Regulation of Pheromone Response in *Saccharomyces* by Ste12-PRE Interaction and TOR-Cdc55 Signaling

by

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Abstract

Ste12 is the key regulator in the yeast pheromone response pathway and works as an important model for understanding gene regulation by MAP kinase cascades. In this thesis I address how the binding strength of pheromone-response element (PRE) sequences, their orientation, and intervening nucleotide distance between two PREs govern the overall response to pheromone. I found that Ste12 binds as a monomer to a single PRE *in vitro*, and that two PREs upstream of a minimal core promoter cause a level of induction proportional to their relative affinity for Ste12 *in vitro*. Although consensus PREs are arranged in a variety of configurations in the promoters of pheromone responsive genes, I found there are severe constraints with respect to how they can be positioned in an artificial promoter to cause induction of gene expression. Two closely-spaced PREs can induce transcription in a directly-repeated or tail-to-tail orientation, while PREs separated by at least 40 nucleotides are capable of inducing transcription when oriented in a head-to-head or tail-to-tail configuration. By comparing the constraints defined by analysis of artifical promoters, I found that a single PRE can cause response to pheromone induction in combination with a properly oriented PRE-like sequence.

By studying Ste12 multimerization, I found that this process might involve dephosphorylation on Ste12 to regulate the expression of pheromone-regulated genes. I discovered that Cdc55, a regulatory subunit of protein phosphatase IIA, can affect pheromone response. In the *cdc55* null mutant I observed decreased expression level of a reporter gene and decreased mating efficiency. Cdc55 directly or indirectly alters the

phosphorylation status of Ste12, as I observed hyperphosphorylated Ste12 in the *cdc55* mutant compared to wild type. The effect of Cdc55 is independent of the pheromone response MAP kinase pathway, but was found to be controlled downstream of TOR. Analysis of artificial reporter genes and a candidate set of pheromone responsive promoters demonstrated that TOR-Cdc55 signaling regulates a distinct subset of pheromone-responsive genes. These results demonstrate a new regulatory circuit for the pheromone response controlled by the TOR signal pathway, which operates to control mating of yeast haploids in response to nutrients.

Preface

Experiment results in chapter 3 were basis of a first author published paper. Su TC, Tamarkina E, Sadowski I (2010) Organizational constraints on Ste12 cis-elements for a pheromone response in Saccharomyces cerevisiae. *FEBS J* 277(15):3235-48 (License Number: 2918910608116). Data in chapter 4 will be submitted for publication with the experiment results currently in progress.

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List of Abbreviations

A	Absorbance
aa	amino acid
ATP	adenosine triphosphate
β-Me	beta-mercaptoethanol
Bp	base pair
cdc	cell division cycle
CDK	cyclin dependent kinase
cDNA	complementary DNA
CTD	carboxyl-terminal domain
dATP	2'-deoxyadenosine triphosphate
dCTP	2'-deoxycytoosine triphosphate
dGTP	2'-deoxyguanosine triphosphate
dTTP	2'-deoxythymidine triphosphate
dNTTP	2'-deoxynucleoside triphosphate
DBD	DNA binding domain
DNA	deoxyribonucleic acid
E. coli	*Escherichia coli*
EDTA	ehylenediaminetetraacetic acid
EGTA	ethylene glycol tetraacetic acid
EMSA	electrophoretic mobility shift assay
ERK	extra-cellular regulated kinase
FRE	filamentous response element
GDP	guanosine diphosphate
GTP	guanosine triphosphate
h	hour
kb	kilobase
kDa	kilodalton
LTR	long terminal repeat
MAPK	mitogen activated protein kinase
MEK	MAPK/ERK kinase
MEKK	MEK kinase
min	minute
MOPS	3-(N-morpholino)propanesulfonic acid
MW	molecular weight
OD	optical density
PAGE	polyacrylamide gel electrophoresis
PAK	p-21 activated kinase
PCR	polymerase chain reaction
RCS	relative competition strength
RNA Pol II	Pol II RNA polymerase II
RNA	ribonucleic acid
RNAse A	ribonuclease A
RT	room temperature

s	second
S. aureus	*Staphylococcus aureus*
SC medium	synthetic complete medium
SDS	sodium dodecylsulfate
SSC buffer	saline-sodium citrate buffer
Ste	sterile
TAF	TBP-associated factor
TBP	TATA-binding protein
TCS	TEA/ATTS consensus sequence
UAS	upstream activating sequence

Nomenclature

Wild-type alleles in *Saccharomyces cerevisiae* are presented in italicized capital letters (e.g. *STE12*), while mutant recessive alleles are shown in lower case italics (e.g. *ste12*). Gene products are written with the first letter capitalized (e.g. Ste12).

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I would also like to acknowledge all the friendships that I have made during my time in Vancouver. Everyone has been fantastically supportive in one way or another, both in my research and personal lives. I would especially like to thank all members of the Sadowski lab, both past and present: Matthew Dahabieh, Wendy Bernhard, Kris Barreto, Sheetal Raithatha, Pedro Lourenco, Tom Malcolm, Alice Kuo and Jennifer Parent.

As an international student from Taiwan, it has been difficult for both my family and me while I have studied abroad here in Vancouver. I would like to dedicate this thesis to my parents, aunt Shirley, uncle Ian, brother Ting-Yi, and fiancée Yu-Ching for all their encouragement, support and sacrifice.

Chapter 1 Introduction

1.1 *Saccharomyces* as a model eukaryotic organism

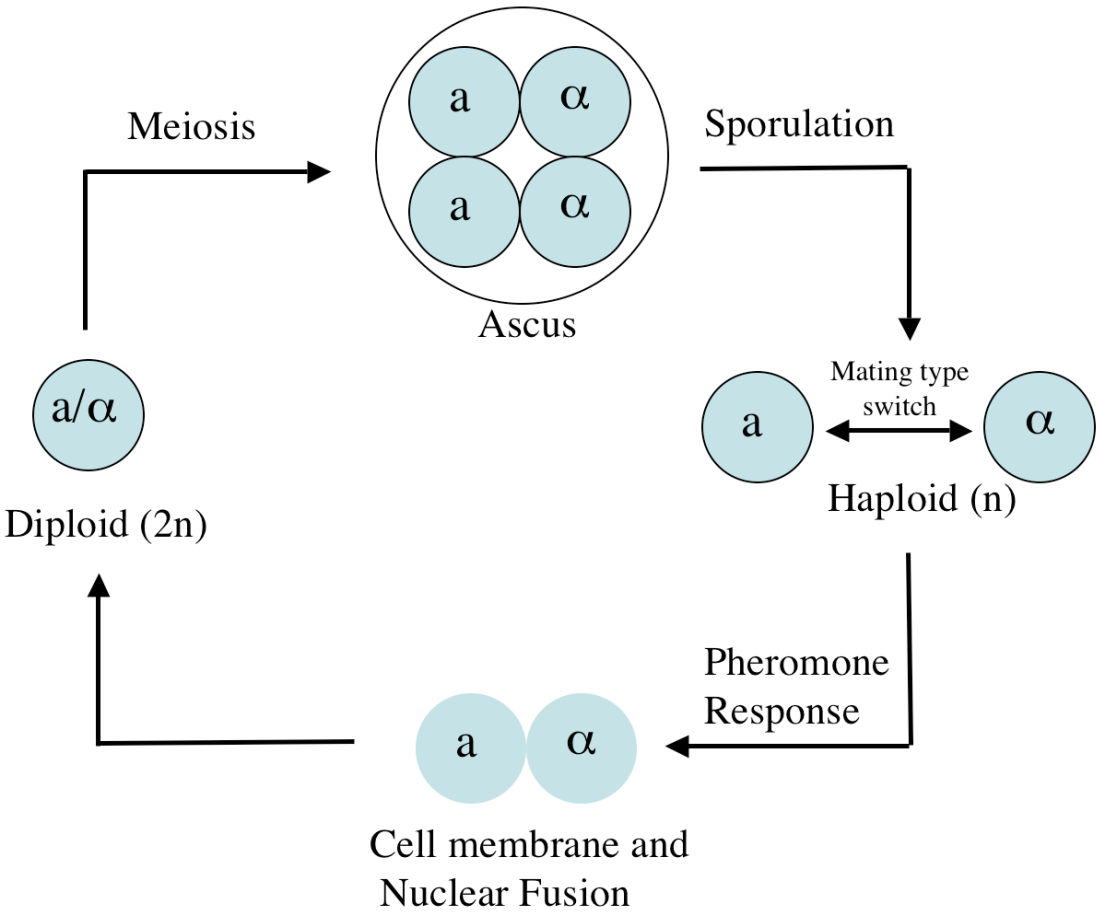
The budding yeast, *Saccharomyces cerevisiae*, is a eukaryotic unicellular microorganism of the fungal kingdom. For thousands of years, this organism has been used by humans for the preparation of food and beverages, such as bread, fermented flour dough for various foods, wine and beer. This is achieved by the natural capacity of yeast to convert carbohydrates into alcohol and carbon dioxide. Yeast also has represented an important model for understanding the molecular biology and genetics of eukaryotic organisms for many decades. The ease with which it can be manipulated genetically, the conservation of many protein functions with higher eukaryotes, and a large selection of genomic-scale strategies has enabled a detailed understanding of many novel protein functions conserved with multicellular eukaryotes such as flies and mammals.

1.2 The *Saccharomyces cerevisiae* life cycle

Saccharomyces cerevisiae can exist in either a diploid or haploid form, but in the wild, yeast are usually maintained in the preferred diploid form. Both haploids and diploids reproduce mitotically, but when diploid cells encounter an inadequate source of carbon and nitrogen, they undergo meiosis to produce four spores by sporulation. The spores are contained within an ascus, which protects them from harsh environments. Spores germinate into haploid offspring when the environmental and nutritional stress is relieved (Fig. 1.1).

Figure 1.1 The yeast life cycle.

Yeast cells exist in either diploid or haploid forms, but mostly as diploids in the wild. When exposed to nutrient deficiency such as limiting carbon and nitrogen sources, the diploid yeast cells undergo meiosis to produce four spores by sporulation. Spores germinate into haploid offspring when the environmental stress is relieved. Haploid yeast can be classified as a or α mating type defined by the presence of the *MATa* or *MATα* allele at the mating locus. Haploid yeast cells, defective for the *HO* endonuclease gene, can maintain their haploid status by mitosis. The diploid status can be restored when they encounter yeast of the opposite mating type. The process is referred to as pheromone response and mating, and occurs most efficiently in a nutrient rich environment.


Meiosis
a
α
a
α
Sporulation
Ascus
a/α
Diploid (2n)
Mating type
switch
a
α
Haploid (n)
Pheromone
Response
a
α
Cell membrane and
Nuclear Fusion

Haploid yeast exist as one of two mating types, designated a and α, and are produced by the presence of an a or α allele at the mating type locus (*MAT)*. Laboratory strains of haploid yeast that are defective for the *HO* endonuclease, can propagate indefinitely by mitosis in culture. But when haploid yeast encounter cells of the opposite mating type, under conditions of adequate nitrogen and carbon, they can fuse in a mating process to form a unicellular body to restore the diploid form. The process is regulated by secreted peptide signaling molecules, known as pheromones, and the corresponding haploid signal transduction response.

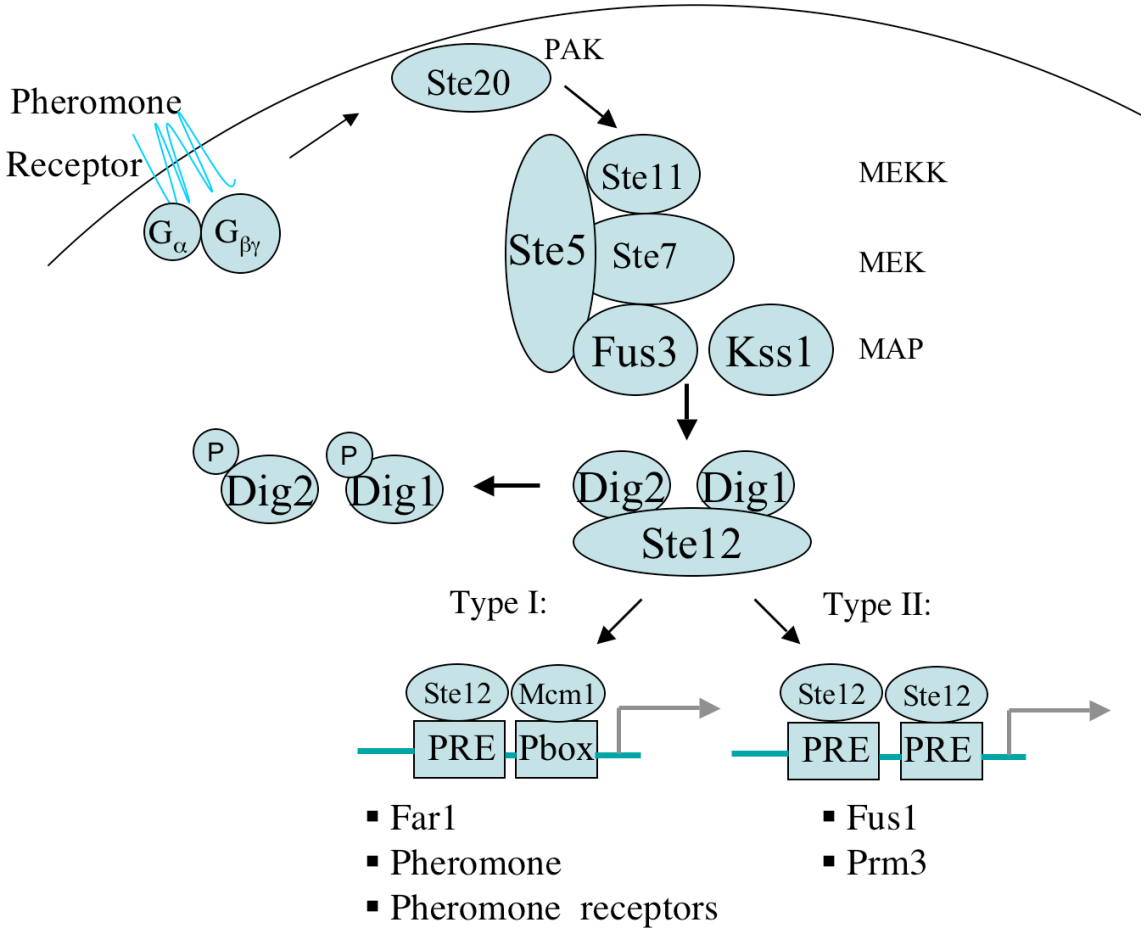
1.3 Pheromone response

Mating is initiated when haploid cells sense pheromone released by the opposite mating type. Both mating types secrete specific pheromones, known as a and α-factor, produced by *MATa* and *MATα* haploid yeast, respectively (99, 123, 169). The mating pheromones are recognized by pheromone receptors located on the cell membrane. Binding of pheromone to the receptor activates a downstream signal transduction pathway, known as the pheromone-response pathway, which is the prototypical example of a mitogen-activated protein kinase (MAPK) cascade (Fig. 1.2).

