

Multiple aspects of Pol II function are coordinated through the C-terminal domain (CTD) of Rpb1p (Dahmus, 1996; Kim et al., 1994; Koleske and Young, 1994; Lu et al., 1991; O'Brien et al., 1994). The phosphorylation state of the CTD reflects the shift of Pol II from initiation to elongation. The CTD has multiple repeats of the heptapeptide sequence YSPTSPS that can be either phosphorylated or unphosphorylated (Conaway et al., 2000; Dahmus, 1996). The switch to the phosphorylated state accompanies the transition of the Pol II complex from initiation to elongation (Dahmus, 1996; Lu et al., 1991; O'Brien et al., 1994). The CTD also interacts with several Srbp components of the Mediator (Nonet and Young, 1989; Thompson et al., 1993), connecting the Pol II and Mediator components of the holoenzyme.
1.4.2.2 The Mediator complex

The Mediator was originally identified as a factor required by transcriptional activators to stimulate transcription with reconstituted Pol II and GTFs in vitro (Flanagan et al., 1991).

The Mediator complex of *Saccharomyces cerevisiae* consists of approximately twenty proteins, including Srb2p, Srb4p to Srb11p, Med1p to Med4p, Med6p to Med8p, Gal11p, Sin4p, Rgr1p and Rox3p (Gustafsson et al., 1998; Kim et al., 1994; Koleske and Young, 1994; Lee et al., 1997; Li et al., 1995b; Myers et al., 1998; Nonet and Young, 1989; Thompson et al., 1993). The exact composition of the Mediator, like the holoenzyme, depends upon the method of purification; for example, one Mediator preparation does not include Srb8p to Srb11p, although it does contain the other Srbps (Myers et al., 1998).

Prior to the identification of the Mediator complex, nearly two thirds of the proteins in the Mediator were identified in disparate genetic screens for genes that affect transcription control in yeast. The *SRB* genes were isolated and characterized as extragenic supressors of *RPB1* CTD truncation mutations (Nonet and Young, 1989; Thompson et al., 1993), while *GAL11* was originally identified as a gene required for maximum expression of galactose-metabolizing enzymes (Suzuki et al., 1988). Although the Mediator has sometimes been classified as a co-activator of transcription, not all components of the Mediator increase transcription. In fact, Srb8p to Srb11p, Sin4p, Rgr1p and Rox3p have all been identified as repressors of transcription in genetic screens (Gustafsson
et al., 1998; Hengartner et al., 1995; Kuchin et al., 1995; Li et al., 1995b; Liao et al., 1995; Song et al., 1996). The presence of repressors in the complex implies that the Mediator may have both positive and negative effects on transcription. As a result, it has been suggested that the Mediator complex integrates both positive and negative regulatory signals and acts as a "control panel" for transcription (Malik and Roeder, 2000).

1.4.2.3 The general transcription factors

The general transcription factors (GTFs) are a group of proteins which are associated with some forms of the holoenzyme and are absolutely required for Pol II transcription (Hampsey, 1998; Koleske and Young, 1994). The GTFs include TFIIB, TFIIF, TFIIE and TFIIH.

TFIIB links many components of the PIC, since discrete domains of TFIIB interact with TBP, TAF\textsubscript{II}s, Pol II, and multiple subunits of TFIIF (Barberis et al., 1993; Buratowski et al., 1989; Buratowski and Zhou, 1993; Fang and Burton, 1996; Gonzalez-Couto et al., 1997; Goodrich et al., 1993; Ha et al., 1993). In addition to contacting multiple PIC components and stabilizing the TFIID-TATA complex, TFIIB influences both transcriptional start site (Leuther et al., 1996; Li et al., 1994) and the unidirectional assembly of the PIC (Lagrange et al., 1998; Littlefield et al., 1999).

In addition to its role in basal transcription, TFIIB is a target of gene-specific activators. The activator-TFIIB interaction influences transcription in two ways. First, activators increase TFIIB recruitment and thereby activate
transcription (Kim and Roeder, 1994; Lin et al., 1991; Roberts et al., 1995; Roberts et al., 1993). Second, the interaction of TFIIB with activators has been demonstrated to induce a conformational change in TFIIB, which may increase its ability to interact with other components of the transcription apparatus (Roberts and Green, 1994; Wu and Hampsey, 1999).

The three remaining GTFs in the PIC are TFIIE, -F and -H. TFIIE interacts with TFIIF, TFIIH and Pol II in the PIC (Flores et al., 1988; Li et al., 1994; Maxon and Tjian, 1994). TFIIF, which is closely associated with TFIIE (Flores et al., 1988; Sawadogo and Roeder, 1985), stabilizes the interaction of Pol II with TFIIB and further stabilizes the PIC by altering DNA structure in the core promoter (Buratowski et al., 1991; Forget et al., 1997; Robert et al., 1998). TFIIH has both helicase and kinase activity and, in addition to its role in initiation, TFIIH is involved in nucleotide excision repair (Feaver et al., 1991; Lu et al., 1992; Schaeffer et al., 1993; Serizawa et al., 1992; Serizawa et al., 1993; Svejstrup et al., 1996; Tirade et al., 1999).

TFIIE, -F and -H are also involved in the transition of the Pol II complex from pre-initiation to transcript elongation (Conaway et al., 2000). TFIIE stimulates TFIIH, a nine-subunit GTF complex, to begin the process of ATP-dependent promoter melting, which is required for promoter clearance (Kugel and Goodrich, 1998). In order to unwind the DNA at the transcription start site, TFIIH exerts torque on the DNA helix several nucleotides downstream of the start site and unwinds the helix (Kim et al., 2000). TFIIF and TFIIH, together, are necessary for the conversion of Pol II from the initiation state to the elongation
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state, which requires phosphorylation of the Rpb1p (Dvir et al., 1997; Feaver et al., 1991; Lu et al., 1992; O'Brien et al., 1994; Serizawa et al., 1992). The phosphorylation of the CTD by TFIIH is also stimulated by TFIIE, in conjunction with Gal11p, a component of the Mediator complex (Sakurai and Fukasawa, 1998).

1.4.3 Accessory complexes alter chromatin structure

The coiling of eukaryotic DNA around histone octamers in nucleosomes represses transcription by occluding the binding sites for transcription factors. In addition, higher order chromatin structure and modification of histones can repress transcription in entire domains of chromatin (Kornberg, 1999; Kornberg and Lorch, 1999). Two families of accessory complexes associated with transcription counteract the effects of chromatin. One class of accessory complex is typified by the SWI/SNF complex, which remodels nucleosomes in an ATP-dependent manner (Peterson and Workman, 2000). The second class of remodeling complex, which includes the SAGA complex, chemically modifies the histone proteins (Brown et al., 2000).

1.4.3.1 The SWI/SNF complex alters chromatin structure

The SWI/SNF complex is a multi-subunit, DNA-dependent ATPase which affects gene transcription by altering chromatin structure (Peterson and Workman, 2000). SWI/SNF reduces the total length of DNA per nucleosome and increases the accessibility of DNA to DNA binding proteins (Bazett-Jones et al.,
The complex is conserved in all higher eukaryotes and is known to be associated with, but not an integral part of, the holoenzyme (Peterson and Workman, 2000). SWI/SNF complexes have been demonstrated to be recruited to promoters by activator proteins and, in vitro, SWI/SNF recruitment stimulates Pol II transcription of nucleosome arrays (Cosma et al., 1999; Krebs et al., 1999; Natarajan et al., 1999; Neely et al., 1999; Yudkovsky et al., 1999).

A recent study shows that SWI/SNF can be recruited to a promoter by one activator and can subsequently participate in the chromatin remodeling that exposes the binding site for a second transcriptional activator, demonstrating that SWI/SNF can affect multiple aspects of activator function (Cosma et al., 1999).

SWI/SNF is a low abundance complex (100-500 copies/cell) and is required for transcription of only five percent of yeast genes (Cote et al., 1994; Holstege et al., 1998). Other chromatin remodeling may be facilitated by the RSC (remodels the structure of chromatin) complex, a higher abundance complex which has similar subunits and identical in vitro activity to the SWI/SNF complex (Cairns et al., 1996).

1.4.3.2 The SAGA complex contains a histone acetyltransferase

Reversible acetylation of lysine residues in the N-terminal tails of histones is often associated with transcriptional activation of associated genes (Brown et al., 2000; Kornberg and Lorch, 1999). It is postulated that the histone acetylation weakens the affinity of histones for DNA, altering the nucleosome conformation.
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and increasing the accessibility of DNA to transcription factors (Kadonaga, 1998; Struhl, 1998; Wade and Wolffe, 1997). One complex which has histone acetyltransferase (HAT) activity and is associated with Pol II transcription is the SAGA complex. SAGA consists of 14 peptides, including the Gcn5p histone acetyltransferase (Brown et al., 2000). The other components of the complex include the Ada proteins, which interact with activators (Grant et al., 1997), the Spt proteins, which interact with TBP (Grant et al., 1997), several TAF_{II}s, which are shared with TFIID (Grant et al., 1998a), and Tra1p, which interacts with activators (Grant et al., 1998b). The other components of SAGA increase the ability of Gcn5p to acetylate nucleosomal histones (Grant et al., 1997; Grant et al., 1999).

The SAGA complex can be recruited to promoters by specific activators. \textit{In vitro}, recruitment by these activators results in increased transcription in nucleosomal arrays (Ikeda et al., 1999; Utley et al., 1998). \textit{In vivo}, transcriptional activation of the HIS3 gene is correlated with acetylation of local histone H3, and both activation and acetylation are dependent upon the acetylase function of Gcn5p (Kuo et al., 1998). Together, these results indicate that recruitment of SAGA to a promoter results in histone acetylation and transcriptional activation.

SAGA shares several TAF_{II} components with TFIID, and global transcription analysis has shown that the shared TAF_{II} subunits collectively contribute to the transcription of seventy percent of yeast genes (Lee et al., 2000). Both SAGA and TFIID have subunits with HAT activity and, since the complexes have common subunits, questions have been raised as to whether
the complexes have overlapping functions (Green, 2000). Global transcription analysis revealed that, although the two HATs can compensate for the loss of one another, the two complexes are not identical in function. The complexes have overlapping functions at some genes, while other genes are specifically dependent on either TFIID or SAGA (Lee et al., 2000).

Other HAT complexes have been identified in yeast (Brown et al., 2000). The other complexes have different histone specificities and one, Elongator, is involved in elongation and not initiation (Otero et al., 1999; Wittschieben et al., 2000; Wittschieben et al., 1999).

1.4.4 Activators recruit the complexes required for transcription to promoters

Activators are known to bind to DNA and to contact multiple components of the transcription machinery; however, a series of protein-protein contacts that leads to increased transcription has not been defined. Presently, activator interactions with the transcription machinery can be classified into two groups: interaction with proteins or complexes that relieve repression by nucleosomes, and interaction with Pol II and its associated factors (Kornberg, 1999). Through these interactions, activators may stimulate a two-part process of activation, first, by facilitating the local rearrangement of the nucleosome complexes that are inhibiting transcription and second, by recruiting the transcriptional machinery (Berk, 1999).
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Several observations support the model that transcriptional activators activate transcription by localizing accessory complexes to promoters. Activators can interact with both the SWI/SNF and SAGA complexes, bringing chromatin remodeling complexes to promoters where they can increase the accessibility of DNA to proteins (see Accessory complexes alter chromatin structure, above). Furthermore, tethering Gcn5p to the DNA as a LexA-Gcn5p fusion can activate transcription of a reporter gene, demonstrating that localization of HAT activity to a promoter activates transcription (Candau et al., 1997).

Multiple lines of evidence support the hypothesis that activators recruit the components of the transcriptional machinery. First, many components of the transcription machinery can interact with activation domains (Gill et al., 1994; Goodrich et al., 1993; Hoey et al., 1993; Klemm et al., 1995; Koh et al., 1998; Roberts et al., 1993; Stringer et al., 1990). Second, when components of the transcription complex are fused to DNA binding domains, thereby increasing their local concentration at selected promoters, the fusion proteins function as artificial activators (Chatterjee and Struhl, 1995; Farrell et al., 1996; Gaudreau et al., 1997; Gonzalez-Couto et al., 1997; Xiao et al., 1995). Third, it has been demonstrated that a high affinity protein-protein interaction mimics the function of an activation domain (Gaudreau et al., 1998). Gal11Pp (potentiator), an altered form of Gal11p, interacts with the dimerization domain of the activator Gal4p. In yeast strains expressing Gal11Pp, the Gal4p dimerization domain, which has no inherent activation function, can substitute for an activation domain in a Gal11Pp dependent manner (Gaudreau et al., 1998).
1.5 Project objectives

The unifying goal of this research was to further define both the domain structure of Ste12p and the mechanisms that regulate Ste12p activity.

The first part of the work focussed on the effects of Ste12p overexpression. The results can be considered in two parts. First, the experiments showed that \textit{STE12} overexpression causes \textit{FAR1}-independent G1 growth arrest. The work also demonstrated that the arrest was independent of transcription from PRE elements. Second, the experiments showed that overexpression of Ste12p can induce transcription of a \textit{FUS1} reporter gene.

Based on these results, it was postulated that Ste12p overexpression might titrate a direct negative regulator or might activate transcription through Ste12p multimerization. The minimal domain of Ste12p that is required for transcriptional induction and growth arrest was determined to be amino acids 262 to 594.

The second part of the work sought to determine whether amino acids 262 to 594 of Ste12p could interact with negative regulators of Ste12p. Overexpression of \textit{RST1} or \textit{RST2}, two negative regulators of Ste12p, was found to inhibit the growth arrest and transcriptional induction that is caused by \textit{STE12} overexpression. Further work also demonstrated direct interactions between both Rst1p and Rst2p and Ste12p. Rst1p interacts with amino acids 309 to 547 of Ste12p, while Rst2p interacts with the DNA binding domain. The interaction between the Ste12p DNA binding domain and Rst2p interferes with the Ste12p-DNA interaction. Taken together, these results demonstrate that Rst1p and
Rst2p interact directly with distinct domains of Ste12p and may regulate Ste12p by different mechanisms.
2 The phenotypes associated with Ste12p overexpression

The goal of this work was to define both the functional domains of Ste12p and the mechanisms that regulate Ste12p activity. In order to characterize the domain structure of Ste12p, the following series of experiments examined the effects of *STE12* overexpression on responses that are normally associated with pheromone treatment. *STE12* overexpression was shown to induce transcription from a pheromone responsive promoter, induce growth arrest prior to the G1-S transition and increase mating efficiency in yeast. By examining the activity of a series of Ste12p deletions in these tests, I demonstrated that overexpression of amino acids 262 to 594 of Ste12p can relieve negative regulation of Ste12p in the absence of pheromone.

2.1 Overexpression of Ste12p results in increased expression of *FUS1* reporter genes

Deletion of amino acids from the carboxyl-terminal region (amino acids 309 to 669) of Ste12p increases the activity of both Ste12p and Ste12p-Gal4p fusions (Kirkman-Correia et al., 1993; Song et al., 1991). One possible explanation for the increased activity of Ste12p might be the elimination of an interaction with a negative regulator. If this model for Ste12p regulation is correct, it is possible that overexpression of Ste12p could also alleviate negative regulation.
Results

In order to evaluate the effects of Ste12p overexpression on transcription, I examined the change in transcription from a \textit{FUS1-LacZ} reporter when Ste12p is overexpressed in yeast. Four Ste12p derivatives were used in these experiments; Wt Ste12p(1 to 688), Ste12p\textDelta DBD(215 to 688), Ste12p(215-473) and Ste12p(473-688). All four proteins were expressed from a yeast episomal (high copy number) plasmid under the control of a \textit{GAL1} promoter.

2.1.1 Induction of \textit{FUS1-lacZ}

When Wt Ste12p and Ste12p\textDelta DBD were expressed in \textit{STE12} yeast, activity from a \textit{FUS1-LacZ} reporter increased more than 100-fold over a period of two hours following galactose induction (Figure 8A). The induction of \textit{FUS1-LacZ} activity did not require the addition of pheromone. When the same constructs were expressed in \textit{ste12} yeast, only Wt Ste12p was able to activate the transcriptional response (Figure 8B), which was expected, since Ste12p\textDelta DBD is not able to bind to PRE elements. An increase in Ste12p activity when Ste12p is overexpressed is consistent with either the titration of a negative regulator or the multimerization of Ste12p leading to transcriptional activation.

Amino acids 473 to 688 of Ste12p, which correspond to the C-terminal one half of Ste12p\textDelta DBD, have been identified as the focus of negative regulation of Ste12p in the absence of pheromone (Kirkman-Correia et al., 1993; Song et al., 1991). Since Ste12p\textDelta DBD overexpression can activate a \textit{FUS1-lacZ} reporter, I sought to determine whether overexpression of Ste12p(473-688) or
Figure 8. Overexpression of Ste12p induces FUS1-LacZ transcription
Yeast (A:SY2585:STE12, FUS1-LacZ; B: W303a::SUL-1:ste12, FUS1-LacZ) transformed with plasmids encoding WT Ste12p or Ste12pΔDBD under the control of a GAL1 promoter were induced with 2% galactose. Expression of FUS1-LacZ was assayed every thirty minutes by measurement of β-galactosidase activity. The vector pYeDP8-1/2, contains no STE12 sequence.
Ste12p(215-473) might also induce \textit{FUS1-LacZ} activity. When either Ste12p(473-688) or Ste12p(215-473) was overexpressed in yeast, neither protein caused significant induction of a \textit{FUS1} reporter construct (Figure 9).

\subsection*{2.1.2 Induction of \textit{FUS1-HIS3}}

In contrast to their weak induction of expression from a \textit{FUS1-lacZ} reporter, both Ste12p(473-688) and Ste12p(215-473) induced expression of a \textit{FUS1-HIS3} reporter (Figure 10). In Figure 10, yeast which are histidine auxotrophs, but which carry a \textit{FUS1-HIS3} reporter gene, were transformed with plasmids that express galactose-inducible Ste12p constructs. As expected, in the absence of histidine, the strain was unable to grow on galactose medium (Figure 10, top slide, vector). In contrast, the yeast which expressed Ste12p(473-688) or Ste12p(215-473) were able to grow on his' galactose, with Ste12p(215-473) supporting more robust growth. This method is a more sensitive assay for \textit{FUS1} promoter activity, since the activation of \textit{FUS1-HIS3} is required for cell survival in medium lacking histidine (C. Boone, pers. comm.). This experiment demonstrated that both Ste12p(473-688) and Ste12p(215-473) retain an ability to relieve the negative regulation of Ste12p, however, that activity is not sufficient to be observed in a \(\beta\)-galactosidase assay of \textit{FUS1-LacZ} activity.

Based on the results in Figures 8 and 9, Wt Ste12p and Ste12p\(\Delta\)DBD would also be expected to induce \textit{FUS1-HIS3} expression. This induction could
Results

Figure 9. Overexpression of some domains of Ste12p induces FUS1-LacZ expression
Yeast (■: SY2585: STE12, FUS1-LacZ; □: W303a::SUL-1: ste12, FUS1-LacZ) transformed with plasmids encoding Wt Ste12p, Ste12pΔDBD, Ste12p(215-473) or Ste12p (473-688) under the control of a GAL1 promoter were induced with 2% galactose. After two hours, expression of FUS1-LacZ was assayed by measurement of β-galactosidase activity. The vector, pYeDP8-1/2, contains no STE12 sequence.
Figure 10. Ste12p(215-473) induces FUS1-HIS3 transcription
Yeast (SY2585: STE12, his3::FUS1-HIS3) transformed with plasmids encoding Wt Ste12p, Ste12pΔDBD, Ste12p(215-473) or Ste12p (473-688) under the control of a GAL1 promoter were grown for three days on glucose- or galactose-containing media lacking histidine. The vector, pYeDP8-1/2, contains no STE12 sequence.
not be observed by monitoring growth (as in Figure 10), however, because overexpression of Wt Ste12p or Ste12pADBD causes growth arrest in yeast (see Overexpression of Ste12p results in growth arrest, below). In contrast, overexpression of either Ste12p(215-473) or Ste12p(473-688), did not result in growth arrest, allowing us to observe the FUS1-HIS3 induction.

On his' glucose media (Figure 10, bottom slide, vector) the unmodified strain is able to grow slowly. This is due to the weak constitutive expression of His3p in the parent strain (SY2585; C. Boone, pers. comm.).

2.1.3 Contribution of the PRP to increased transcription

The basal activity of Ste12p (activity in the absence of pheromone) at some promoters has been shown to be dependent upon the function of the PRP (Fields et al., 1988; Hagen et al., 1991). For example, expression of the FUS1 gene, whose UAS is composed solely of four PRE elements, is dependent upon the activity of multiple components of the PRP, even in the absence of pheromone (Hagen et al., 1991).

To determine whether the function of overexpressed Ste12p is also dependent upon the function of the PRP, I examined whether deletion of the MAP kinases that activate Ste12p, and that are penultimate to Ste12p in the PRP, could reduce transcriptional induction when Ste12p is overexpressed. In Figure 11, FUS1-LacZ induction by Ste12p overexpression was quantified in a fus3 strain and in a fus3 kss1 strain. As expected, induction of transcription by Wt Ste12p and Ste12pADBD was reduced in both of these strains. Previous
Figure 11. Induction of FUS1-LacZ expression by Ste12p overexpression is partially dependent upon the MAP kinases of the PRP Yeast (■:SY2585: STE12, FUS1-LacZ; □:WHY3-1: STE12, fus3, FUS1-LacZ; □:WHY2-7: STE12, fus3, kss1, FUS1-LacZ) transformed with plasmids encoding Wt Ste12p or Ste12pΔDBD under the control of a GAL1 promoter were induced with 2% galactose. After two hours, expression of FUS1-LacZ was assayed by measurement of β-galactosidase activity. The vector, pYeDP8-1/2, contains no STE12 sequence.
work had demonstrated that deletion of \textit{KSS1} in a \textit{fus3} strain further reduced the activity of the PRP (Elion et al., 1991). The trend was repeated here, as deletion of both kinases had a more marked phenotype than deletion of \textit{FUS3} alone. Both of these observations showed that the activity of overexpressed Ste12p is dependent upon PRP activity.

However, the deletion of \textit{FUS3} and \textit{KSS1} does not eliminate the induction of \textit{FUS1} expression altogether, indicating that some of the activity of overexpressed Ste12p is independent of Ste12p modification by the PRP. This observation supports the hypothesis that accumulation of excess Ste12p is sufficient to overcome a physical limitation to activation by Ste12p, such as binding of a negative regulator.

2.2 Overexpression of Ste12p results in growth arrest

According to the current model, Ste12p contributes to G1 arrest by directing increased transcription of Farlp, the CDK inhibitor (see \textit{Far1p is a pheromone-induced CKI}). In order to evaluate the effect of Ste12p overexpression on cell cycle progression, I examined the growth of yeast in which Ste12p derivatives were being overexpressed (Figure 12).

Overexpression of Wt Ste12p or Ste12p\textsubscript{ADBD} prevented growth of both \textit{STE12} and \textit{ste12} yeast (Figure 12, galactose). Neither Ste12p(215-473) nor Ste12p(473-688) elicited a growth arrest response (Figure 12, galactose), however, demonstrating that residues on either side of amino acid 473 of Ste12p are required for growth arrest.
Results

Figure 12. Ste12p overexpression induces growth arrest in both STE12 and ste12 yeast

Yeast (SY2585: STE12; W303a::SUL-1: ste12) transformed with plasmids encoding Wt Ste12p, Ste12pΔDBD, Ste12p(215-473) or Ste12p (473-688) under the control of a GAL1 promoter were grown for three days on glucose- or galactose-containing media. The vector, pYeDP8-1/2, contains no STE12 sequence.
2.2.1  Far1p-independent growth arrest

Although Ste12pΔDBD could not induce $FUS1$-LacZ transcription (Figure 8B) in a ste12 strain, Ste12pΔDBD was able to cause growth arrest in the same strain (Figure 12, lower left panel). If Ste12pΔDBD induced growth arrest without inducing $FUS1$-LacZ, then it is possible that Ste12p overexpression caused growth arrest by a mechanism that is also independent of the induction of FAR1 gene expression.

To test this model, I examined the growth of far1 yeast strains in which Ste12p was being overexpressed (Figure 13). Ste12p overexpression was sufficient to cause growth arrest in both FAR1 and far1 strains. Both Wt Ste12p and Ste12pΔDBD induced this arrest, demonstrating that an intact Ste12p DBD is not required for the phenotype. Based on these data, I concluded that Ste12p overexpression causes growth arrest that is independent of Far1p and independent of the direct interaction of Ste12p with PREs.

2.2.2  G1 growth arrest

Ste12p overexpression results in growth arrest on solid medium, but arrest on solid media cannot be directly attributed to a stage in the cell cycle. Pheromone-induced arrest is limited to the G1 phase of the cell cycle, at the Start checkpoint. Cells in the G1 phase of the cell cycle, such as those arrested by
Figure 13. Ste12p overexpression induces growth arrest in a FAR1-independent manner
Yeast (yA03: ste12, FAR1; yA02: ste12, far1) transformed with plasmids encoding vector pYeDP8-1/2, Wt Ste12p or Ste12pADB under the control of a GAL1 promoter were grown for three days on glucose- or galactose-containing media. The vector, pYeDP8-1/2, contains no STE12 sequence.
pheromone, have not formed a bud and can be readily identified under the microscope based on this morphology (Guthrie and Fink, 1991).

To determine whether Ste12p overexpression causes G1 (pheromone-like) arrest, I examined the change in yeast growing in liquid culture when Ste12p overexpression is induced (Figure 14). Cells growing in mid log phase were isolated and determined to be 25 to 30 percent unbudded, prior to induction of Ste12p expression. Upon induction of Ste12p expression, the cells began to accumulate in the G1 phase, reaching a peak of 75 to 80 percent unbudded (G1) cells after approximately two hours (Figure 14). This accumulation of cells in the G1 phase was independent of both \textit{FAR1} and genomic \textit{STE12}, as both genes could be deleted without eliminating the arrest.

G1 growth arrest in response to mating pheromone occurred within approximately one cell cycle, or ninety to one hundred and twenty minutes, and arrested cultures were generally greater than ninety percent unbudded (Bucking-Throm et al., 1973; Guthrie and Fink, 1991; Hartwell, 1973). The Far1p independent growth arrest caused by Ste12p overexpression was not as complete, but it did occur as rapidly.

\textbf{2.2.3 Overexpression of nuclear localized Ste12pΔDBD causes growth arrest}

Ste12pΔDBD does not have a nuclear localization signal and, when Ste12pΔDBD is overexpressed, it is observed in both the nucleus and the cytoplasm of yeast (Hung et al., 1997). Since Ste12p is a nuclear protein, it is
Figure 14. Ste12p overexpression causes G1 growth arrest in a FAR1-independent manner
Yeast (W303-1B: STE12, FAR1, FUS1-LacZ, SY2587: STE12, far1, FUS1-LacZ; yAO3:ste12, FAR1, FUS1-LacZ; yAO2:ste12, far1, FUS1-LacZ) transformed with plasmids encoding Wt Ste12p or Ste12pΔDBD under the control of a GAL1 promoter were induced with 2% galactose. Samples of each culture were taken every thirty minutes and fixed with formaldehyde. Samples were examined by phase-contrast microscopy and the percentage of yeast that were unbudded was determined. The vector, pYeDP8-1/2, contains no STE12 sequence.
Results

possible that Ste12pΔDBD is only able to cause growth arrest as a consequence of inappropriate localization. When galactose-inducible Ste12pΔDBD with a nuclear localization signal was expressed in yeast, most of the protein was nuclear localized (Hung et al., 1997), and Ste12pΔDBD/NLS induced growth arrest in both STE12 and ste12 strains (Figure 15). This indicates that the growth arrest function of Ste12pΔDBD is not due to inappropriate localization.

2.2.4 Participation of the G1 cyclins

G1 growth arrest in response to pheromone includes inhibition of Cdc28p-cyclin complexes. As shown in Figure 16, overexpression of either Cln1p or Cln2p, but not Cln3p, relieved some of the growth arrest that was observed when Ste12pΔDBD was overexpressed. A vector control showed growth on glucose and no growth on galactose (data not shown). This indicates that overexpression of Ste12p may affect the function of specific Cdc28p-cyclin complexes. The effect may be direct interference with complex function or may be a result of altered expression of the CLN genes.

2.2.5 Ste12p overexpression does not reduce the viability of yeast

One possible explanation for the accumulation of yeast in the G1 phase of the cell cycle when Ste12p is overexpressed is that a high concentration of Ste12p in yeast is lethal during the G1 to S transition. To determine whether Ste12p overexpression resulted in cell death, I measured the viability of yeast in which Ste12p had been overexpressed (Tables 2 and 3).
Figure 15. Growth arrest by overexpression of Ste12pΔDBD/NLS

Yeast (SY2585: STE12; W303a::SUL-1: ste12) transformed with a plasmid encoding Ste12pΔDBD with a nuclear localization signal (Ste12pDBD/NLS) under the control of a GAL1 promoter were grown for three days on glucose- or galactose-containing media. The vector, YEplac112, contains no STE12 sequence.
Figure 16. Overexpression of *CLN1* or *CLN2* partially relieves growth arrest induced by Ste12p overexpression

Yeast (W303a::SUL-1: ste12) transformed with plasmids encoding Ste12pΔDBD and Cln1p, Cln2p or Cln3p under the control of a galactose inducible promoter were grown for three days on either glucose- or galactose-containing media.
### Table 2. G1 growth arrest by Ste12p overexpression does not reflect cell death

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Percent Unbudded $^3$</th>
<th>Colonies/OD$_{600}$ Unit $^4$ (x 10$^6$)</th>
<th>Viability Relative to Vector $^5$</th>
</tr>
</thead>
<tbody>
<tr>
<td>STE12</td>
<td>Vector</td>
<td>26</td>
<td>3.9</td>
<td>1.0</td>
</tr>
<tr>
<td>STE12</td>
<td>WT Ste12p</td>
<td>76</td>
<td>3.3</td>
<td>0.85</td>
</tr>
<tr>
<td>STE12</td>
<td>Ste12pΔDBD</td>
<td>74</td>
<td>3.7</td>
<td>0.95</td>
</tr>
<tr>
<td>ste12</td>
<td>Vector</td>
<td>17</td>
<td>3.8</td>
<td>1.0</td>
</tr>
<tr>
<td>ste12</td>
<td>WT Ste12p</td>
<td>70</td>
<td>2.9</td>
<td>0.76</td>
</tr>
<tr>
<td>ste12</td>
<td>Ste12pΔDBD</td>
<td>76</td>
<td>2.9</td>
<td>0.76</td>
</tr>
</tbody>
</table>

Cells were grown to OD$_{600}$$\approx$0.6 and Ste12p expression was induced with 2% galactose. After cells were grown a further two hours, the optical density and the percent of unbudded cells was determined for each culture. Simultaneously, 100 µL of a 10$^{-4}$ dilution of each culture was plated in duplicate on glucose plates. After three days, colonies formed on each plate were counted.