Several important downstream events occur during the pheromone response. First, cells become arrested in G_1 phase of the cell cycle, and then undergo morphological alterations to form a projection (shmoo) towards their mating partner. These events are controlled through the induction of a large number of genes that are activated by the central regulator Ste12. Upregulation of pheromone-responsive genes by Ste12 is

Figure 1.2 The yeast pheromone MAP kinase pathway.

The pheromone signal transduction pathway is initiated upon sensing the peptide pheromone from the opposite mating type yeast. Binding of pheromone to the receptor activates the pheromone receptor-coupled trimeric G protein complex. The activated $G_{\beta\gamma}$ subunit relays the signal to Ste20 and subsequently to the pheromone MAP kinase pathway, consisting of Ste11, Ste7 and two MAP kinases Fus3 and Kss1. Both MAP kinases can activate Ste12, presumably through phosphorylation. The inhibitory effects of two Ste12 inhibitors, Dig1 and Dig2 are also relieved through activation of the MAP kinases. Activated Ste12 can either by itself, or in combination with Mcm1 up-regulate its downstream target genes. Abbreviations: PAK, p-21 activated kinase; MEKK, MEK kinase; MEK, MAPK/ERK kinase; MAP, mitogen activated protein kinase.


Pheromone
Receptor
Gα
Gβγ
PAK
Ste20
Ste11
MEKK
Ste5
Ste7
MEK
Fus3
Kss1
MAP
P
P
Dig2
Dig1
Dig2
Dig1
Ste12
Type I:
Type II:
Ste12
Mcm1
PRE
Pbox
Ste12
Ste12
PRE
PRE
▪ Far1
▪ Pheromone
▪ Pheromone receptors
▪ Fus1
▪ Prm3

achieved by recognition of specific consensus DNA binding sequences, known as pheromone responsive elements (PREs), on the promoters of pheromone-responsive genes. Once two haploid cells make contact, they undergo cytoplasmic and nuclear fusion to restore the diploid state, and the pheromone response is completed.

1.3.1 The yeast mating types and regulation of mating-type specific genes

The mating type of yeast haploids is determined by the presence of the *MATα* or *a* allele at the mating type locus. In wild type haploid yeast, the mating type loci, *MATa* and *MATα* are inter-convertible, in an event initiated by the HO endonuclease. The HO endonuclease creates a nick at the mating type locus (*MAT*), which initiates a recombination event where the allele at the mating type locus is removed and replaced by one of the silent alleles at *HMR* or *HML*. A switch in mating type occurs when the allele at the *MAT* locus is replaced by an allele representing the opposite mating type (130, 179). The silenced *HMR* and *HML* loci, encode the *MATa* or *MATα* alleles, respectively (reviewed in 129). Transcriptional silencing of both the *HMR* and *HML* cassettes is normally maintained by the Sir2, Sir3 and Sir4 proteins, and inactivation of any one of these genes results in the expression of both silent cassettes (156). However, most laboratory strains bear a defective *ho* allele, which prevents mating type switching to allow stable propagation of *MATa* and *MATα* strains.

The *MATα* allele encodes two proteins, α1 and α2 (4). α1 is a transcriptional activator, which regulates the expression of *MATα*-specific genes, including those for production of pheromone and the pheromone receptor. On the other hand, α2 is a transcriptional repressor. It functions to repress all *MATa*-specific genes. The *MATa*

allele also encodes two proteins, a1 and a2. In haploid cells the *MAT* al protein does not seem to have a function, but functions as a repressor protein in combination with α2 in diploid cells. In contrast, *MAT* a2 protein does not have a defined function in haploids or diploids. Unlike *MATα*-specific genes whose expression is activated by α1, all *MATa*-specific genes are expressed constitutively in *MATa* haploid cells. After mating and formation of a diploid cell, the haploid-specific genes are repressed by an a1/α2 heterodimer which functions as a repressor of haploid-specific genes (72).

1.3.2 Yeast pheromones and their receptors

The yeast mating pheromone secreted from *MATα* cells is referred as α-factor, while a-factor is produced by *MATa* cells; these are short oligopeptides of 13 and 12 amino acids, respectively (11, 178). The α-factor peptide is encoded by two genes *MF*α1 and *MF*α2, where most expression is contributed by *MF*α1 (169). a-factor is also encoded by two genes *MFa1* and *MFa2*, and similar to α-factor, most a-factor is produced by expression from *MFa1* (123). α-factor is an unmodified peptide, is secreted into the medium by *MATα* cells, and can be synthesized artificially. In contrast a-factor is modified by methylation and farnesylation at the C-terminus, and these modifications make it difficult to synthesize a-factor artificially (23, 122).

The mating pheromones bind to membrane-associated receptor proteins on cells of the opposite mating type. Both pheromone receptors belong to the family of seven-transmembrane spanning proteins, and are coupled to a heterotrimeric G protein complex in the cytosol. *STE2* encodes the receptor for α-factor, while *STE3* encodes the a-factor receptor (18, 63). Binding of pheromone causes a conformational change of the

pheromone receptors, which leads to activation of the coupled G-protein complex, and subsequently initiates the pheromone signal transduction pathway. The intracellular C-terminal tail of the receptors is responsible for regulation of the receptor and contributes to interaction with the heterotrimeric G-protein complex. The tail of Ste2 becomes hyperphosphorylated on several Ser and Thr residues after pheromone binding. Pheromone stimulation causes internalization of the receptors by endocytosis, which is required for desensitization from pheromone to allow resumption of growth following G1 arrest (154). Ubiquitylation of the tail is required for endocytosis, and mutation of residue K337 to arginine inhibits ubiquitylation and prevents the subsequent endocyctosis (73, 158).

1.3.3 G proteins

The G proteins coupled to the pheromone receptors form a heterotrimeric $G_{\alpha\beta\gamma}$ protein complex. This consists of the subunits Gpa1(G_α) and Ste4/Ste18($G_{\beta\gamma}$) (34, 196, 124). The Gpa1 subunit is bound to GDP in the unstimulated state, and binding of pheromone to the receptor triggers an exchange of GDP for GTP. The GTP-bound Gpa1 has a decreased affinity for Ste4/Ste18, resulting in dissociation of Ste4/Ste18 from Gpa1 (74). The free Ste4/Ste18 ($G_{\beta\gamma}$) complex interacts with downstream effectors, such as Ste20, Ste5, Cdc42 and the Far1/Cdc24 complexes to transmit the pheromone signal (107, reviewed in 104). The Ste4/Ste18 complex activates Ste20 protein kinase activity and recruits the scaffold protein Ste5 to the cell membrane, which directs the signal to the pheromone MAP kinase pathway. Additionally, Ste4/Ste18 recruits the Far1/Cdc24

complex into the vicinity of Cdc42 to promote shmoo formation (described in more detail below).

1.3.4 Ste20

Ste20 is a protein kinase related to the p21-activated kinases (PAK) from mammalian cells, which are targets for signaling from small GTPase proteins such as Cdc42 (103, reviewed in 109). Ste20 was first identified as a suppressor of a mating defect of a dominant-negative Ste4 mutant, and it acts upstream of the protein kinase Ste11 and the scaffold Ste5. The region of Ste20 responsible for binding Ste4/Ste18 lies in the C-terminal domain ranging from residues 879 to 887. Mutations of this region abolish pheromone response due to a dramatic decrease in the association with Ste4/Ste18 (107). Once activated, Ste20 relays the signal to the pheromone MAP kinase pathway by phosphorylating its downstream effector Ste11 associated with Ste5 (39).

1.3.5 Ste5

The pheromone MAP kinase pathway is composed of three protein kinases, the MEK kinase Ste11 (MEKK), the MAP-ERK kinase (mitogen activated protein kinases, extracellular signal regulated protein kinases) Ste7 (MEK) and two mitogen-activated protein kinases (MAP), Fus3 and Kss1. Ste5 serves as a scaffold protein that works as a platform to bring these components together. This provides an advantage to increase the efficiency of signal transduction, and to maintain specificity along this pathway with respect to other MAPK pathways in the organism (41, 147). In response to pheromone, Ste5 is recruited to the cell membrane through interaction with the $G_{\beta\gamma}$ Ste4/Ste18, this

brings Ste5 and the associated protein kinases into the vicinity of Ste20, which allows phosphorylation and activation of Ste11. Recruitment to the cell membrane is critical for activating the MAP kinase pathway, and artificial targeting of Ste5 to the membrane can activate the pathway even without pheromone or Ste4/Ste18 (149). Ste5, and its associated kinases, cycles from the nucleus to the cytoplasm. In the presence of pheromone, nuclear Ste5 is rapidly exported to the cytoplasm and this is required for activation of the MAP kinase pathway (117). Overexpression of Ste5 is sufficient to activate Ste11 in the absence of pheromone (26), and temporarily blocking import of Ste5 to the nucleus also enhances pheromone response (117). These results suggest a regulatory mechanism involving control of the amount of Ste5 in the cytoplasm to prevent inappropriate activation of the MAP kinase pathway in the absence of pheromone.

1.3.6 Ste11

STE11 encodes a protein kinase related to the MEK kinases of mammalian MAPK signaling cascades, and *ste11* null mutants cause sterility (155, 168). Ste11 is activated by Ste20 during pheromone response through phosphorylation on three residues at Ser302, Ser306 and Ser307. Non-phosphorylatable mutations of these three sites abolish the function of Ste11. Phosphorylation-mimicking mutations on these sites to aspartic acid result in a dominant constitutively active Ste11 that is independent of Ste20 (39). The N-terminal domain of Ste11 causes an auto-inhibitory effect. It inhibits Ste11 activity by binding to the C-terminal catalytic domain, and deletion of this region produces the dominant *STE11-4* allele, which causes a similar phenotype as mutations of

the three Ste20 phosphorylation sites. Phosphorylation by Ste20 on these three sites is thought to relieve the auto-inhibitory effect of the Ste11 N-terminus (39). In addition to Ste20, Ste11 is also regulated by Ste50. Ste50 constitutively binds to Ste11 through the interaction of the sterile alpha motif (SAM) on both proteins. Ste50 acts as an adaptor to link the G protein associated Cdc42-Ste20 complex to Ste11 (199, 187, reviewed in 151)

1.3.7 Ste7

Ste7 is a protein kinase related to the MAP-ERK kinases of mammalian MAPK cascades, and is activated by Ste11. Like other MEKs, Ste7 is a dual-specificity kinase with the capability to phosphorylate substrates on both threonine and tyrosine residues, and typically this involves phosphorylation of MAP kinases on closely-spaced T and Y residues at the active site (47). The N-terminal region of Ste7 contains a docking site for binding of its MAP kinase substrates (8). Ste7 is activated during pheromone response through phosphorylation on Thr363 by Ste11. A Ste7 T363V substitution is defective for mating, and is unable to phosphorylate its downstream substrate MAPK Fus3 *in vitro* (135).

1.3.8 The MAP kinases, Fus3 and Kss1

Fus3 was first discovered in a screen for yeast mutants defective for cell fusion during mating. In contrast, Kss1 was identified from a multi-copy suppressor screen for clones that could force adaptation to pheromone. By sequence analysis, Fus3 shares 54% identity and 75% similarity with Kss1, and both are related to the MAP and ERK kinases of mammalian cells (33, 43). Ste7 activates these MAPK kinases by phosphorylation of

Fus3 at Thr180 and Tyr182 and Kss1 at Thr183 and Tyr185. Both phosphorylations on these proteins are required for activity *in vivo* (52, 114). These two protein kinases were initially thought to be functionally redundant for pheromone response, because individual null mutations of each MAP kinase does not cause sterility, but *fus3 kss1* double mutant strains are sterile (42). Subsequently, several distinct functions have been identified for each. For example, the activity of Fus3, but not Kss1 is responsible for repressing transcription of *CLN1* and *CLN2* (42). Additionally, Kss1 is required for filamentous growth of yeast, whereas Fus3 inhibits this response, an effect that relates to a function of Fus3 in phosphorylation of the transcription factor Tec1 in pheromone treated cells to prevent inappropriate induction of filamentous response genes in nitrogen starved cells (described in more detail below). Additionally, Kss1 can be activated by Ste7 in the absence of the scaffold Ste5, unlike Fus3 whose activity is dependent upon Ste5 (51, 57). *FUS3* expression is also upregulated in pheromone treated cells, but not *KSS1*. In contrast, expression of *FUS3* is inhibited in diploid yeast (43), whereas *KSS1* is expressed at equivalent levels in both haploid and diploid cells (7, 111). These observations are consistent with a role for Kss1 in regulation of filamentous growth. Also, in haploid cells, Fus3, but not Kss1 is required for pheromone-responsive cell cycle arrest in G1 (42).

It has been shown that both Fus3 and Kss1 can phosphorylate the key transcriptional activator Ste12 *in vitro*, along with two Ste12 inhibitory proteins Dig1 and Dig2. Unfortunately, the specific phosphorylation sites on these potential substrates required for pheromone response have not been identified (14, 44). Most other identified Fus3 substrates seem to be involved in down-regulation of pheromone response,

including the pheromone receptor Ste3 (48), the MEKK Ste11 (204), the scaffold protein Ste5 (93), the cytoskeleton protein Bni1 (119), and the GTPase-activating protein Sst2. Additionally, as mentioned above, Fus3 phosphorylates the transcriptional activator Tec1 in pheromone treated cells to prevent expression of filamentous response genes during mating (17, 31). Identification of specific substrates for Kss1 is not as well established as for Fus3.

1.3.9 Dig1 and Dig2, inhibitors of the transcriptional activator Ste12

Dig1 and Dig2 (also known as Rst1 and Rst2, regulators of Ste12) are two inhibitory proteins of Ste12. Dig1 and Dig2 were first identified as negative regulators of yeast filamentous growth, and in a two-hybrid screen (27). *DIG1* is constitutively expressed in both haploid and diploid cells and its expression is not subject to pheromone regulation. On the other hand, *DIG2* is restricted to haploid cells and its expression is induced in pheromone-stimulated cells (27). In a *dig1 dig2* strain, Ste12-regulated genes are expressed constitutively. The inhibitory effects are mediated by interaction with different regions of Ste12. Dig1 binds the central transactivation domain, while Dig2 binds the N-terminal DNA-binding domain of Ste12 (27, 141, 184). Both Dig1 and Dig2 become phosphorylated in response to pheromone. It is believed that phosphorylation relieves their inhibitory effect on Ste12 (27, 184), but the precise effect of phosphorylation has not been established for either protein.

1.3.10 Ste12

1.3.10.1 Ste12 general background

Ste12 is a key regulator of both pheromone response and filamentous growth. Ste12 was initially discovered in a collection of haploid yeast mutants with mating defects (67). Ste12 is a DNA binding protein and transcriptional activator with a length of 688 amino acids (38, 46). Deletion analysis has identified three functional regions on Ste12 protein, including an N-terminal DNA-binding domain, a central trans-activation domain and a C-terminal regulatory domain (87, 173, 200) (Fig. 1.3). The DNA binding domain, spanning residues 1-215, shows some similarity to a homeodomain (200). Residues 214-473 comprise the activation domain, and a Ste12 truncation with a deletion of this region completely abolishes response to pheromone. The activation domain contains at least 4 sites of phosphorylation (77). The C-terminal region, flanking residue 473, can promote Ste12 multimerization *in vitro* (141) and the region spanning residues 471-688 was designated as "regulatory" domain, because it seems to inhibit transactivation by Ste12, and its deletion causes an elevated pheromone response (173).

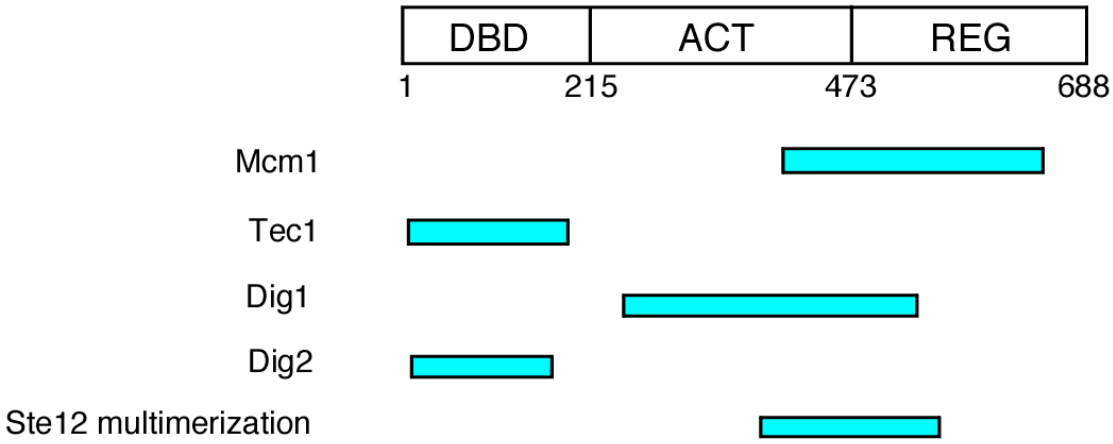
1.3.10.2 Regulation of subsets of genes by Ste12

In addition to interaction with the inhibitors Dig1 and Dig2, Ste12 also regulates specific subsets of target genes in cooperation with other transcriptional activators, including Mcm1, Tec1, and α1. The Ste12/Mcm1 complex activates gene expression for production of the pheromone receptors and pheromones (49, 165). Ste12 interacts with Mcm1 through the C-terminal regulatory domain (46). In contrast, Ste12/Tec1 complexes regulate filamentous growth of yeast in response to nutrient limitation. Ste12,

through its DNA-binding domain, interacts with Tec1 enabling cooperative interaction on promoters of genes that bear binding sites for both of these factors (32). Ste12 also interacts with Mat α1 to upregulate the expression of α–specific genes (50)

Figure 1.3 Ste12 protein structure.

Ste12 is a 688 amino acid protein with three functional domains, the DNA binding (DBD), transcriptional activation (ACT), and regulation domain (REG). Ste12 interacts with various regulatory proteins and additional DNA binding proteins, including Mcm1, Tec1, Dig1, and Dig2. Regions responsible for interactions with these factors are shown, as well as a region involved in Ste12 multimerization.


DBD
ACT
REG
1
215
473
688
Mcm1
Tec1
Dig1
Dig2
Ste12 multimerization

1.3.10.3 Ste12 *cis*-elements, PRE and FRE

The Ste12 typical consensus sequence, known as the pheromone response element (PRE), was first identified as the sequence ATGAAACA or TGAAACA (38, 64, 94, Fig. 1.4). Subsequently, many atypical PRE-like elements were also shown to be functional for conferring pheromone response. For example, there are four PREs in the *FUS1* promoter with different sequences, only one of which represents a consensus PRE. The other three PREs differ by a single nucleotide at various positions within the consensus PRE sequence. A *LacZ* reporter containing a single copy of a PRE is not sufficient to produce a response to pheromone induction, and at least two copies were shown to be required, as determined within the context of the *FUS1* promoter (64).

In addition to regulating response to pheromone, Ste12 is also involved in controlling differentiation of yeast into filamentous forms in cooperation with Tec1 (53). When subjected to conditions of nitrogen or carbon starvation, certain strains of yeast undergo differentiation into elongated filamentous forms that protrude outward from the colony as a mechanism to enable foraging for nutrients. The combination of Ste12 and Tec1 binding to filamentous response genes involves recognition of adjacent binding sites for both Ste12 and Tec1, known as the filament response element (FRE). FREs were characterized in several different promoters, including that of the *TY1* transposon and the *TEC1* promoter itself, which consist of one copy each of a PRE and TCS, the Tec1 consensus binding sequence (9).

Figure 1.4 Typical PRE sequence and variants.

The nucleotide consensus sequence of the pheromone response element (PRE) (line 1). Three PRE variants, shown to contribute to pheromone response of the *FUS1* promoter are listed in lines 2-4. Each PRE variant contains one nucleotide substitution at various positions, as compared to the consensus, and the differences are indicated in lower case.

Typical PRE : 1. ATGAAACA

variants: 2. ATGAAACg

3. tTGAAACA

4. ATGAgACA

1 2 3 4 5 6 7 8

1.3.10.4 Regulation of a subset of pheromone-responsive genes by Ste12 and Mcm1

In general, pheromone responsive genes can be placed into two classes. The first class represents those under control of both Ste12 and Mcm1, whose promoter regions contain one copy each of a PRE and P box, which is the consensus binding sequence for Mcm1 (165). Ste12 regulates these genes by interacting cooperatively with Mcm1. Genes encoding the pheromone receptors, *STE2* and *STE3*, and those encoding the mating pheromones, *MF*α1, *MF*α2, *MF*a1, and *MF*a2, are regulated in this manner. This class of genes has high constitutive levels of expression in the absence of pheromone, and is also pheromone-inducible (49, 165).

For the second class of pheromone inducible genes, whose promoter regions only appear to contain PRE(s), but lack obvious P box elements, expression is believed to be solely dependent upon Ste12 to confer pheromone induction. For genes of this type, expression is unaffected by pheromone in a *ste12* null mutant (153). This class of genes is typically expressed at very low basal levels, and is strongly up-regulated at the transcriptional level during pheromone response. The *FUS1* gene, for example has four PREs within its promoter that mediate pheromone response. Treatment with pheromone causes induction of *FUS1* up to 35 fold (64).

1.3.10.5 Regulation of filamentous response genes by Ste12 and Tec1

Under conditions of nutrient limitation, some yeast strains undergo differentiation to filamentous forms to enable foraging for nutrients. Ste12 and Tec1 are important regulators of the filamentous growth response. These proteins bind cooperatively to an

element known as the FRE (116), containing closely spaced binding sites for Ste12 and Tec1 to activate filamentous response genes, including those required for cell elongation and flocculation. However, although most attention on filamentous response has been focused on several genes bearing a typical FRE, including the *TY1* LTR promoter, and *TEC1* itself (32), most other genes induced during filamentous growth do not contain a similar FRE regulatory element. Instead, many filamentous response genes like *FLO11*, *CWP1*, and *KSS1*, for example, don't contain a PRE, but rather only possess Tec1 binding sites (TCS) in their promoter regions (32). Consistent with this observation, a reporter gene with only TCS sites was shown to confer responsiveness to filamentous growth conditions (91). The Tec1 protein is specifically degraded in pheromone-treated cells as a mechanism to maintain signal specificity during pheromone response. The MAPK Fus3 phosphorylates Tec1 in pheromone treated cells, which leads to ubiquitin-dependent degradation (6, 31). Destruction of Tec1 during pheromone response prevents inappropriate activation of filamentous response genes by pheromone.

Given that Ste12 is involved in both pheromone response and filamentous growth, a major question is how specificity for pheromone and nutrient limitation are distinguished to ensure the appropriate response. One mechanism may involve specificity of the pheromone response MAPKs. For example, Fus3 was shown to inhibit Kss1 activation during pheromone response (160), and as mentioned above, phosphorylates Tec1 to cause its degradation in pheromone treated cells (6, 31). Binding of Ste11, Ste7 and Fus3 to the scaffold protein Ste5 is required for pheromone responsive transcription (26). However, Ste5 is not required for filamentous growth, and Kss1 can be activated by Ste7 in the absence of Ste5 (14).

In addition to regulation by the MAP kinase pathway, it was also shown that Srb10/Cdk8 of the mediator complex phosphorylates Ste12 and targets it for degradation in a rich nutritional environment (the mediator is discussed in more detail below). However, under nitrogen starvation conditions, Srb10/Cdk8-mediated Ste12 degradation is relieved because Srb10/Cdk8 becomes inactivated. This regulation on Ste12 for filamentous growth ensures the activation of its downstream genes in combination with Tec1 (136). Signal specificity conferred by the MAPK pathway and Srb10/Cdk8 for filamentous growth has not been thoroughly elucidated.

1.3.10.6 Ste12 post-translational modifications

It has been shown that Ste12 undergoes multiple post-translational modifications, including phosphorylation, sumoylation, and ubiquitylation (31, 78, 191). Ste12 is heavily phosphorylated prior to and after pheromone treatment (78). However, a function for most phosphorylations on Ste12, and those on the regulatory subunits Dig1 and Dig2 have still not been identified. However, two phosphorylation sites on Ste12 have been identified that are involved in the regulation of filamentous growth. The mediator-associated protein kinase Srb10/Cdk8 phosphorylates Ste12 on residues S261 and S451 (136). These phosphorylations cause instability of Ste12, resulting in degradation. Under conditions of nutrient limitation that induce filamentous growth, Srb10/Cdk8 is inactivated, causing loss of these phosphorylations on Ste12 to allow its accumulation (136). Phosphorylation of S261 and 451 appear to promote degradation by ubiquitylation, but specific lysine residues targeted by ubiquitin ligases have not yet been confirmed (Raithatha and Sadowski, unpublished). Ste12 is known to be phosphorylated

on several additional residues *in vivo*, including S226, S400, and T525 as identified by metabolic labeling and mass spectrometry analysis of phosphopeptides (60, 78), although function(s) for these have yet to be identified. In addition to phosphorylation, Ste12 was reported to undergo sumoylation, and this seems to increase Ste12 activity (191).