$^1$STE12 was SY2585, ste12 was W303a::SUL-1.

$^2$All plasmids had a GAL1 promoter.

$^3$Percent Unbudded was determined as in Materials and Methods.

$^4$Colonies/OD$_{600}$ Unit is the number of colonies counted on the plate relative to the OD$_{600}$ of the culture two hours after induction. Each value reflects an average of two plates.

$^5$Relative viability is defined as Colonies/OD$_{600}$ Unit relative to the vector control.
Table 3. G1 growth arrest by Ste12p overexpression does not reduce viability in far1 yeast

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Colonies/OD&lt;sub&gt;600&lt;/sub&gt; Unit (x 10&lt;sup&gt;6&lt;/sup&gt;)&lt;sup&gt;3&lt;/sup&gt;</th>
<th>Viability Relative to Vector&lt;sup&gt;4&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>STE12, FAR1</td>
<td>Vector</td>
<td>3.3</td>
<td>1.0</td>
</tr>
<tr>
<td>STE12, FAR1</td>
<td>WT ste12p</td>
<td>3.3</td>
<td>1.0</td>
</tr>
<tr>
<td>STE12, FAR1</td>
<td>Ste12ΔDBD</td>
<td>3.2</td>
<td>0.96</td>
</tr>
<tr>
<td>STE12, far1</td>
<td>Vector</td>
<td>3.2</td>
<td>1.0</td>
</tr>
<tr>
<td>STE12, far1</td>
<td>WT ste12p</td>
<td>3.4</td>
<td>1.1</td>
</tr>
<tr>
<td>STE12, far1</td>
<td>Ste12ΔDBD</td>
<td>3.5</td>
<td>1.1</td>
</tr>
<tr>
<td>ste12, FAR1</td>
<td>Vector</td>
<td>3.2</td>
<td>1.0</td>
</tr>
<tr>
<td>ste12, FAR1</td>
<td>WT ste12p</td>
<td>2.8</td>
<td>0.88</td>
</tr>
<tr>
<td>ste12, FAR1</td>
<td>Ste12ΔDBD</td>
<td>2.8</td>
<td>0.88</td>
</tr>
<tr>
<td>ste12, far1</td>
<td>Vector</td>
<td>2.4</td>
<td>1.0</td>
</tr>
<tr>
<td>ste12, far1</td>
<td>WT ste12p</td>
<td>2.3</td>
<td>0.96</td>
</tr>
<tr>
<td>ste12, far1</td>
<td>Ste12ΔDBD</td>
<td>2.5</td>
<td>1.1</td>
</tr>
</tbody>
</table>

Cells were grown to OD<sub>600</sub>≈0.6 and Ste12p expression was induced with 2% galactose. After cells were grown a further two hours, the optical density of the culture was determined. Simultaneously, 100 μL of a 10<sup>-4</sup> dilution of each culture was plated in duplicate on glucose plates. After three days, colonies formed on each plate were counted.

<sup>1</sup>STE12, FAR1 was W3031-B, STE12, far1 was SY2587, ste12, FAR1 was yAO3, and ste12, far1 was yAO2.

<sup>2</sup>All plasmids had a GAL1 promoter.

<sup>3</sup>Colonies/OD<sub>600</sub> Unit is the number of colonies counted on the plate relative to the OD<sub>600</sub> of the culture two hours after induction. Each value reflects an average of two plates.

<sup>4</sup>Relative viability is defined as Colonies/OD<sub>600</sub> Unit relative to the vector control.
Results

To measure viability, the yeast were grown to mid log phase in liquid culture and Ste12p overexpression was induced by the addition of galactose. The yeast were exposed to galactose for two hours, which has been demonstrated to be sufficient time for both G1 growth arrest (Figure 14) and \textit{FUS1-lacZ} induction (Figure 8). Following galactose exposure, the cells were transferred to glucose media and following three days growth, the viability of yeast in the galactose treated cultures was determined. Similar to the results in Figure 14, Ste12p overexpression resulted in the accumulation of greater than seventy per cent unbudded cells in yeast cultures. The yeast in the G1 arrested cultures were found to be 76 to 110 per cent viable relative to the unarrested (vector) controls. If Ste12p overexpression caused cell death in G1 phase, one would predict that only the unarrested cells, which represented 25 to 30 \% of the culture, would have been viable. Similar viability was observed after growth arrest of \textit{STE12}, \textit{ste12}, \textit{FAR1} and \textit{far1} yeast. Taken together, these results indicate that overexpression of Ste12p and Ste12p\text{ADBD} can induce G1 growth arrest in yeast that is independent of \textit{FAR1} and of \textit{STE12}, which implies that the arrest is independent of direct interaction between Ste12p and PREs.

2.3 Overexpression of Ste12p increases mating in both \textit{STE12} and \textit{ste12} strains

Ste12p overexpression results in both transcriptional induction and G1 growth arrest, two components of the pheromone response. As shown in this work, the G1 growth arrest induced by Ste12p is independent of Far1p, the CKI
which has been implicated in pheromone-induced growth arrest, indicating a
novel function for Ste12p. A third measure of PRP and Ste12p function is the
mating efficiency of yeast. In order to further characterize the effects of Ste12p
overexpression, and to compare them to a normal pheromone response, I
examined the mating efficiency of yeast which overexpress Ste12p.

When a cells expressing Ste12p were mated to an α strain (HLY334),
overexpression of Ste12p increased mating efficiency (Table 4). In STE12 yeast,
overexpression of Ste12pΔDBD increased mating efficiency by a factor of 12.9,
compared to yeast expressing only genomic STE12. In a ste12 strain, which
otherwise did not mate, Wt Ste12p overexpression allowed 77% mating
efficiency. As was the case for FUS1-LacZ induction by Ste12p overexpression,
a STE12 gene with an intact DBD was required for mating (Table 4, absolute
mating efficiency of ste12 yeast).

Collectively, these data indicate that Ste12p overexpression increases
mating efficiency through increased transcriptional activation. Since the
increased mating required an intact Ste12p DBD and Ste12p overexpression did
not substitute for pheromone induction of the PRP (data not shown), it is unlikely
that a novel function of Ste12p causes the increased mating efficiency. This is
consistent with previous observations that Ste12p increases mating through
transcriptional activation (Fields and Herskowitz, 1985; Fields and Herskowitz,
1987).
Table 4. Ste12p overexpression increases mating efficiency

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Relative Mating Efficiency of STE12 Yeast</th>
<th>Absolute Mating Efficiency of ste12 Yeast^3 (% mated)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>WT Ste12p</td>
<td>1.6</td>
<td>77</td>
</tr>
<tr>
<td>Ste12pΔDBD</td>
<td>12.9</td>
<td>0</td>
</tr>
</tbody>
</table>

STE12 (SY2585) or ste12 (W303a::SUL-1) yeast was mated to a ten-fold excess of HLY334 (Mat α) cells.

^1 All plasmids had a GAL1 promoter.

^2 Mating efficiency was calculated relative to the number of STE12 yeast added to the mating reactions. Relative mating efficiency was calculated relative to the vector-bearing yeast.

^3 Mating efficiency was calculated relative to the number of STE12 yeast added to the mating reactions. All numbers reflect an average of two plates.
2.4 Nested deletions of STE12 show that amino acids 262 to 594 are sufficient to induce transcription and growth arrest

To define the minimum fragments of Ste12p capable of inducing FUS1-LacZ transcription and/or growth arrest in yeast, I made a set of nested deletion mutants of STE12 expressed from the GAL1 promoter (Figure 17).

I found that sequences C-terminal to amino acid residue 594 or N-terminal to residue 262 can be deleted from overexpressed Ste12p while maintaining elevated FUS1-LacZ expression in a STE12 strain (Figure 18). Overexpression of Ste12p mutants p1(215 to 641), p2(215 to 594), p5(262 to 688), p10(262 to 594) or p11(262 to 641) induced FUS1-LacZ activity in the absence of pheromone (Figure 18). In contrast, Ste12p mutants p3(215 to 547), p4(215 to 500), p6(309 to 688), p7(356 to 688), p8(403 to 688), p9(450 to 688) or p12(309 to 547) did not elevate FUS1-LacZ transcription when overexpressed. The minimum active fragment, amino acids 262 to 594 (p10) was sufficient to elevate transcription of FUS1-LacZ to levels comparable to those induced by much larger fragments of Ste12p (Figure 18). Fragments of Ste12p with amino acids deleted from either extremity of the p10 sequence were not able to activate FUS1-LacZ.
Figure 17. Nested deletions of STE12
Nested deletions of STE12 expressed under the control of the GAL1 promoter. The minimum active fragment (encodes amino acids 262 to 594) is the minimum fragment required to induce FUS1-LacZ activity and growth arrest.
Figure 18. Amino acids 262 to 594 of Ste12p induce FUS1-LacZ transcription when overexpressed

Yeast (SY2585: STE12, FUS1-LacZ) transformed with plasmids encoding Wt Ste12p, Ste12pΔDBD or constructs p1 through p12 (Figure 17) under the control of a GAL1 promoter were induced with 2% galactose. After two hours, expression of FUS1-LacZ was assayed by measurement of β-galactosidase activity. The vector, pYeDP8-1/2, contains no STE12 sequence.
The deletion mutants (Figure 17) were also evaluated for their ability to induce growth arrest in \textit{STE12} and \textit{ste12} strains (Table 5). Constructs p1, p2, p5, p10 and p11 all caused growth arrest in both \textit{STE12} and \textit{ste12} yeast. Parallel to the results for \textit{FUS1-LacZ} induction, constructs p3, p4, p6, p7, p8, p9 and p12 were not able to prevent growth of yeast on galactose. The minimum fragment of Ste12p whose overexpression caused growth arrest is amino acids 262 to 594. This is the same fragment which, when overexpressed, induces the activity of \textit{FUS1-LacZ}. The (+) and (-) signs in Table 5 indicate whether the observed growth was most similar to the vector (+) or WT Ste12p (-) control.

Expression of all of the deletion mutants was confirmed by western blot (data not shown).

Collectively, these results identified amino acids 262 to 594 as the minimum fragment of Ste12p that can induce transcription and cause growth arrest. If there is a negative regulator of Ste12p whose activity can be titrated by an increase in Ste12p expression, amino acids 262 to 594 are sufficient to titrate it. Alternately, Ste12p overexpression might cause activation by Ste12p aggregation. If so, then 262 to 594 must be able to aggregate with Wt Ste12p and cause activation, or must interact with and inhibit factors which prevent aggregation of individual Wt Ste12p molecules with one another. In either case, one can predict that there is a mechanism for negative regulation of Ste12p that acts through amino acids 262 to 594 of Ste12p.
Table 5. Growth arrest by Ste12p deletion mutants

<table>
<thead>
<tr>
<th>Ste12p Construct</th>
<th>Amino acids Expressed</th>
<th>Growth on Galactose</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vector</td>
<td>None</td>
<td>+</td>
</tr>
<tr>
<td>WT Ste12p</td>
<td>1 to 688</td>
<td>-</td>
</tr>
<tr>
<td>Ste12pΔDBD</td>
<td>215 to 688</td>
<td>-</td>
</tr>
<tr>
<td>p1</td>
<td>215 to 641</td>
<td>-</td>
</tr>
<tr>
<td>p2</td>
<td>215 to 594</td>
<td>-</td>
</tr>
<tr>
<td>p3</td>
<td>215 to 547</td>
<td>+</td>
</tr>
<tr>
<td>p4</td>
<td>215 to 500</td>
<td>+</td>
</tr>
<tr>
<td>p5</td>
<td>262 to 688</td>
<td>-</td>
</tr>
<tr>
<td>p6</td>
<td>309 to 688</td>
<td>+</td>
</tr>
<tr>
<td>p7</td>
<td>356 to 688</td>
<td>+</td>
</tr>
<tr>
<td>p8</td>
<td>403 to 688</td>
<td>+</td>
</tr>
<tr>
<td>p9</td>
<td>450 to 688</td>
<td>+</td>
</tr>
<tr>
<td>p10</td>
<td>262 to 594</td>
<td>-</td>
</tr>
<tr>
<td>p11</td>
<td>262 to 641</td>
<td>-</td>
</tr>
<tr>
<td>p12</td>
<td>309 to 547</td>
<td>+</td>
</tr>
</tbody>
</table>

1 All Ste12p species are expressed under the control of a GAL1 promoter.
2 Growth was evaluated after 3 days on galactose and compared to growth of the same transformant on glucose. The growth of all constructs was compared to Vector (+) and WT Ste12p (-) controls which had been grown in the same experiments. Identical results were obtained using the strains SY2585 (STE12) and W303a::SUL-1 (ste12).
3 Rst1p and Rst2p interact with distinct regions of Ste12p

The following experiments examine the roles of Rst1p and Rst2p as functionally redundant negative regulators of Ste12p at PREs. The results in Chapter 2 demonstrated that Ste12p overexpression can activate transcription and/or growth arrest in the absence of pheromone. The following data show that Rst1p and Rst2p can interact with Ste12p in vitro and can decrease Ste12p function in vivo. Further, the data indicate that Rst1p and Rst2p regulate Ste12p by different mechanisms.

3.1 Overexpression of Rst1p and Rst2p abrogates the effects of Ste12p overexpression

Overexpression of Ste12p resulted in the induction of Ste12p-responsive transcription and growth arrest in a manner consistent with the titration of a direct negative regulator from endogenous Ste12p (Dolan, 1996; Dolan and Fields, 1990). If Rst1p and Rst2p act as direct negative regulators of Ste12p, then simultaneous overexpression of RST1 or RST2 should reduce the effect of STE12 overexpression. To determine if this was true, I concurrently overexpressed STE12 and RST1 or RST2.

3.1.1 Rst1p and Rst2p inhibit growth arrest

Both Wt Ste12p and Ste12pΔDBD caused growth arrest when overexpressed in yeast (Figures 12, 13 and 14). Concurrent overexpression of
either RST1 or RST2 prevented growth arrest by Ste12p overexpression in STE12 yeast, as could be predicted for negative regulators of Ste12p function. Overexpressing Wt Ste12p or Ste12pΔDBD caused growth arrest, but the yeast grew vigorously when either RST1 or RST2 was overexpressed simultaneously (Figure 19, galactose). In ste12 yeast, however, overexpression of RST2 did not prevent the growth arrest induced by Ste12pΔDBD as efficiently as overexpression of RST1 did (Figure 20, ste12, galactose). One possible explanation for this phenotype is that Rst1p and Rst2p interact with different parts of Ste12p, and that Rst2p cannot interact efficiently with Ste12p species that do not have an intact DNA binding domain.