1.3.11 Far1 and cell cycle arrest

Pheromone-induced cell cycle arrest in G1 phase is an important event for mating response, because it ensures that only haploid yeast undergo mating to prevent aneuploidy. Far1 is a key regulator of G1 arrest, and acts as a G1 Cdk-cyclin inhibitor to inhibit activity of the Cdc28-Cln complex (20, 146). *FAR1* is expressed in haploid cells but not diploids. Regulation of *FAR1* is under the control of Ste12 and Mcm1, and there are 4 PREs and 2 P boxes in the *FAR1* promoter. *FAR1* is constitutively expressed in untreated cells and is up-regulated 4 to 5-fold in the presence of pheromone (20, 96). In addition, Far1 protein is phosphorylated by Fus3 during pheromone response, which promotes association with the Cdc28-Cln2 complex (44, 145). In addition to its role in cell cycle arrest, Far1 also functions in the establishment of cell polarization during pheromone response. In unstimulated cells, Far1 sequesters Cdc24, the guanine-nucleotide exchange factor (GEF) for the GTPase Cdc42, in the nucleus. During pheromone response, the Far1-Cdc24 complex is exported from the nucleus by Msn5. Far1 works as an adaptor to facilitate interaction between Cdc24 and the activated Gβγ complex. This interaction promotes cell polarization of the actin cytoskeleton to produce a protrusion, or shmoo, towards the mating partner (137, 167). This process is regulated by Cdc42, which is a Rho-type small GTPase with a molecular mass of 21 kD (83).

Cdc42 is active when it is GTP-bound (174), and activation is induced by the Cdc24/Far1 complex during pheromone response (137). Cdc42 is prenylated in its C-terminal domain, a modification that targets the protein to the inner cell membrane (205). When recruited by the Cdc24/Far1complex, Cdc42 can establish shmoo formation through regulation of the downstream effectors Bem1, Bni1, Gic1 and Gic2 which control cytoskeletal organization (15, 103, 148).

1.3.12 Adaptation to pheromone

Following pheromone stimulation, cells need to recover from the effects of pheromone, including G1 arrest, to enable reentry into the cell cycle. High doses of pheromone, or prolonged exposure, can result in the death of yeast (98). Recovery from pheromone is regulated at multiple levels. The G protein subunit Gpa1 is inactivated by Sst2, which stimulates GTP hydrolysis of the activated Gα subunit (2, 35). In the MAP kinase pathway, a phosphatase Msg5 inactivates Fus3 by dephosphorylation of the pT and pY residues at the active site (36). Additionally, pheromone response causes the production and secretion of Bar1, a protease that degrades extracellular pheromone (132). A combination of these mechanisms, which form part of the pheromone response, ensures a transient burst of Ste12-dependent transcription, which is promptly shut off to enable recovery from G1 arrest.

1.4 Transcription regulation in eukaryotes

1.4.1 RNA polymerase II

In eukaryotes, transcription is mediated by three forms of RNA polymerase, designated RNA polymerase I, II, and III (RNA Pol I, II, and III). RNA Pol I is responsible for the transcription of ribosomal RNAs (rRNA), including 28S, 18S and 5.8S (reviewed in 61), RNA Pol II transcribes protein-encoding mRNA and small nuclear RNAs (snRNA), while RNA Pol III mediates the expression of 5S rRNA and the transfer RNAs (tRNA) (193, 194). In yeast, the three RNA polymerases are large complexes composed of multiple subunits. The core of RNA polymerase II contains 12 subunits, of which Rpb1, Rpb2 and Rpb3 constitute the major catalytic subunits which are functionally equivalent to the prokaryotic β, β' and α subunits, respectively, of *E. coli*. RNA Polymerase (198).

The 12 subunit RNA Polymerase II core complex is unable to initiate transcription from specific sites on templates *in vitro*. Rather, purified RNA Pol II core initiates transcription randomly on DNA *in vitro*, and specific initiation from promoters requires a set of accessory factors known as the TFIIs (reviewed in 65).

1.4.2 The TFIIs for RNA Pol II

RNA Pol II is recruited to promoters along with a set of general transcription factors (GTFs) that include TFIID, TFIIB, TFIIA, TFIIE, TFIIF, and TFIIH (reviewed in 65). TFIID is a complex composed of TBP and the TBP-associated factors (TAF_{II}s). TBP recognizes the TATA box of promoters, while the TAF_{II}s interact with additional core promoter elements (21, reviewed in 65). TFIIA contains two protein subunits, and

stabilizes the binding between TFIID and DNA (152). TFIIB acts as a bridge for the interaction between TFIID and RNA Pol II/ TFIIF, and stabilizes association of RNA Pol II with TFIID (118). The role of TFIIF is to destabilize non-specific DNA interactions, and direct RNA Pol II to the preinitiation complex. The two subunits of TFIIE stimulate the kinase and ATPase activities of TFIIH, which is necessary for initiation of transcription (120, 139, 140). TFIIH has multiple roles in transcriptional initiation, including an ATPase/helicase activity that is required for promoter melting, and phosphorylation of the C-terminal domain (CTD) of the largest subunit of RNA Pol II (113, 166, 188).

1.4.3 The RNA Pol II C-terminal domain, CTD

The CTD of RNA Pol II plays an important role in governing association of additional complexes required for transcription and RNA processing. The CTD is comprised of a repeated 7 amino acid sequence, Tyr-Ser-Pro-Thr-Ser-Pro-Ser, with 26 repeats in yeast and 52 in humans (29). The phosphorylation status of the CTD determines which complexes are associated with RNA Pol II. In general, RNA Pol II assembled at the preinitiation complex is unphosphorylated. Serine 5 of the CTD becomes phosphorylated by Kin28 of TFIIH upon initiation (164). Ser2 becomes phosphorylated by CTDK-I during elongation (84, 92, 105, 164). Different combinations of phosphorylations on the CTD are recognized by different complexes involved in RNA processing, transcriptional elongation, chromatin reorganization, and transcriptional termination. For example, Ser 5 phosphorylated CTD is required for recruitment of the RNA capping enzymes and cleavage-polyadenylation factors (25, 108, 121).

1.4.4 The mediator

Mediator is a large multiprotein complex comprised of at least 35 proteins in yeast (128), which is required for transcriptional activation by gene-specific regulatory proteins. Mediator binds the non-phosphorylated CTD, and stabilizes association of the general transcription factors (GTFs) with the core promoter region (86, 128). The mediator is represented by several subcomplexes termed the head, middle and tail modules. Subunits of the head subcomplex are the most highly conserved amongst eukaryotes, and function to stabilize interaction of TFIID and the other GTFs at the core promoter (19). The middle region interacts with the unphosphorylated CTD (128), while the tail subunits are involved in recruitment by transcriptional activator proteins (202). An additional subcomplex comprised of the proteins Srb10/ Cdk8, Srb11/ Cyclin C, Srb8, and Srb9 represents the kinase submodule, which is involved in the regulation of subsets of specific genes, and in most cases plays a repressive role (5, 28, 95). However, Srb10/Srb11 can cause either activation or repression/ inhibition of transcription through the phosphorylation of gene-specific transcriptional activators. For example, phosphorylation of Gal4 on S699 by Srb10/Srb11 is required for the full induction of the *GAL* genes (75). In contrast, as mentioned above, Srb10/Srb11 also responds to nutrient availability, and this plays a role in regulating filamentous growth by phosphorylating the key regulator Ste12 (136).

1.4.5 Co-activator complexes

In addition to the general transcription factors required by RNA Polymerase II, eukaryotic transcription also requires co-activator complexes necessary to manage the organization of genomic DNA into chromatin. The nucleosome is the most fundamental unit of eukaryotic chromatin (142), which is comprised of a histone octamer with 147 nucleotides of DNA wrapped around the protein complex surface (3). The presence of histones on DNA imposes a hindrance as an obstacle for initiation by the transcription machinery (66). Histone modifications at their N-terminal tails, such as acetylation, makes DNA more accessible for other regulator proteins by neutralizing the positive charge and promoting interaction of elongation and chromatin modifying complexes. Histone acetylation is mediated by the action of histone acetyltransferases (HATs) and is opposed by histone deacetylaes (HDACs). These enzymes are recruited to DNA by gene-specific transcriptional regulatory proteins, and in general transcriptional activators recruit HATs (reviewed in 16), whereas transcriptional repressors recruit HDACs (69, 85, reviewed in 97). One example of a HAT is the SAGA complex, of which the Gcn5 subunit represents the histone acetylation catalytic subunit, which acetylates histones mostly on H3 (59, 159). The effect of histone modifications on the pheromone response genes has not yet been established. But considering what is known for other genes, there must be alterations in histone modifications during pheromone induction.

In addition to complexes that produce post-translational modifications on histones, transcriptional activator proteins also recruit ATP-dependent machines that alter the placement of nucleosomes on DNA. One example is the SWI/SNF (Swi tching/ Sucrose Non Fermenting) complex (134, 180), which consists of 11 subunits with a total molecular mass of 200 MD (171). In general, this class of chromatin remodeling complexes has DNA-dependent ATPase activity, and functions to slide nucleosomes along DNA, cause dissociation of histones, and replace histones with other histone variants (102, 125). Interestingly, the SWI/SNF proteins were identified genetically for their requirement for expression of the *HO* gene in mating type switching (SWI) and *SUC2* for sucrose utilization (SNF), but none of the components in the SWI/SNF complex were identified in the sterility (*ste*) screen, suggesting an independence of chromatin remodeling in Ste12-depedent gene expression, or that activation by Ste12 must involve different, or more general, chromatin reorganizing complexes.

1.4.6 Yeast promoter structure

Yeast RNA Pol II promoters generally could have two parts, represented by the core promoter and upstream regulatory elements. For TATA box-containing genes, their core promoters consist of elements that bind the general transcription factor components, and include the TATA box and initiator (Inr) (reviewed in 181). The TATA box functions as a binding site for TBP (of TFIID), while the TAFIIs, and TFIIB/ TFIIA interact with the Inr element, and other sites within the core promoter (170). The Inr is

located close to the transcription start site and functions to define the site of transcription initiation (reviewed in 182).

The upstream regulatory elements can generally be classified as upstream activating sequences (UASs), or upstream repressing sequences (URSs), which are functionally analogous to enhancers and silencers from mammalian cells, respectively (reviewed in 62, 182). UASs represent binding sites for transcriptional activators, while URS elements bind transcriptional repressor proteins. Unlike mammalian cells, where regulatory elements can influence gene expression over long distances on DNA, most UASs and URSs in yeast genes typically reside within 1-2 kb upstream of the transcriptional start site (reviewed in 182).

1.4.7 Transcriptional activator proteins

Transcriptional activators are sequence-specific DNA binding proteins that bind elements within the UAS and cause recruitment of the GTFs and co-activator complexes to promoters. Most activators are comprised of at least two functional regions, a DNA-binding domain and transcriptional activation domain. The DNA-binding domains provide specificity toward specific genes, while the activation domain(s) function to recruit the general transcriptional factors through specific protein-protein interactions. A number of different structural motifs can confer sequence-specific DNA-binding activity, including zinc fingers (144), homeodomains (88), and bZIP/bHLH motifs (45, 115), and others. A structure has not yet been produced for the Ste12 DNA binding domain, but based on sequence comparisons it seems to be most closely related to a homeodomain,

consisting of three α-helices where the third helix serves as the DNA recognition helix (200).

Unlike DNA binding domains, transcriptional activation domains cannot be classified by structural similarity, rather they are often comprised of acidic segments (180) or glutamine (186)- or proline-rich regions (180). Transcriptional activation domains from various proteins have been shown to recruit the GTF and co-activator complexes by forming direct interactions with TBP (180), the TAFs (58), TFIIA (89), TFIIB (110), mediator, and proteins within the SWI/SNF (131) and SAGA complexes (40). The activation domain in Ste12 resides in the central portion of the protein (residues 215-500)(87), but specific interactions of this segment with GTF components have not yet been identified. Interestingly though, a null mutation of the tail mediator subunit *gal11* causes a sterile phenotype, and *GAL11* is also necessary for induction of *FUS1* in pheromone-treated cells (37, 138). The regulatory protein Dig1 binds to a region on Ste12 overlapping the activation domain (141), and it is presumed that this must prevent activation by blocking interaction with the GTFs and co-activator proteins.

1.5 The target of rapamycin (TOR) signaling pathway

The TOR (Target of Rapamycin) signaling pathway plays a conserved function in regulating cellular growth control in yeast, *Drosophila*, and mammals. The TOR pathway regulates transcription, translation, protein degradation, and ribosome biogenesis in response to nutrient signals (reviewed in 79). In yeast, there are two TOR proteins Tor1 and Tor2 (70), which share 67% sequence identity. *TOR1* is not essential, but disruption of *TOR2* causes lethality (71). These genes were identified as dominant alleles

that cause resistance to the fungal immunosuppressive compound rapamycin. Mutations of residue Ser1972 of Tor1 or Ser1975 in Tor2 produce resistance to the growth inhibiting effect of rapamycin in yeast (175). The TOR proteins are organized into two different complexes in yeast. Both Tor1 and Tor2 can be contained in the TOR complex 1 (TORC1), which is involved in nutrient sensing and regulation of transcription and translation, while TOR complex 2 (TORC2) uniquely contains Tor2, and regulates actin polarization during cell cycle progression (162, 163). Interestingly the TORC2 complex is insensitive to the effects of rapamycin (112). Mammalian cells have only one *TOR* gene, *mTOR* (24), which primarily regulates translation through phosphorylation of the translational regulators S6K and 4EBP1. Yeast TORC1 also regulates translation and ribosome biogenesis through phosphorylation of the S6K homolog Sch9, and Eap1, which play similar roles as mammalian S6K and 4EBP1, respectively (30).

Yeast TORC1 has an important role in regulating transcription in response to nutrient availability. This effect seems to involve regulation of downstream protein phosphatases, including Sit4 (10), which impose their regulatory effect on transcription by controlling the sub-cellular localization of transcriptional activators. For example, the activator Gln3 is retained in the cytoplasm with the inhibitor protein Ure2 under ideal growth conditions where Sit4 is inhibited by active TORC1, but is dephosphorylated by Sit4 when TOR is inhibited by nutrient limitation or rapamycin, allowing translocation of Gln3 to the nucleus (10). Activators like Msn2 and Msn4 are also under similar control by TORC1 (10). In yeast, TORC1 activity is activated by high quality sources of nitrogen and carbon, conditions where Msn2 and Msn4 are retained in the cytoplasm, although the mechanisms for regulation of these factors by TORC1 have not been

identified (100).

Another major phosphatase that is regulated by TORC1 signaling in yeast is protein phosphatase 2A (PP2A), which is comprised of the catalytic subunits Pph21 or Pph22, the regulatory subunits Cdc55 and Rts1, along with the structural subunit Tpd3 (68, 81, 172, 189, 201). Cdc55 is 526 amino acids in length with a molecular weight of about 60 kDa. It was first identified in a screen for cold-sensitive mutants. Diploid *cdc55* homozygous null mutant cells have an abnormal morphology and a slower growth rate at 14°C (68). The protein is localized mostly to the nucleus, bud neck and growing bud in dividing cells (54). Cdc55 and Rts1 are not functionally redundant, and provide substrate specificity to the catalytic subunits (201, reviewed in 195).

Phenotypically, the function of Cdc55 involves regulation of mitosis, in particular at the spindle/ kinetochore check point (190) and mitotic exit (192). Cdc55-containing PP2A dephosphorylates the Cdc14 inhibitor Net1 in metaphase. Phosphorylation of Net1 relieves the inhibitory effect of Cdc14. Downregulation of Cdc55 contributes to the initiation of mitotic exit (143). Cdc55 was also shown to have an effect on specific genes by regulating the DNA binding of transcriptional activators. For example, in a *cdc55* null mutant the GATA factors Gln3 and Gat1 are inhibited from binding the *DAL5* promoter in rapamycin-treated yeast (55).

1.6 Research goals and hypothesis

Inspection of the promoters of genes induced by Ste12 during pheromone response, reveals that relatively little is known about the role of Ste12 relating to organization of PREs within promoters, and PRE variants with respect to the level of

pheromone induction. In studies of the *FUS1* promoter, a dogma was established that at least two PREs are necessary, and sufficient, for pheromone induction (64) and subsequently the *FUS1* promoter was widely used for studies of pheromone response. The requirement of two PREs for induction suggests a possible role of Ste12 multimerization for transcriptional activation. However, the stoichiometry for binding of Ste12 protein to a single PRE is still unknown. Comparison of Ste12 binding on the yeast genome by CHIP-chip and CHIP-seq to expression profiles in pheromone-treated yeast cells (157, 203) does not reveal a correlation between the number of PREs and gene expression levels. For example, genes like *FIG1* and *PRM2* contain two and one PRE, respectively in their promoter, and are among the strongest induced genes in pheromone treated cells. On the contrary, genes like *AGA1* and *STE12*, both containing at least three PREs, show much less expression than *FIG1* and *PRM2* (157. 203). Furthermore, genes like *PHD1* with at least four PREs in its promoter is not responsive to pheromone at all (150). Based on these results, I propose that Ste12 may bind a single PRE as a monomer, but that Ste12 multimerization for binding to at least two PREs must be necessary to activate gene expression. Second, because there are genes with multiple PREs that are not pheromone inducible, there may be constraints on how PREs must be organized to dictate the overall pheromone response. Finally, because many pheromone-inducible genes contain only a single consensus PRE, such as *HYM1* and *PRM2*, which contradicts the established dogma, I hypothesize that such promoters should have additional functional *cis*-elements to enable pheromone response.

Chapter 2 Materials and methods

2.1 Oligonucleotides, plasmids and yeast strains

Sequences of oligonucleotides for construction of minimal promoter reporters are detailed in Table 2.1 and yeast strains used are in Table 2.2. Oligonucleotides for construction of reporter genes were annealed and cloned into the *Xho*I/*Xba*I sites of pIS341, which is a *lys2* disintegrator vector (161), bearing the *GAL1* core promoter region upstream of *LacZ* and the *ADH1* terminator. The sequence of the *GAL1* core promoter promoter region is listed as follows with the TATA box underlined: GGGTAATTAATCAGCGAAGCGATGATTTTTGATCTATTAACAGATATATAAA TGGAAAAGCTGCATAACCACTTTAACTAATACTTTCAACATTTTCAGTTTGTA TTACTTCTTATTCAAATGTCATAAAAGTATCAACAAAAAATTGTTAATATACC TCTATACTTTAACGTCAAGGAGAAAAAACTATA. All experiments were performed in the W303-1A strain background (*MATa ade2 leu2 trp1 ura3 can1*). Reporter gene plasmids were linearized by digestion with *Nru*I prior to transformation into yeast using the LiAc technique (56). URA^+ transformants were allowed to grow nonselectively on yeast extract peptone dextrose (YPD) for 3 days to allow rearrangement of the disintegrator, prior to streaking for single colonies on 5-fluoroorotic acid (5-FOA). Strains bearing reporter gene integrants at the *lys2* disruption were identified by replica plating, and single copy integration was verified by analysis of chromosomal DNA using PCR (161). Pheromone responsiveness of strains bearing the reporter genes was assayed in cultures grown in yeast extract peptone dextrose to OD_{600} of 0.6. Pheromone was added at a concentration of 2 μg/mL. The cells were collected

and β-galactosidase activity was assayed as described previously (1). Briefly, for each yeast culture, the OD_{600} was measured and 1ml of yeast cells was collected by centrifuge. Collected cells were re-suspended in 14 μl 0.1% SDS, 21 μl chloroform, and 800 μl Z buffer (60 mM $Na_2HPO_4 \cdot 7H_2O$, 40 mM $NaH_2PO_4 \cdot H_2O$, 10 mM KCl, 1mM MgSO4$\cdot 7H_2O$, 38 mM β-mercaptoethanol, pH 7). Cells were mixed vigorously for 25 s and incubated at 28 °C for 5 min before the addition of 160 μl ONPG (*o*-nitrophenyl-β-D-galactoside, 4 mg/ml in Z buffer) to start the reaction. Reactions were stopped after 30 minutes by adding 400 μl 1M Na_2CO_3. After centrifuging at 13800 g for 1 min, the supernatant from each reaction was collected and the OD_{420} was measured. β-galactosidase activity was calculated by the OD_{420} divided by the OD_{600} and multiplied by 1000. Error bars represent the standard deviation of results from three independent experiments.

Table 2.1 Annealed oligonucleotides used for construction of reporter gene plasmids and yeast strains.			
Oligos[a]	Plasmid[b]	Strain[c]	Sequence (5' to 3')[d]
TS018/019	pTS18	yTS18	TCGAGTCTGTAAT**ATGAAACG**AATAACGTGCGGCCGCT CTAG
TS020/021	pTS20	yTS20	TCGAGTCTGTAAT**ATGAAACA**AATAACGTGCGGCCGCT CTAG
TS022/023	pTS22	yTS22	TCGAGTCTGTAAT**TTGAAACA**AATAACGTGCGGCCGCT CTAG
TS026/027	pTS26	yTS26	TCGAGATG**ATGAAACA**AAC**ATGAAACA**TCTGCGGCCG CTCTAG
TS028/029	pTS28	yTS28	TCGAGATG**ATGAAACG**AAC**ATGAAACG**TCTGCGGCCG CTCTAG
TS030/031	pTS30	yTS30	TCGAGATG**TTGAAACA**AAC**TTGAAACA**TCTGCGGCCGC TCTAG
TS032/033	pYS32	yTS32	TCGAGATG**ATGAGACA**AAC**ATGAGACA**TCTGCGGCCG CTCTAG
TS084/085	pTS84	yTS84	TCGAGCGGAGAGCT**CGTTTCAA**A**ATGAAACA**AACGCC GTCGCGGCCGCTCTAG
TS112/113	pTS112	yTS112	TCGAGAT**TTGAAACA**CAGCATTTCTTTTCGGAGAGCT**C GTTTCAA**A**ATGAAACA**AAGCGGCCGCTCTAG
TS114/115	pTS114	yTS114	TCGAGAT**ATGTCCAA**CAGCATTTCTTTTCGGAGAGCT**C GTTTCAA**A**ATGAAACA**AAGCGGCCGCTCTAG
TS116/117	pTS116	yTS116	TCGAGAT**TTGAAACA**CAGCATTTCTTTTCGGAGAGCT**A TGTCCAA**A**ATGAAACA**AAGCGGCCGCTCTAG
TS118/119	pTS118	yTS118	TCGAGAT**TTGAAACA**CAGCATTTCTTTTCGGAGAGCT**C GTTTCAA**A**ATGTCCAA**AAGCGGCCGCTCTAG
TS122/123	pTS122	yTS122	TCGAGATCAGCATTTCTTTTCGGAGAGCT**CGTTTCAA**A GCGGCCGCTCTAG
TS124/125	pTS124	yTS124	TCGAGAT**CAGCATTTCTTTTCGGAGAGCT**AGCGGCCG CTCTAG
TS126/127	pTS126	yTS126	TCGAGATG**ATGAAACA**AAC**ATGAAACG**TCTGTAAGCG GCCGCTCTAG
TS128/129	pTS128	yTS128	TCGAGATG**ATGAAACG**AAC**ATGAAACA**TCTGTAAGCG GCCGCTCTAG
TS130/131	pTS130	yTS130	TCGAGATG**ATGAAACA**AAC**TTGAAACA**TCTGTAAGCG GCCGCTCTAG
TS132/133	pTS132	yTS132	TCGAGATG**ATGAAACA**AAC**ATGAGACA**TCTGTAAGCG GCCGCTCTAG
TS134/135	pTS134	yTS134	TCGAGATG**TTGAAACA**AAC**ATGAAACG**TCTGTAAGCG GCCGCTCTAG
TS136/137	pTS136	yTS136	TCGAGATG**TTGAAACA**AAC**ATGAGACA**TCTGTAAGCG GCCGCTCTAG
TS138/139	pTS138	yTS138	TCGAGATG**ATGAAACG**AAC**ATGAGACA**TCTGTAAGCG GCCGCTCTAG
TS169/170	pTS169	yTS169	TCGAGCCAC**ATAAAACA**GGCTTGACATTATATTGGCCA TTTGATTTATACT**TGTTTCA**GATCGCGGCCGCTCTAG
TS179/180	pTS179	yTS179	TCGAGATG**ATGAAACA**AAC**ATGGGACA**TCTGTAAGCG GCCGCTCTAG

Table 2.1 (continued) Annealed oligonucleotides used for construction of reporter gene plasmids and yeast strains.

Oligos[a]	Plasmid[b]	Strain[c]	Sequence (5' to 3')[d]
TS181/182	pTS181	yTS181	TCGAGATG**ATGAAACA**AAC**ATGGGGCA**TCTGTAAGCG GCCGCTCTAG
TS183/184	pTS183	yTS183	TCGAGATG**ATGAAACA**AAC**ATGGGGGA**TCTGTAAGCG GCCGCTCTAG
TS185/186	pTS185	yTS185	TCGAGATG**ATGAAACA**AAC**ATCGGGGA**TCTGTAAGCG GCCGCTCTAG
TS191/192	pTS191	yTS191	TCGAGGCTTTTCCGTTTGGC**TGAAGCA**ACTT**TGAAACA** CAACTACGACATTATAGCGGCCGCTCTAG
TS193/194	pTS193	yTS193	TCGAGCGC**ATGAAACA**GTTTTGGTGCGATGTT TTGGTGCGATGTTTTGGTGCGATG**ATGAAACA** AAGCGGCCGCTCTAG
TS195/196	pTS195	yTS195	TCGAGCGC**TGTTTCAT**GTTTTGGTGCGATGTT TTGGTGCGATGTTTTGGTGCGATG**ATGAAACA** AAGCGGCCGCTCTAG
TS197/198	pTS197	yTS197	TCGAGCGC**ATGAAACA**TGCGATGTTTTGGTGC GATG**ATGAAACA**AAGCGGCCGCTCTAG
TS199/200	pTS199	yTS199	TCGAGCGC**TGTTTCAT**TGCGATGTTTTGGTGC GATG**ATGAAACA**AAGCGGCCGCTCTAG
TS201/202	pTS201	yTS201	TCGAGCGC**ATGAAACA**TGGTGCGATG**ATGAAACA**AAG CGGCCGCTCTAG
TS203/204	pTS203	yTS203	TCGAGCGC**TGTTTCAT**TGGTGCGATG**ATGAAACA**AAGC GGCCGCTCTAG
TS206/207	pTS206	yTS206	TCGAGCCACGGCTTGACATTATATTGGCCATTTGATTTA TACT**TGTTTCA**GATCGCGGCCGCTCTAG
TS208/209	pTS208	yTS208	TCGAGGCTTTTCCGTTTGGCACTT**TGAAACA**CAACTACG ACATTATAGCGGCCGCTCTAG
TS215/216	pTS215	yTS215	TCGAGATG**ATGAAACA**AAC**CGTTTCAT**TCTGTGCGGCC GCTCTAG
TS277/278	pTS277	yTS277	TCGAGCGC**TGTTTCAT**TGCGATGTTTTGGTGCGATG**AT GAAACA**AAGCGGCCGCTCTAG
TS279/280	pTS279	yTS279	TCGAGCGC**TGTTTCAT**TGGTGCGATG**ATGAAACA**AAGC GGCCGCTCTAG

[a]Annealed oligonucleotides bearing the indicated WT or mutant PREs, produce XhoI- and XbaI-compatible overhanging ends.
[b]The annealed oligos were cloned into the XhoI/ XbaI sites of pIS341, which is a *lys2* disintegrator plasmid bearing a minimal *GAL1* core promoter upstream of a *LacZ* reporter gene and 5' *ADH1* termination fragment, to produce the indicated reporter plasmids.
[c]Plasmids were integrated at single copy into a *lys2* disruption in W303-1a to produce the indicated yeast strains.
[d]Sequences of the top strand of the annealed double stranded oligos. WT and mutant PRE sequences are indicated in bold lettering.