3.1.2 Rst1p and Rst2p reduce FUS1-lacZ expression

A second phenotype of overexpression of Ste12p is the induction of FUS1-LacZ transcription in the absence of pheromone (Figure 8). Galactose-induced transcription of Wt Ste12p and Ste12pΔDBD may increase FUS1 activity by competing with endogenous Ste12p for interaction with a negative regulator. If this is true, then overexpression of the negative regulator should counteract the effect of increased Ste12p levels in the cell. Expression of either Rst1p or Rst2p under the control of a galactose-inducible promoter reduced the induction of FUS1-LacZ by Wt Ste12p and Ste12pΔDBD in a dose-dependent manner (Figure 21). If Rst1p or Rst2p was overexpressed from an episomal (high copy number) plasmid, either protein reduced activation by Wt Ste12p or Ste12pΔDBD (Figure 21, panel A), while expression from a centromeric (low copy number)
Figure 19. Overexpression of Rst1p or Rst2p relieves growth arrest caused by Ste12p overexpression
Yeast (SY2585: STE12) transformed with plasmids encoding Wt Ste12p DBD or Ste12pΔDBD and Rst1p (1) or Rst2p (2) under the control of a GAL1 promoter were grown for three days on galactose- or glucose-containing media. The vector, pYeDP8-1/2, contains no STE12 sequence. The control (c), YEplac181, contains no RST sequence. pMT556 and pMT558 encode Rst1p and Rst2p, respectively.
Results

Figure 20. Overexpression of Rst2p does not relieve growth arrest by overexpression of Ste12ΔDBD in ste12 yeast
Yeast (SY2585: STE12, W303a::SUL-1: ste12) transformed with plasmids encoding Ste12pΔDBD and Rst1p (1) or Rst2p (2) under the control of a GAL1 promoter were grown for three days on galactose- or glucose-containing media. The vector, pYeDP8-1/2, contains no STE12 sequence. The control (c), YEplac112, contains no RST sequence. pG1T and pG2T encode Rst1p and Rst2p, respectively.
Figure 21. Overexpression of Rst1p or Rst2p reduces induction of FUS1-LacZ by Ste12p overexpression
Yeast (SY2585; STE12, FUS1-LacZ) transformed with plasmids encoding Wt Ste12p DBD or Ste12pDDBD and Rst1p or Rst2p under the control of a GAL1 promoter were induced with 2% galactose. RST plasmids in panel A are episomal, those in panel B are centromeric. After two hours, β-galactosidase activity from a FUS1-LacZ reporter was assayed. The vector, pYeDP8-1/2, contains no STE12 sequence. The controls, YEplac181 (panel A) and YEplac112 (panel B), contain no RST sequence. pMT556 and pMT558 are episomal plasmids that encode Rst1p and Rst2p, respectively. pG1T and pG2T are centromeric plasmids that encode Rst1p and Rst2p, respectively.
Results

plasmid also reduced the induction of FUS1-LacZ, but to a lesser extent (Figure 21, panel B).

*RST1* or *RST2* overexpression reduced induction of FUS1-LacZ expression by Ste12p in both the presence and absence of pheromone (Figures 21 and 22). After a two-hour induction with both pheromone and galactose, overexpression of either *RST* gene reduced FUS1-LacZ activity by nearly one half as compared to a vector control (Figure 22).

The overexpression of single *RST* genes from these vectors was insufficient to eliminate activation by Ste12p. However, if negative regulation by Rst1p and Rst2p is dependent upon the recruitment of a co-regulator, such as the MAPK kinase Kss1p, the amount of co-regulator in the cell may limit the effects of RstXp overexpression. Alternately, the expression vectors may express less than stoicheometric amounts of Rst1p and Rst2p.

3.2 *Rst1p and Rst2p interact with distinct regions of Ste12p*

Rst1p and Rst2p reside in complexes with Ste12p, Kss1p and/or Fus3p (Cook et al., 1996; Tedford et al., 1997). Since overexpression of Rst1p or Rst2p inhibits the effects of Ste12p overexpression, I investigated whether Rst1p or Rst2p could interact directly with Ste12p.

In Figure 23, recombinant GST-Rst1p and GST-Rst2p were added to [35S]-methionine labeled yeast extracts expressing Ste12p constructs and recovered with glutathione agarose. GST-Rst1p interacted with both Wt Ste12p
Figure 22. Overexpression of Rst1p or Rst2p reduces induction of FUS1-LacZ in response to pheromone.

Yeast (SY2585: STE12, FUS1-LacZ) transformed with plasmids encoding Rst1p or Rst2p under the control of a GAL1 promoter were induced with 2% galactose and 2 μg/ml α-factor. After two hours, β-galactosidase activity from a FUS1-LacZ reporter was assayed. The vector, pYeDP8-1/2, contains no STE12 sequence. The control (c), YEplac181, contains no RST sequence. pMT556 and pMT558 are episomal plasmids that encode Rst1p and Rst2p, respectively.
Figure 23. Recombinant Rst1p and Rst2p interact with Ste12p in yeast extracts
Yeast (W303a::SUL-1: ste12) expressing Wt Ste12p (A) or Ste12pΔDBD (B) under the control of a GAL1 promoter were labeled with [\textsuperscript{35}S]-methionine. Ste12p derivatives were recovered from yeast lysates by immunoprecipitation with α-Ste12p antibodies (lanes 1, 4 and 7) or by interaction with 5μg of either GST-Rst1p (lanes 2, 5 and 8) or GST-Rst2p (lanes 3, 6 and 9) and subsequent recovery with glutathione agarose. Recovered proteins were resolved by electrophoresis and visualized by autofluorography. The vector, pYeDP8-1/2, contains no STE12 sequence.
and Ste12pΔDBD proteins (Figure 23, lanes 2 and 5; labeled A and B) in yeast extracts. In contrast, GST-Rst2p was able to interact with Wt Ste12p (Figure 23, lane 3) but was unable to bind to Ste12pΔDBD (Figure 23, lane 6). These results show that recombinant GST-Rst1p and GST-Rst2p can interact with Ste12p in yeast extracts, but that the two inhibitors bind to different domains of Ste12p. Alternately, conformational changes to Ste12p caused by the DBD deletion may affect the structure of distal domains and thereby disrupt the interaction of Rst2p with Ste12pΔDBD.

When GST-Rst1p and GST-Rst2p were mixed with extracts of yeast containing no Ste12p (Figure 23, lanes 8 and 9) and recovered with glutathione agarose, Rst1p bound a single protein of approximately 85 kDa, while Rst2p bound two proteins, one of approximately 85 kDa and another of approximately 92 kDa. These proteins have not been identified.

Rst1p and Rst2p could also interact with recombinant Ste12p in baculovirus-infected insect cell extracts in the absence of any other yeast proteins (Figure 24, lanes 1 and 3). This indicates that Rst1p and Rst2p can interact directly with Ste12p. This result, however, may be complicated by the fact that insect cells express MAP kinases. The inactive form of Kss1p regulates Ste12p and the Kss1p interaction with Ste12p is dependent upon the presence of Rst1p and Rst2p (Bardwell et al., 1998b). Thus it is possible that the interaction of GST-Rst1p and GST-Rst2p with Ste12p from insect cells is mediated by insect MAP kinases.
Figure 24. Rst1p and Rst2p interact with Ste12p in insect cell lysates
5 μg of GST-Rst1 (lanes 1 and 2), GST-Rst2p (lanes 3 and 4) or GST (lanes 5 and 6) were added to crude 100 μg of Sf9 (*Spodoptera frugiperda*) lysates containing Wt Ste12p (+) or no Ste12p (-) expressed from a baculovirus and protein complexes were recovered with glutathione agarose. After washing, the complexes were resolved by electrophoresis and Wt Ste12p (A) was detected by western blot with α-Ste12p antibodies. The input is crude Sf9 (*Spodoptera frugiperda*) lysate from cells infected with the Ste12p virus (+) (lane 8) or the wild-type virus (-) (lane 7).
3.2.1 **Rst1p interacts with amino acids 262 to 594 of Ste12p**

Overexpression of Ste12p(262 to 594) results in *FUS1-lacZ* induction which can be reduced by the overexpression of Rst1p or Rst2p. In the experiment shown in Figure 25, I examined whether the same region of Ste12p can interact with Rst1p or Rst2p. I determined that recombinant GST-Rst1p could interact with Ste12p fragments including p1(215 to 641), p2(215 to 594) and p5(262 to 688) in yeast extracts (Figure 25, p1, p2, p5, lanes 2). GST-Rst1p did not recover fragments p4(215 to 500) or p7(356 to 688) and recovered p3(215 to 547) and p6(309 to 688) with reduced efficiency (Figure 25, p3, p4, p6, p7; lanes 2). These results show that amino acids 262 to 594 of Ste12p can interact with Rst1p, while a smaller fragment, Ste12p(309-547) can also interact, but with lower affinity.

3.2.2 **Rst2p interacts with the DBD of Ste12p**

GST-Rst2p did not interact with any Ste12p fragment that did not include the Ste12p DNA binding domain (Figure 25, lanes 3). Since GST-Rst2p can interact efficiently with Wt Ste12p, I examined whether or not Rst2p interacts directly with amino acids 1 to 215 of Ste12p. In this experiment, both GST-Rst2p and GST-Rst1p interacted with purified recombinant 6-His-Ste12pDBD from *E. coli* (Figure 26, lanes 1 and 2), but the Rst2p-Ste12pDBD interaction was very...
Figure 25. Rstlp interacts with amino acids 262 to 594 of Ste12p
Yeast (W303a::SUL-1: ste12) expressing Ste12p constructs p1 through p7 (Figure 17) under the control of a GAL1 promoter were labeled with [³⁵S]-methionine. Ste12p derivatives were recovered from yeast lysates by immunoprecipitation with α-Ste12p antibodies (lanes 1) or by interaction with 5 μg of either GST-Rst1p (lanes 2) or GST-Rst2p (lanes 3) and subsequent recovery with glutathione agarose. Ste12p constructs encode the following amino acids: p1(215 to 641), p2(215 to 594), p3(215 to 547), p4(215 to 500), p5(262 to 688), p6(309 to 688), and p7(356 to 688). Recovered proteins were resolved by electrophoresis and visualized by autoradiography. Please see Figure 17 for a schematic of the Ste12p constructs.
strong whereas the Rst1p interaction was weak. The interaction of GST-Rst1p
with Ste12pDBD was not consistently detected when this assay was repeated.
6-His-Gal4pDBD did not interact with either Rst1p or Rst2p (Figure 26, lanes 5
and 6) and GST alone failed to interact with either Gal4pDBD or Ste12pDBD,
confirming that the Rst1p and Rst2p interactions with 6-His-Ste12pDBD are
specific. This result also demonstrates that Rst2p and Rst1p can interact directly
with Ste12p in the absence of other proteins.

Rst2p could interact with smaller fragments of the Ste12p DNA binding
domain (Figure 27). The smallest fragment of the DNA binding domain that was
found to interact with GST-Rst2p was amino acids 21 to 195 (Figure 27, lanes 13
to 16), the region of Ste12p implicated in DNA binding (Yuan and Fields, 1991).
When the DNA binding domain was bisected, neither of the resulting halves was
able to interact with Ste12p (Figure 27, lanes 17 to 24), indicating that Rst2p
must contact amino acids at either end of the Ste12pDBD. When further
deletions were made at either end of Ste12p, I found that Ste12pDBD fragments
that contain amino acids 21 to 170 or amino acids 45 to 195 did not interact with
GST-Rst2p (data not shown). This confirms that amino acids from both ends of
the DBD are required for Rst2p interaction. Consistent with previous results,
neither GST nor GST-Rst1p interacted with the DNA binding domain of Ste12p.

3.2.3 Rst1p interacts directly with Ste12p

GST-Rst1p was able to recover Ste12p from yeast extracts (Figure 23)
and interacted efficiently with amino acids 262 to 594 (Figure 25). However,
**Figure 26. Rst2p interacts with the DNA binding domain of Ste12p**

A two-fold molar excess of GST-Rst2p (lane 1), GST-Rst1p (lane 2) or GST (lane 3) was mixed with 6-His-Ste12pDBD (labeled A). Proteins were recovered from solution by glutathione agarose, washed and resolved by electrophoresis. 6-His-Ste12pDBD was detected by western blot with polyclonal α-histidine antibodies. The 6-His-Ste12pDBD in lane 4 is equivalent to 1/12 of the 6-His-Ste12pDBD used in the interaction assays. In lanes 5 to 8, 6-His-Gal4pDBD was treated in the same manner. All proteins used in this assay were purified from *E. coli.*
Results

amino acids: | 1 to 215 | 1 to 195 | 21 to 215 | 21 to 195
---|---|---|---|---
GST-Rst1p | GST-Rst2p | GST | input | GST-Rst1p | GST-Rst2p | GST | input | GST-Rst1p | GST-Rst2p | GST | input
MW | 57 | 39 | 34

Figure 27. Rst2p interacts with amino acids 21 to 195 of the DNA binding domain of Ste12p

3 μg of GST-Rst1p (lanes 1, 5, 9, 13, 17 and 21), GST-Rst2p (lanes 2, 6, 10, 14, 18 and 22) or GST (lanes 3, 7, 11, 15, 19 and 23) was mixed with 1 mg of crude E. coli extracts expressing 6-His-Ste12pDBD constructs. Proteins were recovered from solution by glutathione agarose, washed and resolved by electrophoresis. 6-His-Ste12pDBD proteins were detected by western blot with polyclonal α-histidine antibodies. The 6-His-Ste12pDBD in lanes 4, 8, 12, 16, 20 and 24 is equivalent to 1/20 of the 6-His-Ste12pDBD used in the interaction assays. E. coli extracts in lanes 1 to 4 contain 6-His-Ste12pDBD(1 to 215); lanes 5 to 8 contain 6-His-Ste12pDBD(1 to 195); lanes 9 to 12 contain 6-His-Ste12pDBD(21 to 21); lanes 13 to 16 contain 6-His-Ste12pDBD(21 to 195); lanes 17 to 20 contain 6-His-Ste12pDBD(1 to 108), lanes 21 to 24 contain 6-His-Ste12pDBD(109 to 215). All GST proteins used in this assay were purified from E. coli.
since Rst1p simultaneously interacts with other proteins from the whole cell extract, it was not clear whether Rst1p interacted directly with Ste12p(262 to 594). To determine whether Rst1p could interact directly with the C-terminus of Ste12p, I expressed a trpE-Ste12pΔDBD fusion in E. coli. GST-Rst1p recovered trpE-Ste12ΔDBD from solution, but GST-Rst2p did not (Figure 28, lanes 2 and 3). From this result, I concluded that recombinant Rst1p can interact with the C-terminus of Ste12p in the absence of other eukaryotic proteins.

3.3 Rst2p interacts with the Ste12p DNA binding domain in vivo

Collectively, the results in Figures 21 to 28 demonstrate that Rst1p and Rst2p can interact directly with separate regions of Ste12p. Rst1p interacts with residues 262 to 594 the region of Ste12p which, when overexpressed, activates pheromone-like responses in yeast and Rst2p interacts with the Ste12p DBD. The data is insufficient, however, to establish that the Ste12p DNA binding domain is the domain through which Rst2p inhibits the activity of Ste12p.

To examine the effect of Rst1p and Rst2p on the DNA binding domain of Ste12p, I used a fusion of the Ste12p DNA binding domain (Ste12pDBD, amino acids 1 to 215) to the potent transcriptional activation domain of HSV-1 VP16 (Sadowski et al., 1988). The fusion, DBD-VP16, was expressed from a galactose-inducible promoter. Expression of DBD-VP16 in yeast activated transcription of FUS1-LacZ approximately 25-fold more than expression of the Ste12p DBD alone (Figure 29). When Rst2p was co-expressed with DBD-VP16,
Figure 28. Rst1p interacts directly with Ste12pΔDBD
5 μg of GST (lane 1), GST-Rst1p (lane 2) or GST-Rst2p (lane 3) were added to 100 μg of E. coli lysates containing a trpE-Ste12pΔDBD fusion (Ste12p amino acids 216 to 688) and protein complexes were recovered with glutathione agarose. After washing, the complexes were resolved by electrophoresis and trpE-Ste12pΔDBD was detected by western blot with α-Ste12p antibodies. The input is crude E. coli lysate containing the trpE-Ste12pΔDBD fusion.
Figure 29. Rst2p inhibits the function of the Ste12p DNA Binding Domain
Yeast (W303A::SUL-1: ste12, FUS1-LacZ) transformed with plasmids expressing Ste12pDBD or DBD-VP16 and Rst1p or Rst2p under the control of a GAL1 promoter were induced with 2% galactose. After two hours, β-galactosidase activity from a FUS1-LacZ reporter was assayed. The vector, pYeDP8-1/2, contains no STE12 sequence. The control, YEplac1112, contains no RST sequence. pG1T and pG2T are centromeric plasmids that encode Rst1p and Rst2p, respectively.
the activation of \textit{FUS1-LacZ} was reduced (Figure 29). The overexpression of \textit{RST1}, however, had no effect on the induction of \textit{FUS1-LacZ} by DBD-VP16 (Figure 29).