Table 2.2 Additional yeast strains.		
Strain names	Genotype	Reference/source
SY2585	*MATa leu2 trp1 ura3 ade2 mfa2Δ::FUS1-lacZ his3Δ::FUS1-HIS3*	Charles Boone
yAO6	*MATa leu2 trp1 ura3 ade2 his3 can1 ste12Δ*	Amy Olson
W303-1A	*MATa ade2 his3 leu2 trp1 ura3 can1*	
y970	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU2*	Alan Myers
y227	*MATα lys1*	David Mitchell
yTS227C	*MATα lys1cdc55::Kan*	This study
yTS126F	*MATa ade2 his3 leu2 trp1 ura3 can1 fus3::TRP1 lys2::2-PRE-GAL1-LacZ(pTS126)*	This study
yTS126K	*MATa ade2 his3 leu2 trp1 ura3 can1 kss1::TRP1 lys2::2-PRE-GAL1-LacZ(pTS126)*	This study
YTS126R	*MATa ade2 his3 leu2 trp1 ura3 can1 rts1::HIS5 lys2::2-PRE-GAL1-LacZ(pTS126)*	This study
yTS971	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU2 lys2::2-PRE-GAL1-LacZ(pTS126)*	This study
yTS971F	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU2 fus3::TRP1 lys2::2-PRE-GAL1-LacZ(pTS126)*	This study
yTS971K	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU21 kss1::TRP1 lys2::2-PRE-GAL1-LacZ(pTS126)*	This study
YTS971R	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU21 rts1::HIS5 lys2::2-PRE-GAL1-LacZ(pTS126)*	This study
yTS972	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU2 lys2::2-PRE-GAL1-LacZ(pTS84)*	This study
yTS973	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU2 lys2::2-PRE-GAL1-LacZ(pTS169)*	This study
yTS974	*MATa ade2 his3 leu2 trp1 ura3 cdc55::LEU2 lys2::2-PRE-GAL1-LacZ(pTS191)*	This study
yTSCCW	*MATa, leu2-3,112, ura3-52, trp1, his4, rme1, HMLa lys2::2-PRE-GAL1-LacZ(pTS26)*	This study
yTSCC1	*MATa, leu2-3,112, ura3-52, trp1, his4, rme1, HMLa, cmp1::LEU2 lys2::2-PRE-GAL1-LacZ(pTS26)*	This study
yTSCC2	*MATa, cmp1::LEU2, cmp2::Kan, his4, leu2-3,112, trp1, ura3-52, rme1, HMLa lys2::2-PRE-GAL1-LacZ(pTS26)*	This study
yTSCC3	*MATa, leu2-3,112, ura3-52, trp1, his4, rme1, HMLa, cnb1::LEU2 lys2::2-PRE-GAL1-LacZ(pTS26)*	This study

2.2 Recombinant proteins and EMSA

Full-length Ste12 and Ste12(1-595) proteins were expressed as N-terminal 6-His fusions in insect cells using baculovirus in the Sf21 insect cell line (136). Tec1 was expressed with a 6-His-N-terminal and C-terminal flag epitope tag using the Bac-to-Bac system (Invitrogen, Carlsbad, CA, USA). Antibodies A3, B3 and F3 were raised against *Escherichia coli* TrpE fused to Ste12 residues (265–688), (314–688) and (1–215), respectively. Sf21 cells infected with Ste12 and Tec1 virus were collected and washed in ice-cold lysis buffer (20 mM Tris, pH 8.0, 40 mM NaCl, 1 mM dithiothreitol, 5% glycerol, 2.5 mM $MgCl_2$, 1 mM Na_3VO_4, 5 mM EGTA, 50 mM NaF and 20 mM β-glycerol phosphate). The cells were lysed by forcing through a 27-gauge needle ten times, and then sonicated for 10 s. A clarified supernatant was obtained by centrifugation at 13400 ***g*** for 10 min and used without purification for EMSA. Ste12 proteins were produced by *in vitro* transcription and translation using the TNT T7 Quick Coupled Transcription/Translation System (Promega, Madison, WI, USA). Briefly, plasmid pSC4, which contains a full-length genomic clone of *STE12*, was used as template for amplification with oligonucleotide oIS1144, in combination with oVT2, oET30 and oIS1146 (Table 2.3), to produce fragments with a 5' T7 RNA polymerase promoter and encoding Ste12 (1–215), Ste12 (1–350) and Ste12 (1–479), respectively. The Ste12 derivatives were also produced individually or by co-translation in 50 μL reactions containing 1 μL of T7 RNA polymerase and 40 μL of rabbit reticulocyte lysate. The reactions were carried out at 30 °C for 90 min and then assayed immediately for DNA binding activity.

Oligonucleotides used for the electrophoretic mobility shift assays (EMSA) are detailed in Table 2.4, and were annealed and labeled using Klenow (New England Biolabs, Beverly, MA, USA) with [^{32}P]αdATP and [^{32}P]αdTTP, as described previously (18). The 5' overhangs of unlabeled competitor oligonucleotides were filled in using Klenow and an unlabeled dNTP mixture. EMSA reactions contained 1 μL of labeled oligonucleotide probe (2 pmol), 2 μg of poly(dI-dC) (Sigma, St Louis, MO, USA), 2.5 mM $MgCl_2$, 1% glycerol, 20 mM Tris (pH 8.0), 40 mM NaCl and 1 μL of Sf21 extract or *in vitro* translation reaction in a total volume of 20 μL. Labeled oligonucleotide probes were added to the binding reactions after a 30 min pre-incubation on ice with unlabeled competitor oligos or specific antibodies. Binding reactions were performed at room temperature for 30 min and the reactions were resolved on nondenaturing polyacrylamide gels containing 0.5 X TBE (89 mM Tris, 89 mM Boric acid, 2 mM EDTA, pH 8.0) buffer and 1% glycerol at 200 V for 3 h. Signals produced in the EMSA reactions were quantified using Imagequant software (GE Healthcare, Milwaukee, WI, USA).

Table 2.3 Oligonucletides for production of templates for *in vitro* transcription and translation reactions.	
Oligos	Sequence (5’ to 3’)
IS1144/STE12 T7F	CCCTCGAGTAATACGACTCACTATAGGGAGCCACCATGAAAGT CCAAATAACCAATAGT
VT2/STE12 215R	TCGAATTCTCATCTAGAATCTAAATGTTGAAGTAA
ET30/STE12 350R	CCGGTCAATCCCTTTCATTCACGTTATCATAGGAAATAG
IS1146/STE12 479	ATATCAAAAGGCTTGATCGAGTAGAG

Table 2.4 Annealed double stranded oligonucleotides for use as probes and competitors in EMSA reactions.	
Oligos	Sequence (5' to 3')
RS010/11	TCGAACATGATGAAACACATAT
RS012/13	TCGAACATGATAAAACACATAT
RS012/13-1	TCGAACATGATCAAACACATAT
RS012/13-2	TCGAACATGATTAAACACATAT
RS014/15	TCGAACATGATGAGACACATAT
RS016/17	TCGAACATGAAGAAACACATAT
RS018/19	TCGAACATGATGCAACACATAT
RS020/21	TCGAACATGATGGAACACATAT
RS022/23	TCGAACATGATGAAGCACATAT
RS024/25	TCGAACATGATGAAAGACATAT
IS1428/1429	TTAATCTGTAATTTGAAACAAATAACGTTTAA
IS1430/1431	TTAATCTGTAATATGAAACAAATAACGTTTAA
IS1432/1433	TTAATCTGTAATATGAAACGAATAACGTTTAA
IS1434/1435	TTAATCTGTAATATGAGACAAATAACGTTTAA
CN140/141	TAGACGTTTCAGCTTCCAAAACAGAAGAATG
ET1/2	ATGATGAAACAAACATGAAACGTCTG

2.3 Western blots

Polyclonal anti-Ste12 antisera A3 and B3 (Sadowski lab) were used at 1:10000 dilutions. The blots were probed overnight in 1X TBS-T (0.1 M Tris-HCl pH 8.0, 0.9 % NaCl, 0.05 % Tween 20) with 5% skim milk at 4 ºC.

2.4 Flow cytometry

Yeast cells were harvested at OD_{600} between 0.5 and 1.0. For pheromone treated cells, yeast were treated with 2 μg/ml alpha-factor for 1 h before harvesting. Cells were then fixed in 70% ethanol and 30% 1M sorbitol for 30 min on ice for 1 hour at RT. Cells were recovered by centrifugation and the yeast pellets were resuspended in 200 μl 0.2 M TrisHCl (pH 7.5) with 1 mg/ml RNAse A, and incubated at 37 °C overnight. Five microliters 20 mg/ml proteinase K was added and the mixtures incubated at 50 °C for 1 h. The cells were then centrifuged and the pellets were washed two times with 400 μl 0.2 M TrisHCl (pH 7.5), and resuspended in 600 μl 1μg/ml propidium iodide. The cells were stained at 4 °C in the dark overnight and sonicated briefly before analysis by flow cytometry (FACScan, BD).

2.5 Quantitative mating assay

From overnight cultures of cells grown in YPD, 6 X 10^6 *MAT*α cells (Y227α or Y227α *cdc55*) were mixed with 2 X 10^6 *MATa* cells (wildtype W303-1A or Y970 *cdc55*). The mixture was spotted onto a 0.45 μm pore, 25-mm diameter nitrocellulose filter disk (Millipore) on an agar plate [YEPD, synthetic complete (SC), or YEPD with 20 ng/μl

rapamycin], and incubated in a 30 °C incubator for 2 h. After incubation, the yeast was flushed off the filter and collected with 1 ml of *his⁻ lys⁻* minimal media. The collected yeast was sonicated for 25 s to disperse any clumps. 200 µl of a 10^{-4} serially diluted sample was spread on *lys⁻* plates and *his⁻ lys⁻* plates. Mating efficiency was calculated by the colony number from the *his⁻ lys⁻* plate divided by the resulting colony number from the *lys⁻* plate. All assays were performed in triplicate, and the error bars represent standard deviation. For examining the effect of rapamycin on mating, the yeast mixtures were treated with rapamycin at a final concentration of 20 ng/µl for 1 h prior to spotting onto filters on plates containing the same concentration of rapamycin.

2.6 ^{35}S Ste12 labeling and immunoprecipitation

Both the wild type yeast strain W303-1A and *cdc55* null strain Y970 were transformed with pJL01, which expresses full length wild type Ste12 under control of the *GAL10* promoter on a *URA3* marked plasmid. Before labeling, the strains were grown overnight in *ura⁻* raffinose media. The yeast were diluted, and re-grown at a starting OD_{600} of 0.5 in 30 ml *ura⁻* raffinose culture for a further 4 h at 30 °C. Before collecting the yeast, the cultures (30 ml) were divided into 15 ml aliquots, one of which was treated with pheromone during labeling. The yeast were collected by centrifugation, washed with 1ml *met⁻ ura⁻* raffinose media, and then resuspended in 100 µl *met⁻ ura⁻* raffinose media, to which 75 µl ^{35}S labeling mix (N.E.N.) and 20 µl 20% galactose was added. For the pheromone-treated samples, 15 µl of alpha-factor (2 mg/ml) was added. The labeling reaction was carried out at 30 °C for 1 hour, during which the mixture was resuspended every 15 minutes. After labeling, 1 ml ice cold Yeast Lysis buffer [YLB, 50 mM Tris-

HCl pH8.0, 5mM $MgCl_2$, 150 mM NaCl, 5 mM NaF, 2 mM $ZnCl_2$, and 1X Protease Inhibitor Cocktail (Sigma)] was added and mixed. The cells were collected by centrifugation, and washed with 1ml ice cold YLB twice more. The cells were resuspended in 400 µl YLB and 400 µl of acid washed glass beads were added. The cells were lysed by mixing vigorously in six 1 min bursts, with the yeast placed on ice for 1 min between mixing. Four hundred microliters 2X RIPA buffer (20 mM Tris-HCl pH8.0, 200 mM NaCl, 2 mM EDTA, 2% NP-40, 1% sodium deoxycholate, 0.2% SDS) was then added and the yeast mixed vigorously for a further 1 min. The mixtures were then centrifuged at 13800 g for 30 min, and the supernatant was transferred to a new tube. The supernatant was first pre-cleared with 1 µl pre-immunized rabbit serum and 50 µl 10% formalin fixed *S. aureus* (Zymed) on a rolling platform for 30 min at 4 °C. After the pre-clear, the lysate was centrifuged and the supernatant removed to a new tube. To immunoprecipitate Ste12, the supernatant was first incubated on ice with 5.5 µl anti-Ste12 serum B3 for 30 min. Sixty-five microliters of 10% *S. aureus* was added to the samples, and mixed on a rolling platform for 2 h at 4 °C. The immunoprecipitates were then washed subsequently with 1 ml of buffers W1 (10 mM Tris-HCl pH8.0, 1M NaCl, 0.1% NP-40), W2 (10 mM Tris-HCl pH8.0, 0.1M NaCl, 0.1% NP-40, 0.1% SDS), W3 (10 mM Tris-HCl pH8.0, 0.1%SDS), and then 1X RIPA buffer. The final *S. aureus* pellet was resuspended in 50 µl 1X SDS sample buffer, and incubated at 37 °C for 10 min. The samples were spun down for 5 min, and the supernatant was removed and boiled for 2 min before analysis on SDS-PAGE. For SDS-PAGE, the samples were run on 7.5 % acrylamide gels at 200 V/20 mA for 10 h to separate phosphorylated Ste12 species. The gels were dried and exposed to X-ray film to detect labeled Ste12 protein.