The expression of DBD-VP16 also activated transcription of endogenous \textit{FUS1}. Consistent with the experiments using the \textit{FUS1-LacZ} reporter gene (Figure 29), overexpression of Rst2p reduced \textit{FUS1} activation by DBD-VP16, while overexpression of Rst1p did not (Figure 30 and data not shown). When DBD-VP16 was expressed in either \textit{RST2} or \textit{rst2} yeast, \textit{FUS1} expression was increased (Figure 30, lanes 2 and 4). When \textit{RST2} was expressed under the control of a galactose promoter in the same yeast, \textit{FUS1} activity was reduced (Figure 30, compare lanes 1 to 4 to lanes 5 to 8).

These results demonstrate that Rst2p is able to inhibit Ste12p function through the specific interaction of Rst2p with the DNA binding domain of Ste12p. Consistent with the finding that Rst1p interacts with amino acids 262 to 594 of Ste12p, Rst1p was unable to regulate Ste12p through its DNA binding domain alone.

3.4 \textbf{Rst2p disrupts the interaction of Ste12p with DNA in vitro}

Rst2p interacts specifically with the DNA binding domain of Ste12p and inhibits Ste12p function. Based on these observations, it is possible that Rst2p inhibits Ste12p DNA binding. I assayed this by observing the effects of Rst2p in an electrophoretic mobility shift assay (EMSA) of Ste12p.
**Figure 30. Rst2p inhibits the function of the Ste12p DNA Binding Domain**

Yeast (W3031a: RST2; MTy1147: rst2) transformed with plasmids expressing Ste12pDBD or DBD-VP16 and a vector (lanes 1 to 4) or Rst2p (lanes 5 to 8) under the control of a GAL1 promoter were induced with 2% galactose. After two hours, RNA was prepared from the cells and the expression of both FUS1 (top) and ACT1 (bottom) was detected by northern blot.
Results

The DNA binding domain of Ste12p, when expressed and purified from *E. coli*, bound specifically to a pheromone responsive element in an EMSA (Figure 31 and 32, lanes 1 to 5). When Rst2p was added to the EMSA reaction, however, the interaction of Ste12pDBD with the DNA was reduced (Figure 31, lane 9). The efficiency of the Ste12pDBD interaction with DNA was inversely proportional to the amount of Rst2p present in the EMSA reaction (Figure 32, lanes 6 to 9). In contrast, the presence of Rst1p in the EMSA did not affect the binding of the Ste12pDBD to DNA (Figure 31 lanes 6 and 7). It should be noted that, in Figure 32, equal masses of GST-Rst2p and GST were used to titrate Ste12p. As GST is a much smaller protein than Rst2p (approximately one fifth the size), the molar quantities of GST were higher than the molar quantities of GST-Rst2p. It should also be noted that the upper band in lane 13 is Ste12p-specific (data not shown), indicating that the GST is not disrupting Ste12p binding, but stabilizing a larger Ste12p-DBD complex.

These results demonstrate that Rst2p can prevent the binding of Ste12pDBD to DNA *in vitro*, indicating that Rst2p may inhibit Ste12p function by impeding its ability to bind to PREs.

3.5 Accumulation of Ste12p is unnecessary for FUS1 induction in response to pheromone

Ste12p bound to PREs mediates pheromone responsive transcription. Many of the genes encoding pheromone response pathway components and PRP regulatory proteins, including *STE12* and *RST2*, have multiple upstream
Figure 31. Rst2p disrupts the interaction of Ste12pDBD with DNA in EMSA
3 μg of 6-His-Ste12pDBD was incubated in the presence of competitors
(oligonucleotides or protein) on ice. After one hour, an oligonucleotide probe
containing two PRE elements arranged tail to tail was added and the reactions
were incubated for a further 30 minutes on ice. Reactions were resolved as
described in Materials and Methods. A indicates the 6-His-Ste12pDBD-
oligonucleotide complex. Lane 1 contains no inhibitors. Lanes 2 and 3 contain
10- and 100-fold excesses of unlabelled PRE oligonucleotide. Lanes 4 and 5
contain 10- and 100-fold excesses of unlabeled Gal4p UAS oligonucleotide (see
Materials and Methods), respectively. Lanes 6 and 7 contain 1 and 5 μg of GST-
Rst1p. Lanes 8 and 9 contain 1 μg and 5 μg of GST-Rst2p. Lanes 10 and 11
contain 4 μg and 16 μg of GST.
Figure 32. Disruption of DNA binding with increasing amounts of Rst2p
Reactions were performed as described in Figure 31. A indicates the 6-His-
Ste12pDBD-oligonucleotide complex. Lane 1 contains no inhibitors. Lanes 2
and 3 contain 10- and 100-fold excesses of unlabeled PRE oligonucleotide.
Lanes 4 and 5 contain 10- and 100-fold excesses of unlabeled Gal4p UAS
oligonucleotide (see Materials and Methods), respectively. Lanes 6 to 9 contain
1 μg, 2 μg, 4 μg and 10 μg of GST-Rst2p and lanes 10 to 13 contain 1 μg, 2 μg,
4 μg and 10 μg of GST.
PREs. Pheromone-stimulated transcription of genes encoding PRP components results in an amplification of signaling sensitivity in response to pheromone. Since \textit{STE12} has five consensus PREs in its promoter, I examined whether Ste12p accumulation in response to pheromone relieves inhibition of Ste12p by Rst1p and Rst2p.

To determine whether Ste12p accumulation is required for activation of transcription in response to pheromone, I measured induction of \textit{FUS1} transcription in cells treated with cycloheximide. As measured by northern blot, inhibition of protein synthesis by cycloheximide did not prevent \textit{FUS1} induction. Instead, cycloheximide treatment caused an approximately 100-fold superinduction of \textit{FUS1} transcription following a 30-minute treatment with \( \alpha \) mating pheromone (Figure 33, \textit{FUS1}). This result demonstrates that nascent Ste12p synthesis is not required for induction of transcription in response to pheromone.

In the absence of cycloheximide, \textit{STE12} transcription increased two-fold in response to pheromone (Figure 33, \textit{STE12}, lanes 3 and 4, or 7 and 8). In contrast, when yeast are treated with pheromone over a 30-minute period, Ste12p protein levels remained approximately constant (Figure 34, compare lanes 5 and 6). These results are consistent with the conclusion that \textit{FUS1} induction is not dependent upon novel synthesis of Ste12p.

The increased \textit{FUS1} induction in response to pheromone when cycloheximide is present indicates that one or more proteins, whose synthesis is
Figure 33. *FUS1* induction in response to pheromone does not require new protein synthesis

Yeast (W3031a: *RST2*, MTy1147: *rst2*) were treated for ten minutes with 0.1 mg/ml cycloheximide (CYC: lanes 1, 2, 5 and 6) followed by a thirty minute treatment with α-factor (α: lanes 1, 3, 5, and 7). Yeast in lanes marked (-) were grown for the same period but were not treated with either cycloheximide or α-factor. Gene expression of *FUS1*, *RST2*, *RST1*, *STE12* and *ACT1* was evaluated by northern blot.
stimulated in pheromone-treated yeast, inhibit pheromone induction. Rst1p is not a likely candidate for this protein, as \textit{RST1} expression is not affected by \( \alpha \)-factor treatment (Figure 33, \textit{RST1}). Consistent with previous observations, however, the transcription of \textit{RST2} is slightly elevated in response to pheromone (Figure 33, \textit{RST2}). A failure to accumulate Rst2p cannot be the sole mechanism for increased \textit{FUS1} expression in cycloheximide treated yeast, since superinduction of \textit{FUS1} is observed in both \textit{RST2} and \textit{rst2} strains. Other negative regulators of the PRP, which do not interact directly with Ste12p (see Recovery from pheromone response), are also known to be induced by pheromone treatment. Cycloheximide treatment may inhibit synthesis of multiple proteins required for attenuation of pheromone-responsive transcription. Alternatively, cycloheximide treatment may result in the loss of unstable regulatory proteins.

Two other mechanisms may be involved in \textit{FUS1} superinduction. First, cycloheximide may enhance the stability of \textit{FUS1} mRNA, perhaps by trapping the RNA molecules on polysomes and thereby shielding them from cytoplasmic ribonucleases (Cochran et al., 1983; Edwards and Mahadevan, 1992; Oleinick, 1977). Alternately, the superinduction may be the result of transcriptional induction in response to cycloheximide, as has been observed for other promoters (Hu and Hoffman, 1993; Koshiba et al., 1995; Li et al., 2001). For example, cycloheximide may alter the chromatin structure adjacent to the \textit{FUS1} gene (Cesari et al., 1998).
Figure 34. Ste12p does not accumulate in yeast cells in response to pheromone induction

Yeast (W3031a: STE12; yA06: ste12) were treated with α-factor (pheromone: lanes 1, 3, 5 and 7). Yeast in lanes marked (-) were grown for the same period but were not treated with α-factor. Immediately after pheromone treatment (t=0) or thirty minutes later (t=30), protein extracts were prepared from the cells and the amount of Ste12p (A) in 1 mg of each extract was determined by western blot with α-Ste12p polyclonal antibodies (TOP). α-Ste12p antibodies were preabsorbed onto extracts from ste12 yeast (see Materials and Methods). Antibody complexes were subsequently removed from the blot and the amount of Gal4p (B) present in the extracts was determined using α-Gal4p antibodies (BOTTOM).
4 Discussion

The unifying goal of this work was to further define both the domain structure of Ste12p and the regulatory mechanisms that control Ste12p activity. The results of this study revealed that overexpression of Ste12p(262 to 594) induces transcription, growth arrest and increased mating in yeast that is independent of Far1p. The results also showed that overexpression of STE12 can cause growth arrest that is independent of transcription from pheromone responsive elements.

The stimulation of Ste12p-dependent responses by overexpression of amino acids 262 to 594 indicated that there may be a direct negative regulator of Ste12p that can be titrated by excess Ste12p. Alternately, the overexpression of Ste12p may result in the formation of Ste12p multimers which can activate transcription. If activation of transcription in response to pheromone by Ste12p is dependent upon the formation of Ste12p multimers, the presence of excess Ste12p might be sufficient to cause multimerization and activation in the absence of the pheromone stimulus.

Overexpression of RST1 or RST2, two negative regulators of Ste12p, was shown to counteract the effects of STE12 overexpression. Additionally, Rst1p and Rst2p, which reside in complexes with Ste12p in vivo, were demonstrated to interact directly with distinct domains of Ste12p.

Ste12p is a transcription factor which functions in a complex network of kinases, negative regulators and other transcription factors. This work
demonstrated a new role for Ste12p in growth arrest and showed that the two negative regulators, Rst1p and Rst2p, must regulate Ste12p by separate mechanisms.

4.1 A mechanism for Far1p-independent G1 growth arrest

Previously published work has shown that overexpression of Ste12p can induce G1 growth arrest that is FAR1-independent (Dolan, 1996; Dolan and Fields, 1990). This work demonstrates that overexpression of Ste12p amino acids 262 to 594 is sufficient to induce G1 growth arrest that is independent of Far1p. Further, the growth arrest occurs in both STE12 and ste12 yeast, indicating that the arrest is independent of transcription from pheromone responsive elements. Like pheromone-induced arrest, the arrest occurs within one cell cycle. Also consistent with a PRP-like response, this arrest can be counteracted by the overexpression of CLN1 and CLN2, two G1 cyclins whose expression and activity is inhibited in pheromone response (see The pheromone response and the cell cycle).

It is possible that Ste12p overexpression inhibits growth by activating transcription in a PRE-independent manner. For example, Ste12p might interact with DNA indirectly by interacting with another transcription factor, such as Tec1p or Mcm1p (see DNA binding and protein-protein interactions of Ste12p). The FAR1 promoter, for example, contains two putative PREs, but it also includes binding sites for Mcm1p, which may facilitate activation by overexpressed Ste12p (SCPD; http://cgsigma.cshl.org/jian/).
Discussion

Some observations from this work support the model that Ste12p induces growth arrest by activating transcription. When deletions were made to define the smallest domains of Ste12p that could induce either G1 arrest or $FUS1$-$LacZ$ induction, both phenotypes were linked to the same domain, amino acids 262 to 594. Amino acids 262 to 594 overlap with amino acids 216 to 356, which are known to function as an activation region of Ste12p and with the pheromone induction domain, amino acids 301 to 335 (Olson et al., 2000; Pi et al., 1997; Song et al., 1991). Since the residues that induce G1 arrest have not been differentiated from the residues that activate transcription, it is possible that the G1 arrest function requires that Ste12p act as an activator. In addition, overexpression of $RST1$ and $RST2$, two negative regulators of Ste12p, can counteract the overexpression of $STE12$ and restore growth. Rst1p and Rst2p may counteract activation of transcription by Ste12p.

Alternatively, Ste12p may be inhibiting growth by a transcription-independent mechanism. It is noteworthy that Rst1p and Rst2p, two cognate negative regulators of Ste12p, were cloned in a two hybrid screen as proteins that interact with Cln1p and Cln2p (Tedford et al., 1997). No explanation or function for this interaction has ever been demonstrated. Perhaps Ste12p interferes with cell cycle progression by either promoting or disrupting the interaction of Rstps and G1 cyclins.

Regardless of whether or not Ste12p must direct transcription in order to facilitate growth arrest, it must inhibit cell cycle progression through a novel mechanism, since the arrest is independent of $FAR1$, the pheromone inducible
Discussion

CKI. In addition, the mechanism is less potent than Far1p-induced arrest, since only 75 to 80 percent of cells appear to arrest when Ste12p is overexpressed, while almost all cells arrest during pheromone response in response to Far1p (Guthrie and Fink, 1991).

Future work must identify which gene or protein targets are affected by Ste12p in the process of causing growth arrest. Global gene expression analysis could compare Ste12p overexpression with pheromone induction (Roberts et al., 2000) to determine which genes are up-regulated when Ste12p is overexpressed in \( far1 \) cells. Global protein-protein interaction by Repressed Trans-Activator (RTA™) analysis (US Patent No. 5,885,779) or "protein chip" analysis (MacBeath and Schreiber, 2000) could identify proteins that interact with Ste12p. Collectively, these data could identify the gene products required for \( FAR1 \)-independent growth arrest.

4.2 Interaction of Ste12p with negative regulators

In this work, I have demonstrated that overexpression of \( STE12 \) increases transcription from a \( FUS1 \) reporter in both \( STE12 \) and \( ste12 \) yeast, that Ste12p\( \Delta \)DBD induces transcription in \( STE12 \) yeast and that amino acids 262 to 594 correspond to the minimal domain of Ste12p that can induce \( FUS1 \)-lacZ transcription. Previous results have shown that deletion of regions of the \( STE12 \) gene encompassed by amino acids 262 to 594 results in increased activity of both Ste12p and Ste12p-Gal4p fusions in yeast (Kirkman-Correia et al., 1993;
Song et al., 1991). All of these results are consistent with the model that a titratable, direct negative regulator inhibits Ste12p.

Two known negative regulators of Ste12p activity are Rst1p and Rst2p. Both of the Rstps are found in complexes that interact with Ste12p, Kss1p and Fus3p, and deletion of the RST1 and RST2 genes promotes both invasive growth and pheromone-response phenotypes (Cook et al., 1996; Tedford et al., 1997). Overexpression of the RST genes counteracts the effects of STE12 overexpression in a dose-dependent manner, indicating that these regulators may be the factors that are titrated when excess Ste12p is present in cells. Also consistent with the function of direct negative regulators, both Rst1p and Rst2p bind directly to recombinant Ste12p and interact with Ste12p in yeast extracts.

Rst1p and Rst2p cannot function as titratable negative regulators, however. Deletion of RST1 and RST2 from the genome does not eliminate the activation of transcription that is observed when Ste12p is overexpressed (Olson et al., 2000). Therefore, excess Ste12p cannot simply be competing with endogenous Ste12p for Rst1p and Rst2p.

Although these results show that Rst1p and Rst2p bind directly to separate domains of Ste12p and inhibit its function, they do not define a mechanism for inhibition of Ste12p by Rst1p and Rst2p.

4.3 A mechanism for regulation of Ste12p by Rst2p

Rst2p interferes with the interaction between Ste12p and the PRE in EMSA assays, indicating that Rst2p may inhibit Ste12p by preventing DNA
Discussion

binding. Rst2p can also inhibit the function of the Ste12p DBD in vivo, since Rst2p inhibits the function of Ste12pDBD-VP16 fusions. These results show that Rst2p interacts with the DNA binding domain of Ste12p, but data presented in Figure 21 and in two-hybrid analysis (Pi et al., 1997) show that RST2 can inhibit the function of Ste12pΔDBD.

The results in Figure 21 do not imply that Rst2p can inhibit Ste12pΔDBD directly, however. In this experiment, FUS1-lacZ transcription is measured in a STE12 strain, as Ste12pΔDBD alone cannot activate transcription. If, as demonstrated in recent work, Ste12p overexpression results in multimerization that activates transcription (Olson et al., 2000), the Rst2p may inhibit Ste12p by interacting with the DNA binding domain of the endogenous Ste12p, not the overexpressed Ste12pΔDBD. It is also possible that Rst2p interacts with Ste12pΔDBD as a member of a complex. Rst2p is required for the regulatory functions of Kss1p, indicating that a Ste12p-Kss1p-Rst2p complex may exist (Bardwell et al., 1998b). If this is true, a direct interaction between Rst2p and Ste12pΔDBD may not be required for inhibition.

A further result that argues that Rst2p does not interact with the Ste12pΔDBD is shown in Figure 20. Rst2p has limited ability to counteract the growth arrest induced by Ste12pΔDBD in a ste12 strain, although it does prevent arrest in a STE12 strain.

Independent of an interaction of Rst2p with the C-terminus of Ste12p, Rst2p inhibits the function of the Ste12p DBD at PREs and disrupts DNA binding

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Discussion

*in vitro*. Based on these results, it is possible to postulate that Rst2p inhibits Ste12p simply by masking its DNA binding residues. However, alternate mechanisms may also be considered. Ste12p is postulated to bind to DNA as a dimer, although the dimerization domain has not been identified in amino acids 1 to 215. It is possible that Rst2p eliminates Ste12p dimerization, like the PIAS inhibitors of Stat transactivation (Chung et al., 1997; Liu et al., 1998).

Another important function of amino acids 1 to 215 of Ste12p is interaction with Tec1p, which is required for cooperative binding to FRE elements (Madhani and Fink, 1997). Since Rst2p is also a negative regulator of invasive growth, it is possible that interaction with the Ste12p DBD may disrupt Ste12p-Tec1p interactions. In this case, Rst2p may function to maintain MAP kinase cascade specificity. The increased expression of *RST2* in the presence of pheromone could result in reduced Ste12p-Tec1p interaction or increased Ste12p-Kss1p interaction, resulting in reduced activation from PREs.

Incomplete understanding of the Ste12p DNA binding domain complicates the process of defining the function of Rst2p in the regulation of Ste12p. Although the DNA binding domain of Ste12p is generally defined as amino acids 1 to 215, the minimum fragment required for DNA binding is actually amino acids 40 to 204 (Yuan and Fields, 1991). As a result, the nominal DBD of Ste12p includes at least 51 amino acids that may have other functions, including dimerization and protein-protein interaction. The Rst2p interaction with amino acids 21 to 195 of Ste12p overlaps with 20 of the amino acids (aa 21 to 40) not implicated in DNA binding, indicating that it could be regulating other Ste12p...
functions. The interaction does not overlap, however with amino acids 1 to 19 of Ste12p, which were recently identified as a negative regulatory domain of Ste12p (Crosby et al., 2000).

4.4 Mechanisms for regulation of Ste12p by Rst1p

Rst1p interacts with the C-terminus of Ste12p, amino acids 309 to 547, a region that includes the pheromone induction domain (amino acids 301 to 335) and overlaps with amino acids 214 to 473 of Ste12p, which are known to activate transcription (Kirkman-Correia et al., 1993; Pi et al., 1997; Song et al., 1991). Rst1p may inhibit Ste12p by masking the activation domain in a pheromone-reversible manner.

Recent observations have shown that Rst1p, and not Rst2p, inhibits the function of Ste12p∆DBD in the absence of pheromone (Olson et al., 2000). When a LexAp-Ste12p(216 to 688) fusion is expressed in RST1 or rst2Δ cells in the absence of pheromone, only a low level of activity is observed from a LexA-LacZ reporter. When the same fusion is expressed in an rst1 strain, however, the LexA-LacZ reporter has a very high level of activity (Olson et al., 2000). This indicates that, in the absence of pheromone, Rst1p does, indeed, regulate the activity of Ste12p∆DBD in vivo.

Based on the observed functions of other negative regulators, Rst1p could inhibit Ste12p by several mechanisms. Rst1p could function like Gal80p, the inhibitor of Gal4p, and mask the activation domain of Ste12p in the absence of pheromone (Ma and Ptashne, 1987; Yano and Fukasawa, 1997). Rst1p might
also interfere with interaction of Ste12p and other DNA binding proteins, such as Mcm1p and α1p. One other strong possibility is that Rst1p stabilizes the interaction between Ste12p and a Ste12p-specific inhibitor, such as an inactive MAP kinase. This possibility is consistent with the observation that Kss1p inhibition of Ste12p is dependent upon Rst1p (Bardwell et al., 1998a).

Rst1p interacts in yeast extracts with Ste12p amino acids 309 to 547, a smaller fragment of Ste12p than is required for activation by overexpression. Recent work has demonstrated that this discrepancy may be observed because Rst1p preferentially interacts with Ste12p multimers (Olson et al., 2000). Several regions in the C-terminus spanning amino acids 216 to 688 are required for multimerization of Ste12p and the region 262-356 is required for activation. So, although amino acids 309 to 547 can support multimerization and Rst1p interaction, they may lack the activation domain that would be required to increase $FUS1$-lacZ expression when Ste12p is overexpressed.

4.5 Rst1p and Rst2p interact with different regions of Ste12p

Previous observations about Rst1p and Rst2p have suggested that these two proteins function in the same manner. First, when $RST1$ and $RST2$ were cloned, it was suggested that the two genes were redundant, as both genes had to be deleted from the yeast genome before the phenotypes of invasive growth, slow growth and increased expression of pheromone responsive genes could be detected (Cook et al., 1996; Tedford et al., 1997). Second, sequence comparison showed that the proteins are 22% identical over their entire
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sequence, sharing a 60 amino acid segment with 64% similarity (Tedford et al., 1997). Third, immunoprecipitation and kinase assays showed that both proteins are located in complexes with Ste12p, Kss1p and Fus3p and that the proteins are MAP kinase substrates. Collectively, these results led to the conclusion that Rst1p and Rst2p inhibit Ste12p by the same mechanism.

One difference that was observed in the original studies of RST1 and RST2 may account for their apparent redundancy. RST2 expression is pheromone-induced, while RST1 is constitutively expressed (Tedford et al., 1997). As a result, the two genes might appear to have a single function when, in fact, they do not. Since RST2 is expressed only at low levels in the absence of pheromone, deleting RST2 will have little effect in vegetatively growing cells, where Rst1p is required for regulation of Ste12p activity. The effects of deletion of RST1, on the other hand, might be masked by increased activity of Ste12p resulting in the increased expression of RST2.

This work shows that Rst1p and Rst2p, in fact, do not work by identical mechanisms. Rst1p and Rst2p interact with different parts of the Ste12p protein and only Rst2p can inhibit the function of the Ste12p DNA binding domain. Rst1p, in contrast, interacts with and may inhibit the function of the Ste12p activation domain. These observations support the hypothesis that the apparent redundancy of Rst1p and Rst2p may be due to their complementary, but non-identical, functions.
4.6 Pheromone-responsive activation of transcription by Ste12p

In these experiments, I have demonstrated that overexpression of \textit{STE12} is sufficient to overcome Ste12p inhibition. It is unlikely, however, that the sole mechanism of Ste12p activation in pheromone response is the accumulation of excess Ste12p. When yeast are treated with both cycloheximide and pheromone, \textit{FUS1} transcription increases one hundred fold in 30 minutes, demonstrating that Ste12p accumulation is not required for activation. In addition, when yeast are treated with pheromone, Ste12p levels remain approximately the same and \textit{STE12} transcription increases only two-fold. \textit{FUS1} transcription is known to increase more than 10-fold under the same conditions (McCaffrey et al., 1987; Trueheart et al., 1987). This demonstrates that the level of expression of Ste12p is not reflected in the level of induction of expression of pheromone responsive genes.

Ste12p activation, therefore, must reflect a change in the state of Ste12p or of its associated negative regulators. Ste12p is activated by MAP kinases and Ste12p, Rst1p and Rst2p are all known targets of Fus3p and Kss1p (Cook et al., 1996; Tedford et al., 1997). Initially, activation of Ste12p could be mediated by changing the nature of the Ste12p-Rst1p interaction. For example, phosphorylation may dissociate the Ste12p-Rst1p-kinase interaction or alter it to expose the Ste12p activation domain. This model is supported by the observation that Rst1p interacts reversibly with the pheromone induction domain (Pi et al., 1997).
After a period of pheromone induction, Rst2p may accumulate in the cell. Since Rst2p is a pheromone-induced negative regulator of transcription, it is possible that Rst2p is required for recovery from pheromone and that, upon induction, Rst2p binds to Ste12p and inhibits transcription of pheromone responsive genes. Alternatively, Rst2p may function to maintain MAP kinase cascade specificity, by ensuring that Ste12p does not interact with Tec1p and activate transcription from FREs. If either of these models proves to be true, phosphorylation of Ste12p and Rst2p may, in fact, facilitate interaction and inhibition.

4.7 A revised model for Ste12p regulation

Recent work in the field of MAP kinase regulation has revealed new aspects of Ste12p function. It is now clear that Ste12p is an effector of two separate MAP kinase cascades and that it has DNA binding partners that are specific to both pheromone response and filamentation (Gustin et al., 1998; Madhani and Fink, 1998). In addition, Ste12p has also been demonstrated to be the target of negative regulation, in complexes with Rst1p, Rst2p and the MAPK Kss1p (Bardwell et al., 1998a; Bardwell et al., 1998b; Cook et al., 1997; Tedford et al., 1997).

Ste12p can no longer be simply visualized as a transcription factor that binds to PREs and activates transcription upon MAP kinase phosphorylation. Any model of Ste12p function must now include the aspects of negative
regulation, relief of inhibition in response to MAP kinase stimulation and mechanisms for maintaining specificity of signaling.

Based on my observations of Ste12p and its regulation by Rst1p and Rst2p, I propose the model in Figure 35 for Ste12p regulation in response to pheromone. In the absence of pheromone, a complex of Kss1p and Rst1p inhibits Ste12p bound to PREs by binding to the activation domain of Ste12p.

Upon pheromone induction, Ste12p and Rst1p become phosphorylated, disrupting the complex and revealing the Ste12p activation domain. After a period of pheromone responsive transcription, Rst2p binds to Ste12p, disrupts DNA binding and stimulates recovery. Rst2p bound to the Ste12p DBD also disrupts the FRE complex, ensuring that there is no crosstalk between the FIP and the PRP.

Several approaches could be used to address this model for Rst2p function. To determine whether pheromone induction of \textit{RST2} is required for Rst2p function, gene expression could be monitored in yeast with a non-pheromone inducible \textit{RST2}. If pheromone induction of \textit{RST2} is required for recovery, yeast with non-pheromone inducible \textit{RST2} should show increased expression of pheromone responsive genes after extended exposure to pheromone. If pheromone-induced \textit{RST2} prevents inappropriate activation from FREs, FIP gene expression should be induced by pheromone in yeast with non-pheromone inducible \textit{RST2}. To explore whether or not Rst2p prevents the interaction of Ste12p with PRE elements \textit{in vivo}, Ste12p interaction with DNA could be compared by chromatin immunoprecipitation or \textit{in vivo} footprinting.
NO PHEROMONE

Kss1p
Rst1p
Ste12p
Ste12p
PRE
PRE

PHEROMONE INDUCTION

Kss1p
Rst1p
Ste12p
Ste12p
PRE
PRE

RECOVERY

Fus3p
Ste12p
Ste12p
P
PRE
PRE

Fus3p
Ste12p
Ste12p
P
PRE
TCS

Figure 35. A new model for Ste12p regulation
Based on the results in this thesis, I propose a new model for Ste12p regulation. For details, see text.
Discussion

in both RST2 and rst2 strains. It is also possible that Rst2p inhibits the function of the Ste12pDBD by preventing its oligomerization. Experiments similar to those published for Rst1p (Olson et al., 2000), could determine whether there is an oligomerization domain in the Ste12pDBD and whether it is the domain of Ste12p that interacts with Rst2p.

This model does not include a function for Rst1p at FREs, due to a lack of data, but it may block the activation domain of Ste12p in the same manner. In filamentation-invasion, one might predict that Rst1p inhibition would be relieved by Kss1p-dependent phosphorylation.

4.8 Future work - Ste12p as a model transcription factor

Ste12p is a transcription factor and an effector in multiple signal transduction pathways, and Ste12p activity is modulated by a web of protein-protein interactions. Because of its genetic accessibility and readily modulated activity, Ste12p has been and should continue to be an excellent model transcriptional activator.

To date, the characterization of Ste12p has focused on the interaction of Ste12p with the pheromone and filamentation MAP kinase cascades. The work has resulted in a significant number of observations about how signal transduction impacts upon the activity of a transcriptional activator.

What has not been directly addressed, however, is how the activator, Ste12p, transmits an extracellular signal to the RNA polymerase II transcriptional machinery. If the component(s) of the transcriptional machinery with which
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Ste12p interacts were identified, it might be possible to define more precisely how Ste12p activates transcription. Consequently, it might also be possible to understand how specific protein-protein interactions modulate Ste12p activity.

Such an approach to understanding Ste12p regulation might provide insight into the functions of multiple transcriptional activators. Several mechanisms that regulate activity are also observed for other transcription factors. Like the Ets-1 family of activators, Ste12p is directed to specific promoters by interactions with other sequence-specific transcription factors (Wasylyk et al., 1998). Like the Myc proto-oncogene, Ste12p directs transcription in response to multiple signal transduction pathways that control diverse developmental programs (Gustin et al., 1998; Sakamuro and Prendergast, 1999). If we understood how Ste12p interacts with the transcriptional machinery, we might then be able to understand how these modes of regulation affect transcription factor activity, both collectively and individually.

Future study of Ste12p will also explore the regulation of Ste12p by Rst1p and Rst2p. Rst1p and Rst2p, which bind directly to Ste12p, interact with different regions of Ste12p and appear to function by different mechanisms. If we understood how Ste12p interacts with the Pol II transcriptional machinery, it might help us to decipher precisely how these negative regulators function and why two separate mechanisms are required.