2.7 RNA preparation

Ten milliliters of a yeast culture grown to OD_{600} 0.8-1.0 was harvested and resuspended in 600 μl RNA extraction buffer (500 mM NaCl, 20 mM Tris-Cl pH 7.5, 10 mM EDTA and 1% SDS). Four hundred microliters of glass beads and 500 μl acid phenol were added and the mixtures were mixed vigorously at 4 °C for 25-30 min. The samples were centrifuged at 16200 g for 5 min and the supernatants removed to a new tube. The supernatants were extracted by mixing with 60 μl 3M NaOAc (pH 5.2) and 400 μl buffer-saturated phenol (Sigma), and then centrifuged at 16200 g for 5 min. The supernatants were removed and the extraction repeated one more time. The supernatant was removed to a new tube and 500 μl chloroform was added for a further extraction. The samples were mixed thoroughly and centrifuged at 16200 g for 5 min. The supernatant was added to 900 μl 95% ethanol to precipitate the RNA for 1 h on ice. The samples were centrifuged at 16200 g for 10 min to pellet the RNA, which was washed with 1 ml of 70% ethanol. The RNA was resuspended in 200 μl RNAse free dH_2O. The samples were stored by adding 400 μl 95% ethanol, mixing well and placing at –20 °C.

2.8 Northern blots

For each sample, 15-20 μg RNA was used. RNA was first precipitated for at least 30 min by mixing with 2 volumes of 95% ethanol and 1/10 volume 3M NaOAc (pH 5.2). The RNA pellets were washed with 70% ethanol and resuspended in 10.9 μl formaldehyde/formamide solution [200 μl formamide, 70 μl formaldehyde and 20 μl 10X MOPS buffer (400 mM MOPS pH7.0, 100 mM NaOAc, 10mM EDTA)]. One microliter of ethidium bromide (0.5 mg/ml) and 5μl MOPS loading dye was added, and the samples

loaded onto an RNA agarose gel (1.8 g agarose, 108 ml dH_2O, 27 ml formaldehyde, and 15 ml 10X MOPS buffer) and run at 100V for 3.5 h (about 10 cm). After electrophoresis, migration of the RNA was checked by visualizing rRNA bands under U.V. irradiation, and if satisfactory the gels were placed in dH_2O for at least 30 min. The RNA was transferred to nylon membranes overnight in 20X SSC (3 M NaCl, 0.3 M sodium citrate pH 7.0) buffer. The membrane was air-dried for 1 h the following day, and RNA was U.V. cross-linked to the membrane using a Bio-Rad cross-linking instrument. The membranes were placed in a hybridization tube with 25 ml hybridization buffer (0.4 M NaCl, 100 mM $NaPO_4$ pH6.5, 4 mM EDTA, 0.2% SDS and 10% dextran sulfate), and pre-hybridized at 65 °C for 2-3 hours. After pre-hybridization, the denatured ^{32}P-labeled DNA probe, produced as described below, was added and hybridized at 65 °C overnight. The next day, the probe solution was discarded and the membranes washed with a 2XSSC/0.2% SDS solution for 20-30 min at RT. The membranes were then washed with 0.2XSSC/0.2% SDS solution at 65 °C for 15 min twice before wrapping in plastic wrap and exposing to X-ray film.

Probes for Northern blots were prepared using the Random Primer DNA Labeling System (Invitrogen). Briefly, 25 ng DNA was prepared in 24 µl dH_2O, denatured at 95°C for 10 min, and immediately placed on ice. The denatured template was mixed with a labeling mix [15 µl Random Primers buffer mixture (0.67 M HEPES, 0.17 M Tris-HCl, 17 mM $MgCl_2$, 33 mM 2-mercaptoethanol, 1.33 mg/ml BSA, 18 OD_{260} units/ml oligodeoxyribonucleotide hexamer primers, pH 6.8), 2 µl 1 mM dATP, 2 µl 1 mM dGTP, 2 µl 1 mM dTTP, and 5 µl ^{32}p α-dCTP]. 2 µl of Klenow DNA polymerase was added and the reactions incubated at 30 °C for 60 min. After incubation, 5 µl stop buffer (0.5 M

EDTA, pH 8.0) was added along with 5 µl 3M NaOAc (pH 5.2), 4 µl 10 mg/ml salmon sperm DNA and 200 µl 95% ethanol, and the labeled probe was precipitated on ice for 1 h. The probe was recovered by centrifuging at 16200 g for 10 min, and the pellet washed 2-3 times with 70% ethanol. The air-dried pellet was dissolved in 130 µl RNAse free dH_2O. Seventy microliters of 10 mg/ml denatured salmon sperm DNA was added to the probe, which was stored at –20°C. 100 µl of the probe solution was used for each hybridization.

2.9 Quantitative RT-PCR

Purified RNA samples were first converted to single strand cDNA using the M-MuLV First Strand cDNA Synthesis kit (New England BioLabs) following the manufacturer's protocols. Quantitative PCR reactions were performed in triplicate using IQ SYBR Green supermix (Bio-Rad), cDNA template and PCR primers (Table 2.5) for specific individual genes in a final 20 µl reaction volume. PCR amplification was performed and detected with the StepOnePlus Real-Time PCR System (Applied Biosystems). The parameters for the PCR reaction is as followed: denature at 95°C for 30 s, annealing at 52°C for 45 s, and elongation at 72°C for 45 s for a total cycle number of 45. To quantify relative transcripts I used the comparative Ct method, also referred as the Ct method. The relative fold of each test group to control group is given by the arithmetic formula $2^{-\Delta\Delta Ct}$. For each sample, the cycle threshold (Ct) values for *PDA1* and genes of interest were determined and values obtained were normalized relative to *PDA1* RNA. The Ct values are represented by subtraction of the average *PDA1* Ct value from Ct values of the gene of interest. Relative transcript expression levels for each gene is depicted as $2^{-\Delta\Delta Ct}$.

Table 2.5 Quantitative PCR primers.		
Primer name	Sequence (5' to 3')	Notes*
TS340	GATGGTGCCTCTAATCAAGGTC	*PDA1*F
TS341	AATGGCCACCGTACCTATAGG	*PDA1*R
TS348	GGCAGAAGTGAACTTCCATAGC	*CIK1*F
TS349	CATTGGCATACGCAAAGCATCG	*CIK1*R
TS350	GAGCTGAACACTTCAGTTCC	*AFR1*F
TS351	GGC CTGAATTAG TTGTTG GC	*AFR1*R
TS352	GACTCAGGAACCTCGCTATTG	*BAR1*F
TS353	CAATAGCTGCCTTCTGTC TCG	*BAR1*R
TS358	GAGTACATCTTCGCAGCCAAC	*SCW11*F
TS359	GCC TTC AACATTGGCTTCACC	*SCW11*R
TS360	CCTCTCCAGCGGTATTATTC	*PRM4*F
TS361	CTGAATGGATTCCAAGGCAG	*PRM4*R
TS364	CAAACTCTGCTGATGGGTTC	*PRM3*F
TS365	GTAGTTACAGCAGCACCAAG	*PRM3*R
TS366	CTGCGGAAGTTGCTCTACAAC	*HYM1*F
TS367	GATGTGCAGTAAATGCGGCAC	*HYM1*R
* F: Forward primer R: Reverse primer		

Chapter 3 Characterization of Ste12 binding sites for pheromone response

3.1 Promoters of pheromone-responsive genes

Haploid yeast treated with mating pheromone induce ~600 genes within 30 minutes to an hour (157, 203). Studies examining pheromone response have typically used a *FUS1* promoter- *LacZ* reporter gene to monitor strength of the response. The *FUS1* promoter contains four PREs with different sequences in a span of ~400 nucleotides. It was shown that at least two copies of the PREs from this promoter are necessary to produce a response to pheromone (64). Although widely used for studying pheromone response, little is known about how Ste12 interacts with the PREs of this promoter to direct pheromone response. Furthermore, the stoichiometry of Ste12 protein for binding a single PRE is still unknown, and it is not clear how multiple Ste12 proteins interact to produce a response.

In examining promoters of the most strongly inducible pheromone-responsive genes (Fig. 3.1)(157, 203), I noted that there are no obvious "rules" that can predict response levels to pheromone. For example, it appears there is no simple correlation between the number of PREs in the promoter and the amount of induction. Consequently, I proposed that pheromone responsiveness must be influenced by the potential interaction strength of PRE for binding of Ste12 protein, in combination with their number and orientation relative to each other.

This chapter is based on Su TC, Tamarkina E, Sadowski I (2010) Organizational constraints on Ste12 cis-elements for a pheromone response in Saccharomyces cerevisiae. *FEBS J* 277(15):3235-48 (License Number: 2918910608116).

Interestingly as well, although at least two copies of a PRE are required for pheromone response of the *FUS1* promoter, I noticed that some strongly inducible genes, including *PRM2*, *CIK1* and *PRM4* contain only one PRE (Fig. 3.1), and there are a number of pheromone responsive genes that lack obvious binding sites for Ste12 (not indicated on Fig 3.1).

In this chapter, I address these issues by investigating how the binding strength of PRE sequences, their orientation, and intervening nucleotide distance between two PREs govern the overall response to pheromone within an artificial promoter.

Figure 3.1 Organization of a selection of strongly inducible pheromone-responsive promoters.

Schematic representation of the organization of consensus PREs within nine of the 35 most strongly induced pheromone response genes (excluding pseudogenes and genes without obvious PREs), as identified by global expression analysis (30 min after α-factor treatment). Numbers between any two PREs indicate the spacing in nucleotides, whereas the number furthest to the right indicates the distance to the translation start site). *STE12* is within the top 100 pheromone-inducible genes, and was included here because I have examined this promoter in some detail (below).

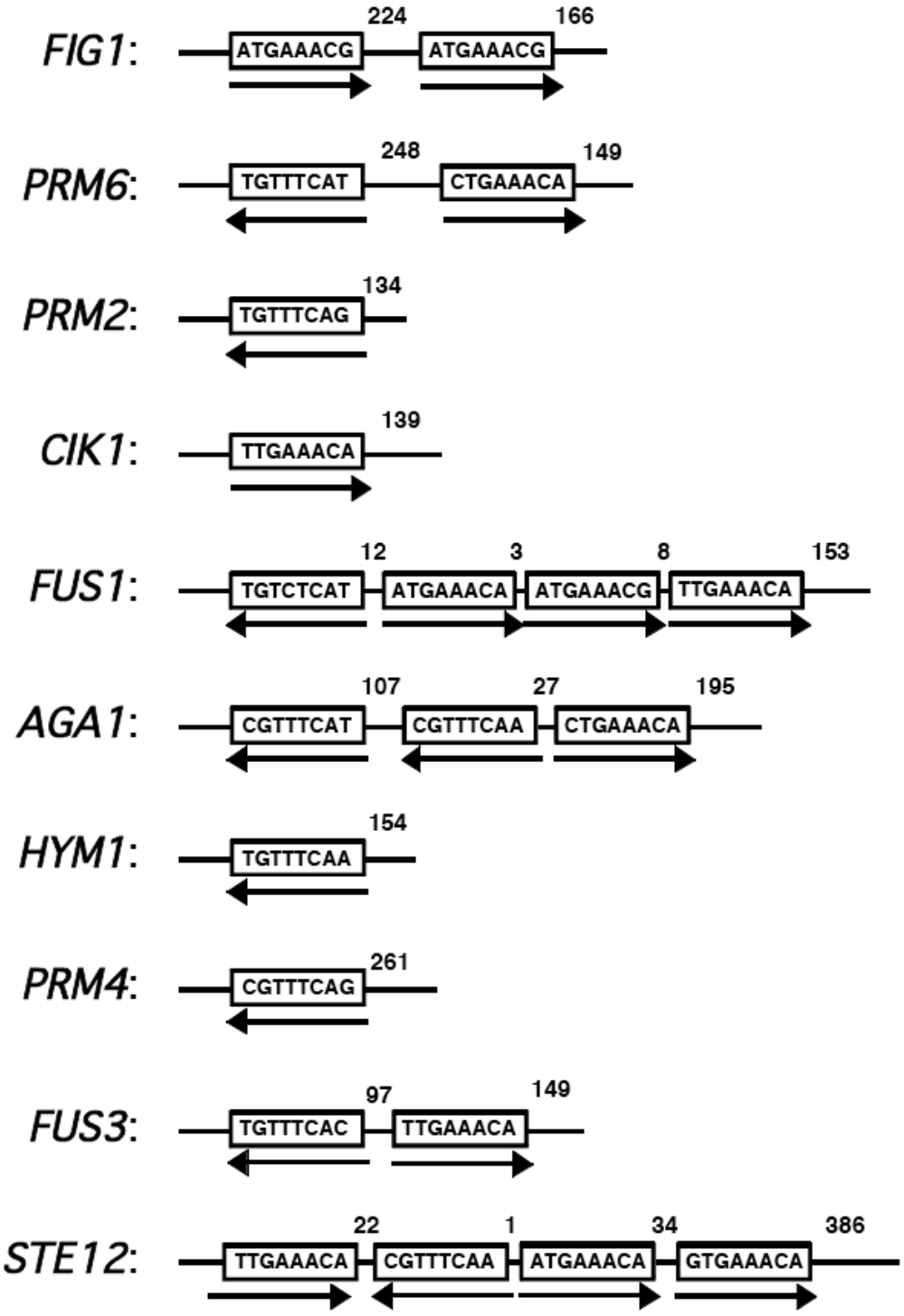


FIG1:
224
166
ATGAAACG
ATGAAACG
PRM6:
248
149
TGTTTCAT
CTGAAACA
PRM2:
134
TGTTTCAG
CIK1:
139
TTGAAACA
FUS1:
12
3
8
153
TGTCTCAT
ATGAAACA
ATGAAACG
TTGAAACA
AGA1:
107
27
195
CGTTTCAT
CGTTTCAA
CTGAAACA
HYM1:
154
TGTTTCAA
PRM4:
261
CGTTTCAG
FUS3:
97
149
TGTTTCAC
TTGAAACA
STE12:
22
1
34
386
TTGAAACA
CGTTTCAA
ATGAAACA
GTGAAACA