Ste12p could also be used as a model to develop novel methods to study transcription factors. Protein microarray technology may provide a practical method to determine the targets of Ste12p within the transcription machinery.
(MacBeath and Schreiber, 2000; Zhu et al., 2000). Protein microarrays of the components of the transcription machinery could be probed with Ste12p to identify Ste12p targets. Interaction could then be confirmed by more traditional assays of protein-protein interaction, such as co-immunoprecipitation. If such a method were successful, it would be applicable to many transcription factors.

4.9 Conclusion

This work has demonstrated that overexpression of STE12 can induce growth arrest and activate transcription. The growth arrest induced by Ste12p is Farlp-independent but otherwise reminiscent of pheromone-induced arrest, which suggests that Ste12p may have a second role in pheromone-induced growth arrest. This work has also demonstrated that the induction of transcription by Ste12p overexpression can be inhibited by overexpression of Rst1p and Rst2p and that Rst1p and Rst2p can bind directly to Ste12p.

The observation that Rst1p and Rst2p interact with different domains of Ste12p suggests that, although the two proteins are genetically redundant, they do not have identical functions. Rst2p is likely an inhibitor of Ste12p DNA binding and Rst1p may inhibit the function of the Ste12p activation domain. Further work will be required to fully characterize the mechanisms of Ste12p inhibition by Rst1p and Rst2p in combination with the PRP and FIP MAP kinases. Future work will also be required to determine how the inhibition is relieved to allow Ste12p to activate transcription.
Discussion

The overriding goal for future study of Ste12p will be to understand how specificity of signaling is maintained when a single activator stimulates the genes required for two different signal transduction cascades. New technologies that explore global gene expression and global protein-protein interactions should facilitate reaching that goal.
5 Materials and Methods

5.1 Plasmids, strains and media

Plasmids and strains used in these experiments are listed in Tables 6, 7 and 8. Yeast media was made as described (Ausubel et al., 1998; Guthrie and Fink, 1991; Kaiser et al., 1994). α-Factor (Sigma Chemical Company) was added at 2 µg/ml to liquid cultures. Cycloheximide was used at a concentration of 0.1 mg/mL. Plasmid transformations into yeast used the method of (Kaiser et al., 1994). Unless otherwise stated, E. coli were grown in LB media, supplemented with 100 µg/mL ampicillin when appropriate.

DNA manipulation techniques including amplification by polymerase chain reaction (PCR), restriction enzyme digestion, DNA purification and ligation followed standard procedures (Ausubel et al., 1998; Sambrook et al., 1989). The plasmid pSC4 (Table 6) was used as the template for PCR amplification of STE12 sequences. The E. coli strain DH5α was used for the propagation of plasmids (Table 8).

Plasmids used for this study are described in Table 6. Oligonucleotides used for PCR amplification of STE12 are described in Table 9. The sequences of plasmids containing PCR-amplified sequences were confirmed by DNA sequencing. Manual DNA sequencing with Sequenase T7 DNA polymerase (United States Biochemical Corporation) and automated sequencing using an
<table>
<thead>
<tr>
<th>Plasmid Name</th>
<th>Plasmid Description</th>
<th>Plasmid Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>PYeDP8-1/2</td>
<td>Episomal plasmid with galactose-inducible promoter.</td>
<td>(Cullin and Pompon, 1988)</td>
</tr>
<tr>
<td>pJL1</td>
<td>Episomal plasmid, expresses WT Ste12 from a galactose-inducible promoter.</td>
<td>W. Hung</td>
</tr>
<tr>
<td>Ye/STE12ΔXbal</td>
<td>Episomal plasmid, expresses Ste12ΔDBD, amino acids 216 to 688, from a galactose-inducible promoter.</td>
<td>W. Hung</td>
</tr>
<tr>
<td>pSC4</td>
<td>Plasmid containing STE12 genomic DNA; used as PCR template for STE12 cloning.</td>
<td>(Fields and Herskowitz, 1987)</td>
</tr>
<tr>
<td>pAO12</td>
<td>Episomal plasmid, expresses Ste12, amino acids 215 to 473, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO1 and AO2, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>pAO34</td>
<td>Episomal plasmid, expresses Ste12, amino acids 473 to 688, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO1 and AO2, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>pUNK</td>
<td>Integrating plasmid, removes STE12 promoter and DBD when transformed into yeast.</td>
<td>Deleted Xbal fragment of pSC4, then cloned the Sphl/SacI fragment into Ylplac211.</td>
</tr>
<tr>
<td>YEpnLs</td>
<td>Episomal plasmid; expresses Ste12ΔDBD, amino acids 216 to 688, with a nuclear localization signal from a galactose-inducible promoter.</td>
<td>W. Hung</td>
</tr>
<tr>
<td>YEplac112</td>
<td>Episomal plasmid, TRP1 marker.</td>
<td>D. Gietz</td>
</tr>
<tr>
<td>YEplac181</td>
<td>Episomal plasmid, LEU2 marker.</td>
<td>D. Gietz</td>
</tr>
<tr>
<td>Ylplac204</td>
<td>Integrating plasmid, TRP1 marker.</td>
<td>D. Gietz</td>
</tr>
<tr>
<td>Ylplac211</td>
<td>Integrating plasmid, URA3 marker.</td>
<td>D. Gietz</td>
</tr>
<tr>
<td>p1</td>
<td>Episomal plasmid, expresses Ste12, amino acids 215 to 641, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO1 and AO15, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p2</td>
<td>Episomal plasmid, expresses Ste12, amino acids 215 to 594, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO1 and AO14, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>-----</td>
<td>---------------------------------------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>p3</td>
<td>Episomal plasmid, expresses Ste12, amino acids 215 to 547, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO1 and AO13, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p4</td>
<td>Episomal plasmid, expresses Ste12, amino acids 215 to 500, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO1 and AO12, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p5</td>
<td>Episomal plasmid, expresses Ste12, amino acids 262 to 688, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO7 and AO4, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p6</td>
<td>Episomal plasmid, expresses Ste12, amino acids 309 to 688, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO8 and AO4, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p7</td>
<td>Episomal plasmid, expresses Ste12, amino acids 356 to 688, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO9 and AO4, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p8</td>
<td>Episomal plasmid, expresses Ste12, amino acids 403 to 688, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO10 and AO4, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p9</td>
<td>Episomal plasmid, expresses Ste12, amino acids 450 to 688, from a galactose-inducible promoter.</td>
<td>PCR amplified STE12 with oligonucleotides AO11 and AO4, cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>p10</td>
<td>Episomal plasmid, expresses Ste12, amino acids 262 to 594, from a galactose-inducible promoter.</td>
<td>BamHI insert of p2, cloned into the BamHI-digested backbone of p5.</td>
</tr>
<tr>
<td>p11</td>
<td>Episomal plasmid, expresses Ste12, amino acids 262 to 641, from a galactose-inducible promoter.</td>
<td>BamHI insert of p1, cloned into the BamHI-digested backbone of p5.</td>
</tr>
<tr>
<td>p12</td>
<td>Episomal plasmid, expresses Ste12, amino acids 309 to 547, from a galactose-inducible promoter.</td>
<td>BamHI insert of p3, cloned into the BamHI-digested backbone of p6.</td>
</tr>
<tr>
<td>Plasmid Name</td>
<td>Description</td>
<td>Author</td>
</tr>
<tr>
<td>--------------</td>
<td>-------------</td>
<td>--------</td>
</tr>
<tr>
<td>pMT556</td>
<td>Episomal plasmid, expresses RST1 under the control of a galactose-inducible promoter.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pMT558</td>
<td>Episomal plasmid, expresses RST2 under the control of a galactose-inducible promoter.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pG1T</td>
<td>Centromeric plasmid, expresses RST1 under the control of a galactose-inducible promoter.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pG2T</td>
<td>Centromeric plasmid, expresses RST2 under the control of a galactose-inducible promoter.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pJL1/trp</td>
<td>Episomal plasmid, expresses WT Ste12 from a galactose-inducible promoter. TRP1 marker.</td>
<td>W. Hung</td>
</tr>
<tr>
<td>pΔXba/trp</td>
<td>Episomal plasmid, expresses Ste12ADBD (amino acids 216 to 688) from a galactose-inducible promoter. TRP1 marker.</td>
<td>W. Hung</td>
</tr>
<tr>
<td>pMT485</td>
<td>2 μ plasmid, URA3 marker, expresses CLN1 under the control of a galactose-inducible promoter.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pMT979</td>
<td>2 μ plasmid, URA3 marker, expresses CLN2 under the control of a galactose-inducible promoter.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pMT42</td>
<td>2 μ plasmid, URA3 marker, expresses CLN3 under the control of a galactose-inducible promoter.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pMT580</td>
<td>Expresses GST-Rst1p in E. coli.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pMT581</td>
<td>Expresses GST-Rst2p in E. coli.</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>pGEX-4T3</td>
<td>GST expression vector for E. coli</td>
<td>Pharmacia Biotechnology</td>
</tr>
<tr>
<td>pSTVP/235</td>
<td>Episomal expression vector for E. coli</td>
<td>W. Hung</td>
</tr>
<tr>
<td>pAO001</td>
<td>PCR amplified FUS1 with oligonucleotides AO23 and AO24, cloned into pGEM3Z(f+) at the BamHI site.</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>---</td>
<td>---</td>
<td>---</td>
</tr>
<tr>
<td>pAO002</td>
<td>Same as pAO001, opposite orientation.</td>
<td>As for AO001.</td>
</tr>
<tr>
<td>pAO003</td>
<td>Episomal plasmid, expresses Ste12, amino acids 1 to 215, from a galactose-inducible promoter.</td>
<td>EcoRI/BamHI insert of pV1.4 cloned into pYeDP8-1/2.</td>
</tr>
<tr>
<td>pV1.4</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 1 to 215, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides VT1 and VT2, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pV2</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 1 to 195, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides VT1 and SDB2, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pV3</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 21 to 215, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides SDB1 and VT2, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pV4</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 21 to 195, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides SDB1 and SDB2, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pAO006</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 1 to 108, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides VT1 and AO26, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pAO007</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 109 to 215, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides VT1 and AO25, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pAO008</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 21 to 170, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides SDB1 and AO27, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pAO009</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 21 to 155, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides SDB1 and AO28, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pAO010</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 70 to 195, in <em>E. coli</em>.</td>
<td>PCR amplified STE12 with oligonucleotides AO30 and SDB2, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>pAO011</td>
<td>Expresses 6-His-Ste12pDBD, amino acids 45 to 195, in <em>E. coli</em>.</td>
<td>PCR amplified <em>STE12</em> with oligonucleotides AO29 and SDB2, cloned into pRSETA (Invitrogen).</td>
</tr>
<tr>
<td>-----------</td>
<td>-------------------------------------------------------------</td>
<td>----------------------------------------------------------------------------------</td>
</tr>
<tr>
<td>pMR8</td>
<td>PHD1 clone, use EcoRI/MluI fragment as probe for Northern and Southern blot analysis.</td>
<td>W. Hung</td>
</tr>
<tr>
<td>pTES314</td>
<td>Expresses TRPE-Ste12p, amino acids 220 to 688, fusion in <em>E. coli</em>.</td>
<td>W. Hung</td>
</tr>
<tr>
<td>pDF33</td>
<td>850 nucleotide fragment of <em>ACT1</em> in pUC18.</td>
<td>D. McMaster</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td>Source</td>
</tr>
<tr>
<td>----------</td>
<td>---------------------------------------------------------------------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>SY2585</td>
<td>Mat a, leu2, trp1, ura3, ade2, mfa2Δ::FUS1-LacZ, his3::FUS1-HIS3</td>
<td>C. Boone</td>
</tr>
<tr>
<td>W303a::SUL-1</td>
<td>Mat a, leu2, trp1, ura3, ade2, mfa2Δ::FUS1-LacZ, his3::FUS1-HIS3, ste12::LEU2</td>
<td>W. Hung</td>
</tr>
<tr>
<td>W3031a</td>
<td>Mat a, leu2, trp1, ura3, ade2, his3, can1</td>
<td>E. Leberer</td>
</tr>
<tr>
<td>W3031b</td>
<td>Mat a, leu2, trp1, ura3, ade2, his3, can1</td>
<td>E. Leberer</td>
</tr>
<tr>
<td>SY2587</td>
<td>Mat a, far1, leu2, trp1, ura3, ade2, his3, can1</td>
<td>C. Boone</td>
</tr>
<tr>
<td>yAO1</td>
<td>Mat a, ste12, leu2, trp1, ura3, ade2, mfa2Δ::FUS1-LacZ, his3::FUS1-HIS3</td>
<td>This study</td>
</tr>
<tr>
<td>yAO2</td>
<td>Mat a, ste12, far1, leu2, trp1, ura3, ade2, his3, can1</td>
<td>This study</td>
</tr>
<tr>
<td>yAO3</td>
<td>Mat a, ste12, leu2, trp1, ura3, ade2, his3, can1</td>
<td>This study</td>
</tr>
<tr>
<td>MTy1147</td>
<td>Mat a, rst2::HIS3, leu2, trp1, ura3, ade2, his3, can1</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>MTy1154</td>
<td>Mat a, rst1::TRP1, leu2, trp1, ura3, ade2, his3, can1</td>
<td>M. Tyers</td>
</tr>
<tr>
<td>WHY2-7</td>
<td>Mat a, fus3::LEU2, kss1::URA3, leu2, trp1, ura3, ade2, mfa2Δ::FUS1-LacZ, his3::FUS1-HIS3</td>
<td>W. Hung</td>
</tr>
<tr>
<td>WHY3-1</td>
<td>Mat a, fus3::LEU2, leu2, trp1, ura3, ade2, mfa2Δ::FUS1-LacZ, his3::FUS1-HIS3</td>
<td>W. Hung</td>
</tr>
<tr>
<td>HLY334</td>
<td>Mat a, ura3-52</td>
<td>G. Fink</td>
</tr>
<tr>
<td>SY991</td>
<td>Mat a, ade2, his3, leu2, trp1, ura3, can1, mfa1::LEU2, mfa2::LEU2</td>
<td>C. Boone</td>
</tr>
<tr>
<td>SY2625</td>
<td>Mat a, bar1, ade2, leu2, trp1, ura3, can1, his3::FUS1-HIS3</td>
<td>C. Boone</td>
</tr>
<tr>
<td>Strain</td>
<td>Genotype</td>
<td></td>
</tr>
<tr>
<td>--------</td>
<td>----------</td>
<td></td>
</tr>
<tr>
<td>DH₅α</td>
<td>supE44, endA1, hsdR17 (rk-, mk+), thi1, recA1, gyrA96, relA1(F80lacZdM15)</td>
<td></td>
</tr>
<tr>
<td>JM101</td>
<td>supE, thi, d(lac-proAB), [F', traD36, proAB, laclqZdM15], [rk+, mk+], mcrA(+)</td>
<td></td>
</tr>
<tr>
<td>NM522*</td>
<td>F'[(proAB+laclqZdeltaM15), supEthi1delta(lacproAB)delatahsd(r-m-)]lambda-deoR+]</td>
<td></td>
</tr>
<tr>
<td>RR1</td>
<td>SupE44, hsdS20(r-Bm-B), ara14, proA2, lacY1, galK2, rpsL20, xy15, mtl1</td>
<td></td>
</tr>
</tbody>
</table>

* Gift from D. Kilburn
### Table 9. Oligonucleotides for construction of STE12 deletions

<table>
<thead>
<tr>
<th>Oligonucleotide name</th>
<th>Ste12p amino acid residue</th>
<th>Oligonucleotide sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td>AO1</td>
<td>215F</td>
<td>5'-GGTACCATGTCTAGAAGACCACATCTAGTACAACA</td>
</tr>
<tr>
<td>AO7</td>
<td>262F</td>
<td>5'-GGTACCATGCCCCTCTCAAATTAATGATTTTTATT</td>
</tr>
<tr>
<td>AO8</td>
<td>309F</td>
<td>5'-GGTACCATGGACTATATTTTTCCTGTATCTGTTGAA</td>
</tr>
<tr>
<td>AO9</td>
<td>356F</td>
<td>5'-GGTACCATGTCTCTTTTAATAGATACCCCTAT</td>
</tr>
<tr>
<td>AO10</td>
<td>403F</td>
<td>5'-GGTACCATGGACCCCTACCAGCTACATGAAATGAT</td>
</tr>
<tr>
<td>AO11</td>
<td>450F</td>
<td>5'-GGTACCATGGAATCTTACCCAAACGGAATGTT</td>
</tr>
<tr>
<td>AO12</td>
<td>500R</td>
<td>5'-GAATTCTCATTGAGATACAGCATATTGTTATC</td>
</tr>
<tr>
<td>AO13</td>
<td>547R</td>
<td>5'-GAATTCTCAGGGCTGAGTGAAGGTTTTTATC</td>
</tr>
<tr>
<td>AO14</td>
<td>594R</td>
<td>5'-GAATTCTCAATTTTCTGTGAGACCTTCC</td>
</tr>
<tr>
<td>AO15</td>
<td>641R</td>
<td>5'-GAATTCTCAAGAATCTTCTGTCACCAGCATTG</td>
</tr>
<tr>
<td>AO4</td>
<td>688R</td>
<td>5'-GAATTCTCAGGGCTCTAGTGAAGGTTTTTATC</td>
</tr>
<tr>
<td>VT1</td>
<td>1F</td>
<td>5'-CTGGATCCATGAAGTCCAAATAACCAATAGT</td>
</tr>
<tr>
<td>SDB1</td>
<td>21F</td>
<td>5'-CTGGATCCATGAAAACGATGAAATGCTAAAGCT</td>
</tr>
<tr>
<td>AO29</td>
<td>45F</td>
<td>5'-CTGGATCCATGTTTTTTAGCCACAGCG</td>
</tr>
<tr>
<td>AO30</td>
<td>70F</td>
<td>5'-CTGGATCCATGGGCTTTTGTCTTGTGATTTT</td>
</tr>
<tr>
<td>AO25</td>
<td>109F</td>
<td>5'-CTGGATCCATGTTCTGAGAGGCTTTTTTC</td>
</tr>
<tr>
<td>AO26</td>
<td>108R</td>
<td>5'-TCGAATTCTCATTCTTTTTTCTTTGACTACTTTCTCT</td>
</tr>
<tr>
<td>AO28</td>
<td>155R</td>
<td>5'-TCGAATTCTAAAAATATCTTTTCTGCTTTTTT</td>
</tr>
<tr>
<td>AO27</td>
<td>170R</td>
<td>5'-TCGAATTCTCATTCCCAACGCACCTG</td>
</tr>
<tr>
<td>SBD2</td>
<td>195R</td>
<td>5'-TCGAATTCTCATGAAAAGATAAGGCAGGCTCATT</td>
</tr>
<tr>
<td>VT2</td>
<td>215R</td>
<td>5'-TCGAATTCTCATCTGAATCTAAATGTTGAAATGAA</td>
</tr>
</tbody>
</table>

*The number indicates the terminal amino acid encoded by the PCR amplification product. F oligonucleotides prime in the forward orientation, R oligonucleotides in reverse.*
Materials and Methods

ABI Prism Genetic Analyzer (Applied Biosystems (Canada) Inc.) were performed according to the manufacturer’s instructions.

Saccharomyces cerevisiae strains yAO1, yAO2 and yAO3 (Table 7) were made by deletion of STE12 from the genome of SY2585, SY2587 and W3031-B using the plasmid pUNK (Table 6). To confirm the identity of the strains, potential ste12 strains were tested for their ability to respond to pheromone in a halo assay (a strains) or for their ability to secrete pheromone (α strains (Guthrie and Fink, 1991)). The genotypes of new strains were confirmed by Southern blot (Ausubel et al., 1998; Sambrook et al., 1989).

5.2 β-galactosidase assays

For β-galactosidase assays, yeast were grown to OD_{600} ≈ 0.8 in selective minimal media, induced with 2 % (% w/v) galactose and/or 2 μg/ml α-factor and then grown for two hours, unless otherwise stated. β-galactosidase activity was determined as described (Ruby et al., 1983). First, OD_{600} of the culture was determined. Then, 500 μL of the culture were harvested and resuspended in 250 μL of Z buffer (100 mM sodium phosphate (pH 7.0), 10 mM potassium chloride, 1 mM magnesium sulphate and 0.27% β-mercaptoethanol), 50 μL 0.1% SDS (sodium dodecyl sulphate) and 50 μL chloroform. Samples were vortexed briefly and incubated for five minutes at 30 °C. The assay was started by addition of 200 μL ONPG (o-nitrophenylgalactoside, 0.004 μg/mL in Z-buffer) and stopped
Materials and Methods

by addition of 500 μL of 1M sodium carbonate (pH 11). Absorbance of the final sample at 420 nm was determined.

The formula for calculation of β-galactosidase activity is as follows:

\[ \beta\text{-galactosidase activity} = \frac{1000 \times A_{420}}{(OD_{600} \times v \times t)} \]

Where:

- \( v \) = sample volume in mL (0.5 mL)
- \( t \) = assay time in minutes.

β-galactosidase activity values are reported as an average of three trials and error bars represent standard deviation of those three trials. For those values for which no error is reported, the standard deviation value is not large enough to distinguish with respect to the scale of the graph.

5.3 G1 Growth Arrest Assay

The method of (Guthrie and Fink, 1991) was used to determine the percentage of yeast cells in a culture that were in the G1 phase of the cell cycle. Briefly, a 1 mL aliquot of yeast from a mid-log suspension culture was fixed in 10% formaldehyde and sonicated for 3 seconds on low power. Fixed cells were washed in 100 μL of PBS and stored in PBS at 4°C until they could be counted. The ratio of unbudded to total cells was determined and expressed as a percentage for a minimum of 200 cells per sample.

5.4 Quantitative Mating Assays

Mating assays were performed by standard techniques (Guthrie and Fink, 1991). The two strains to be mated were grown to OD₆₀₀ ≈ 0.8 and filtered onto a
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common support. The yeast were transferred to galactose-containing plates and incubated at 30 °C for 4 to 5 hours. Yeast were then transferred to diploid-selective media and grown for two or three days, at which time the number of colonies formed was counted.

One haploid strain was added to the mating reaction at a lower (10-fold diluted) frequency than the other. The number of yeast in the mating reaction from the limiting strain is defined as the “number of haploids” in the determination of mating efficiency. Mating efficiency is defined as the ratio of diploids formed to the number of haploids added to the mating reaction.

5.5 Expression and purification of recombinant proteins from *E. coli*

6-His-Ste12pDBD, GST-Rst1p, GST-Rst2p and GST were expressed in NM522 and trpE-Ste12pΔDBD was expressed in RR1 as previously described ((Ausubel et al., 1998), strains in Table 8). RR1 *E. coli* were propagated in M9 minimal media (Ausubel et al., 1998) NM522 cells were infected with T7 RNA poll phage (gift from D. Kilburn) for 6-His-Ste12pDBD expression. Following induction of protein expression, *E. coli* were washed twice in lysis buffer (1mM dithiothreitol, 0.1% Nonidet P40, 250 mM NaCl, 50 mM NaF, 5 mM EDTA, 50 mM Tris (pH 7.5), 1 mM PMSF, 1 µg/ml leupeptin, 1 µg/ml pepstatin, 10 µg/ml soybean trypsin inhibitor, 10 µg/ml TPCK and 0.6 mM dimethylaminopurine) and lysed by sonication. Crude lysates were clarified by centrifugation for five minutes at 10 000xg.
Materials and Methods

The GST-recombinant proteins were batch purified with glutathione agarose as described by the manufacturer (Sigma Chemical Company). Similarly, 6-His-Ste12pDBD was batch purified with Ni-agarose (Invitrogen). Lysates with trpE-Ste12pΔDBD were used without further treatment.

C. Perelli-Hentschel used the method described for 6-His-Ste12pDBD to prepare 6-His-Gal4pDBD (Ausubel et al., 1998).

5.6 Expression of recombinant Ste12p in Spodoptera frugiperda (Sf9) cells

Ste12p was expressed in Sf9 cells from a recombinant AcMNPV virus as described (Olson et al., 2000). At 72 hours post-infection, the cells were washed twice in SF9 extract buffer (20 mM Tris (pH 8.0), 150 mM NaCl, 1 mM EDTA, 1% Nonidet P40, 0.5% sodium deoxycholate, 1 mM PMSF, 3 mM dithiothreitol, 0.7 mM leupeptin, 2 μM pepstatin, 2 mM benzamidine, 2 μg/mL chymostatin, 100 μg/ml TPCK) and lysed in the same buffer by Dounce homogenization. The lysate was clarified by centrifugation at 12,000xg for 20 minutes and used without further purification.

5.7 Protein affinity precipitation of recombinant proteins

Interaction of recombinant 6-His-Ste12pDBD, trpE-Ste12pΔDBD and Wt Ste12p from insect cells with GST-Rst1p and GST-Rst2p was detected by incubation of selected proteins in lysis buffer at 4°C for one hour, followed by addition of glutathione agarose and agitation for ninety minutes. The glutathione
agrose and associated proteins were recovered and washed, and the proteins were eluted in 1 X SDS sample buffer (Sambrook et al., 1989). Proteins were detected by western blot (see **SDS-PAGE and western blot for detection of proteins**). For these assays, 5 µg each of GST-Rst1p, GST-Rst2p, GST or 6-His-Ste12pΔDBD were used. One hundred micrograms each of the crude trpE-Ste12pΔDBD and Wt Ste12p (Sf9) extracts were used, and the Wt Ste12p (Sf9) extract was supplemented with 1 mg/mL bovine serum albumen.

### 5.8 SDS-PAGE and western blot for detection of proteins

Proteins were resolved by SDS-PAGE as previously described (Ausubel et al., 1998) and detected by Coomassie blue staining or by western blot. ECL reagents were used as described by the manufacturer (Amersham Pharmacia Biotech) for luminescent detection. 2 % (%w/v) powdered skim milk was added to both primary and secondary antibody incubations.

Three α-Ste12p antibodies were used in this work. Two polyclonal α-Ste12p antibodies, which interact with the C-terminal 472 and 215 amino acid residues of Ste12p, have been described previously (Hung et al., 1997). The third α-Ste12p antibody was generated against 6-His-Ste12pΔDBD in a New Zealand white rabbit by standard techniques (Olson et al., 2000). The antibody is specific to the N-terminal 215 residues of Ste12p. α-Ste12p antibodies were used at a 1:20 000 dilution.

6-His proteins were detected with polyclonal α-His antibodies from Santa Cruz Biotechnology. GST proteins were detected with polyclonal α-GST.
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antibodies from Santa Cruz Biotechnology. Primary antibodies from Santa Cruz were used at a 1:2000 dilution. Secondary antibody was horseradish-peroxidase-labeled goat- or donkey- anti-rabbit IgG serum from Gibco-BRL (Life Technologies); it was used at a 1:10,000 dilution.

Denatured yeast protein extracts were prepared using the method of (Chang and Herskowitz, 1992). Cultures were grown to OD$_{600}$ ≈ 0.8 and cells were harvested by centrifugation. Cells were lysed in SDS Sample Buffer (100 mM Tris pH 6.8, 4 % SDS, 10 % glycerol, 20 % β-mercaptoethanol) with acid-washed glass beads by heating the samples at 95 to 100 °C for five minutes. Samples were chilled on ice and, following centrifugation, analyzed by western blot. Approximately 20 μg of extract were used per lane.

For the detection of endogenous Ste12p levels following α-factor treatment, polyclonal α-Ste12p antibodies were pre-absorbed onto extracts from ste12 yeast prior to incubation with the STE12 blot. Following Ste12p detection, antibodies were removed from the blot by washing in distilled water, then 0.2 M sodium hydroxide, followed by a final wash in distilled water, for five minutes each. The same blot was subsequently probed with α-Gal4p polyclonal antibodies (Sadowski et al., 1991).

5.9 Metabolic labeling and affinity precipitation of Ste12p

Yeast transformed with galactose-inducible STE12 constructs were grown to OD$_{600}$ ≈ 0.8 and starved for methionine for 20 minutes. Following starvation,
1.2 mCi of $[^{35}\text{S}]$-methionine and 2% (% w/v) galactose were added and cultures were labeled for two hours at 30 °C. $[^{35}\text{S}]$-methionine-labeled extracts were made as described (Tedford et al., 1997). Yeast were lysed with acid-washed glass beads by vigorous vortexing in lysis buffer (see Expression and purification of recombinant proteins from *E. coli*) followed by centrifugation at 17 000 x g for 30 minutes at 4 °C.

Each extract was pre-cleared by incubation with 20 μg of purified GST protein (above) and 50 μL of glutathione agarose (Sigma Chemical Company) for one hour at 4°C followed by brief centrifugation at 2000 rpm. Supernatants were incubated for 1 hour on ice with 5 μg of GST-Rst1p or GST-Rst2p followed by 1 hour of agitation at 4°C with 25 μL of glutathione-agarose. Glutathione-agarose beads and associated proteins were recovered and washed three times in lysis buffer. Proteins were eluted for 30 minutes at 37°C in lysis buffer supplemented with 5 mM glutathione. Eluted proteins were resolved by SDS-PAGE (above) and detected by autofluorography.

Immunoprecipitations with polyclonal α-Ste12p antibodies were performed as previously described (Hung et al., 1997; Olson et al., 2000).

5.10 Northern and Southern blots

Genomic DNA or total RNA was isolated from yeast as described (DNA (Kaiser et al., 1994); RNA (Schmitt et al., 1990)). 10 μg of DNA or 20 μg of RNA was resolved by electrophoresis and transferred to nitrocellulose or nylon as
Materials and Methods

described (Sambrook et al., 1989). Probes for northern and Southern analysis were random prime labeled using the Oligolabeling Kit (Promega) as described by the manufacturer.

5.11 Electrophoretic Mobility Shift Assays

Electrophoretic mobility shift assays were performed as described in (Ausubel et al., 1998). The probe, which contains two PREs arranged tail to tail consisted of two oligonucleotides (WH65A: 5'-
\[ \text{TCGACATGTTTCATTGAAACAAAGC-3'} \] and WH66A: 5'-
\[ \text{TCGAGCTTTGTTTCAAATGAAACATG-3'} \] ) which were annealed and labeled by Klenow end fill-in in the presence of \(^{32}\text{P}\)-dATP. Approximately 20 pmol of probe was used per lane. Non-specific competitor oligonucleotide consisted of a Gal4p DNA binding site formed by annealing GS3: 5'-TCGACGGAGTACTGTCCTCCG-3' and GS4: 5'-TCGACGGAGGACAGTACTCCG-3'. All DNA binding reactions were performed for one hour on ice in 1X EMSA buffer (20 mM Tris (pH 8), 40 mM NaCl, 4 mM MgCl\(_2\), 1 mM DTT and 5% glycerol (%v/v) in the presence of 10 \(\mu\text{g}\) of sheared salmon sperm DNA and 2\(\mu\text{g}\) dldC (Promega). DNA binding reactions were resolved by electrophoresis on a 6% acrylamide gel and visualized by autoradiography.
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