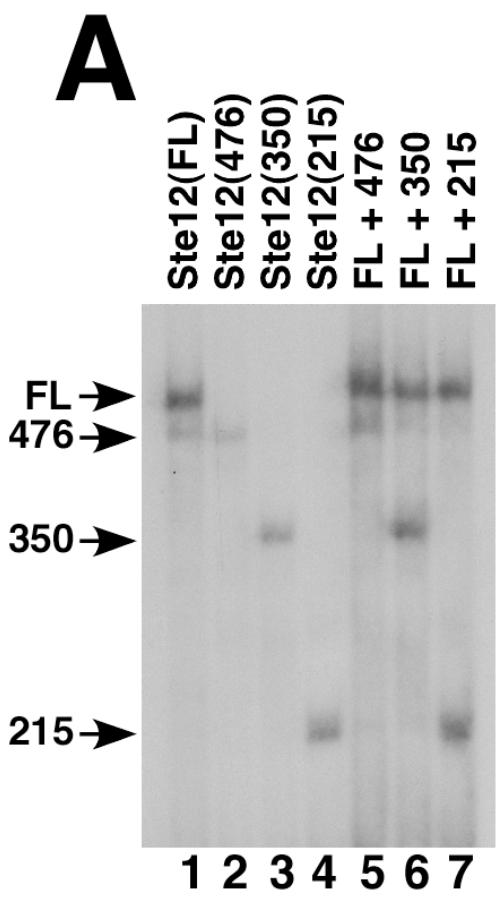
3.2 Ste12 likely binds the consensus PRE as a monomer

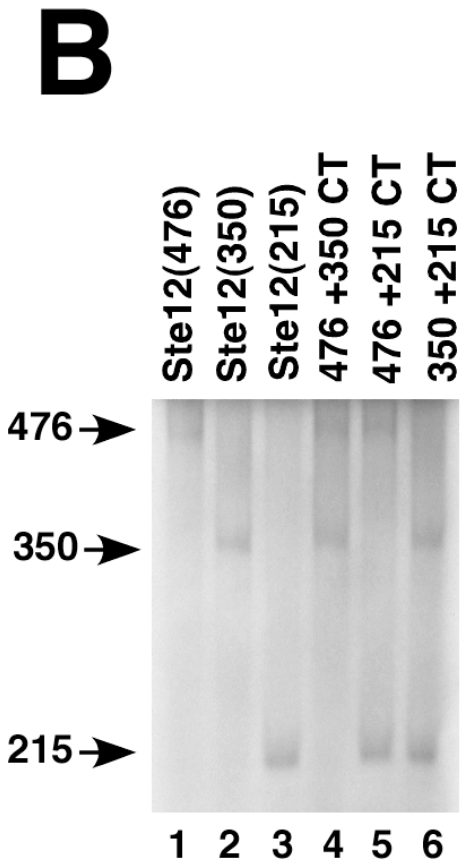
By sequence comparisons, the Ste12 DNA-binding domain seems to most closely resemble a homeodomain (200). Most homeodomain DNA-binding proteins bind DNA as dimers, but there are several examples, including the POU subfamily, including Oct1 and 2, which bind DNA as a monomer. There is currently no information on the stoichiometry of Ste12 protein for binding a single PRE. To examine this, I determined whether Ste12 DNA binding domain fragments were capable of forming heterodimers in EMSA. A set of Ste12 derivatives with various C-terminal truncations were produced by *in vitro* transcription and translation, Ste12(1-476), Ste12(1-350), and Ste12(1-215). For technical reasons, full-length wild type Ste12 could not be produced *in vitro*, and instead was expressed in insect cells, using baculovirus (Ste12-FL). Ste12(FL) and the other three truncated Ste12 derivatives were first examined for their DNA binding capability using a double stranded oligonuclotide probe with only one PRE in EMSA reactions. All forms of Ste12 were capable of binding the oligo, and produced complexes with mobility proportional to their different molecular weights (Fig. 3.2A, lanes 1-4). I then examined whether co-incubation of Ste12(FL) with the truncated Ste12 forms produced complexes of intermediate mobility, which would be indicative of multimer formation (Fig. 3.2A, lanes 5-7). If Ste12 dimerization is required for binding the consensus PRE, an intermediate complex produced from interaction between Ste12(FL) and the truncated Ste12 protein should be observed as was shown for Gcn4 (76) . However, none of the Ste12(FL) - Ste12 truncation combinations produced such intermediate complexes. This result suggests that Ste12 likely binds a single PRE as a monomer. Some transcription factors form such tight dimer interactions, required for DNA binding, that do not

dissociate and reassociate at a sufficient rate that can be observed *in vitro*. To examine whether this is the case for Ste12, I co-translated Ste12(1-476) with the Ste12(1-350), and Ste12(1-215) derivatives, and examined whether intermediate complexes could be observed in EMSA reactions (Fig. 3.2B, lane 4-6). In these experiments I again did not observe intermediate complexes. This result excludes the possibility that co-translation is required for formation of heterocomplexes between the Ste12 derivatives. Taken together, these results indicate that Ste12 must bind a single PRE as a monomer. This conclusion is supported by the fact that truncated Ste12 derivatives lacking the putative multimerization region (Fig 1.3) are capable of binding a single PRE.

Figure 3.2 Ste12 likely binds to a PRE as a monomer.

(A) EMSA reactions were performed with a labeled oligo containing a single PRE (IS1430/1431) and full-length Ste12 (lane 1), Ste12 1–476 (lane 2), Ste12 1–350 (lane 3) and Ste12 1–215 (lane 4). Full-length Ste12 was mixed with 1–476 (lane 5), 1–350 (lane 6) or 1–215 (lane 7) prior to adding the labeled oligo and performing the binding reaction. (B) Reactions were performed with *in vitro* translated Ste12 1–476 (lanes 1, 4 and 5), 1–350 (lanes 2, 4, and 6) or 1–215 (lanes 3, 5, and 6). The Ste12 derivatives were *in vitro* co-translated (lanes 4-6).


A
Ste12(FL)
Ste12(476)
Ste12(350)
Ste12(215)
FL + 476
FL + 350
FL + 215
FL
476
350
215
1 2 3 4 5 6 7

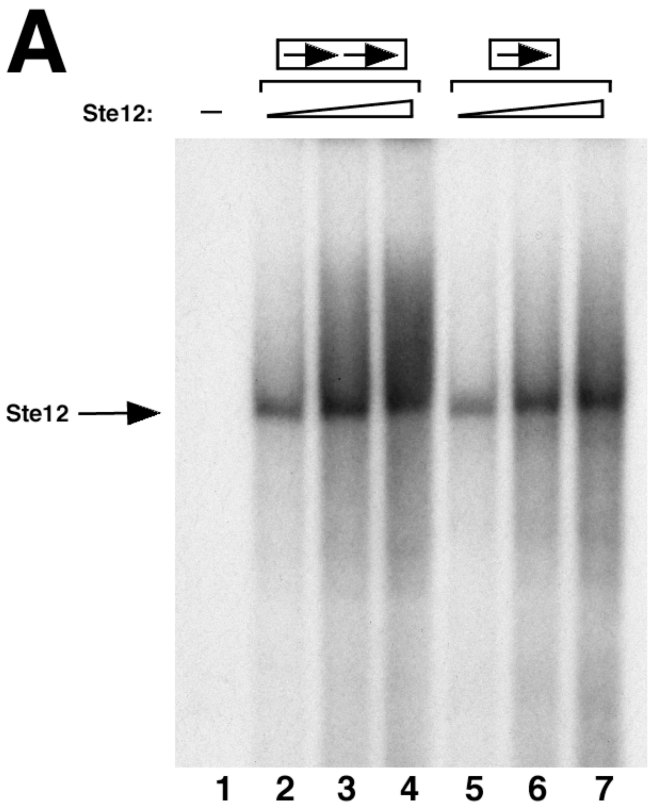

B
Ste12(476)
Ste12(350)
Ste12(215)
476 +350 CT
476 +215 CT
350 +215 CT
476
350
215
1 2 3 4 5 6

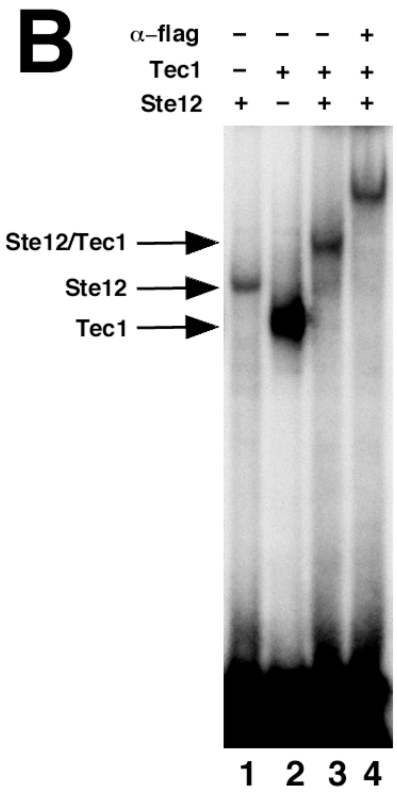
3.3 Recombinant Ste12 seems to be incapable of efficiently forming multimers on two PRE-containing oligos *in vitro*

During pheromone response, promoters such as that of *FUS1* seem to be solely dependent upon Ste12 bound to four PREs. Based on observations that at least two copies of a PRE are required for pheromone response, and that Ste12 binds a single PRE as monomer, I examined Ste12 binding to an oligo containing two PREs in EMSA. For these experiments I used an oligonucleotide representing two directly repeated PREs from the *FUS1* promoter, which were shown to confer response to pheromone *in vivo* (Fig. 3.3A, lanes 2-4). The results showed that, even with excess Ste12, the reactions did not produce a distinct slower migrating complex that could represent two Ste12 monomers bound to DNA. Instead, with increasing amounts of Ste12 protein I observed a slower migrating "smear" of complexes that were also produced with an oligo containing only a single PRE (Fig. 3.3A, lanes 5-7). This suggests that our recombinant Ste12 is capable of specific binding to only one PRE on DNA *in vitro*. These results are consistent with previous results from our lab, where it was shown using competitions that recombinant full length Ste12 produced in insect cells does not form multimers that can be resolved by EMSA with two closely spaced PREs on oligos representing sequences from the *FUS1* promoter *in vitro* (Tamarkina, E, described in Su et al., 2010). Because Ste12 likely binds to a single PRE as a monomer, these observations indicate that there might be spatial constraints between Ste12 molecules, at least for Ste12 produced in insect cells, that prevent access for binding adjacent PREs *in vitro*.

Figure 3.3 Recombinant Ste12 produced in insect cells binds to a single PRE *in vitro*.

(A) EMSA reactions were performed with extracts of Sf21 insect cells producing recombinant Ste12 protein. An oligonucleotide probe containing two directly-repeated PREs (sites II and III from the *FUS1* promoter, ET1/2, lines 2-4) and oligonucleotide probe with one PRE (sites II from the *FUS1* promoter, IS1430/143, lines 5-7) was titrated with an increasing amount of Ste12. (B) Full-length recombinant Ste12 and Tec1 form a complex on an FRE *in vitro*. EMSA reactions using a labeled FRE probe (CN140/141) derived from the *TY1* LTR were performed with Ste12 (lane 1), Tec1-flag (lane 2) or both Ste12 and Tec-1 flag (lanes 3 and 4). Anti-flag antibodies were added to the binding reaction in lane 4.


A
Ste12:
—
Ste12
1 2 3 4 5 6 7


B
α–flag – – – +
Tec1 – + + +
Ste12 + – + +
Ste12/Tec1
Ste12
Tec1
1 2 3 4

Ste12 is also involved in regulating filamentous growth under nitrogen starvation conditions. Ste12 and Tec1 work cooperatively for this response through binding to filamentous response elements (FREs) on the promoters of at least some downstream target genes. The FRE is comprised of adjacent binding sites for Ste12 (PRE) and another transcription factor Tec1 (TCS). Ste12 and Tec1 were previously shown to bind cooperatively to a combination of these elements (FRE) *in vitro* (9, 116). Because our recombinant Ste12 does not seem to form complexes that represent distinct multimers *in vitro*, I wondered whether it was capable of forming complexes with Tec1. To examine this, I used EMSA in reactions with Tec1 and an FRE oligonucleotide probe. Both recombinant Ste12 and Tec1, expressed in insect cells, were capable of binding the FRE oligo (Fig. 3.3B, lanes 1 and 2). However, in reactions where Ste12 and Tec1 were incubated together, a slower migrating Ste12-Tec1 complex was observed (Fig. 3.3B, lane 3). The recombinant Tec1 protein is tagged with a Flag epitope, and consistently, inclusion of antibodies against the Flag–tag caused a supershift of the Ste12-Tec1 complex (Fig. 3.3B, lane 4). Based on this result, I conclude that Tec1 is not excluded from forming complexes with our recombinant Ste12 for binding *in vitro*. Consequently, based on these observations it seems some feature of our recombinant Ste12 prevents formation of multimers with itself, but not complexes with another transcription factor Tec1. This suggests that there might be additional factors or post-translational modifications required *in vivo* to efficiently promote binding of Ste12 to multiple PREs for pheromone response.

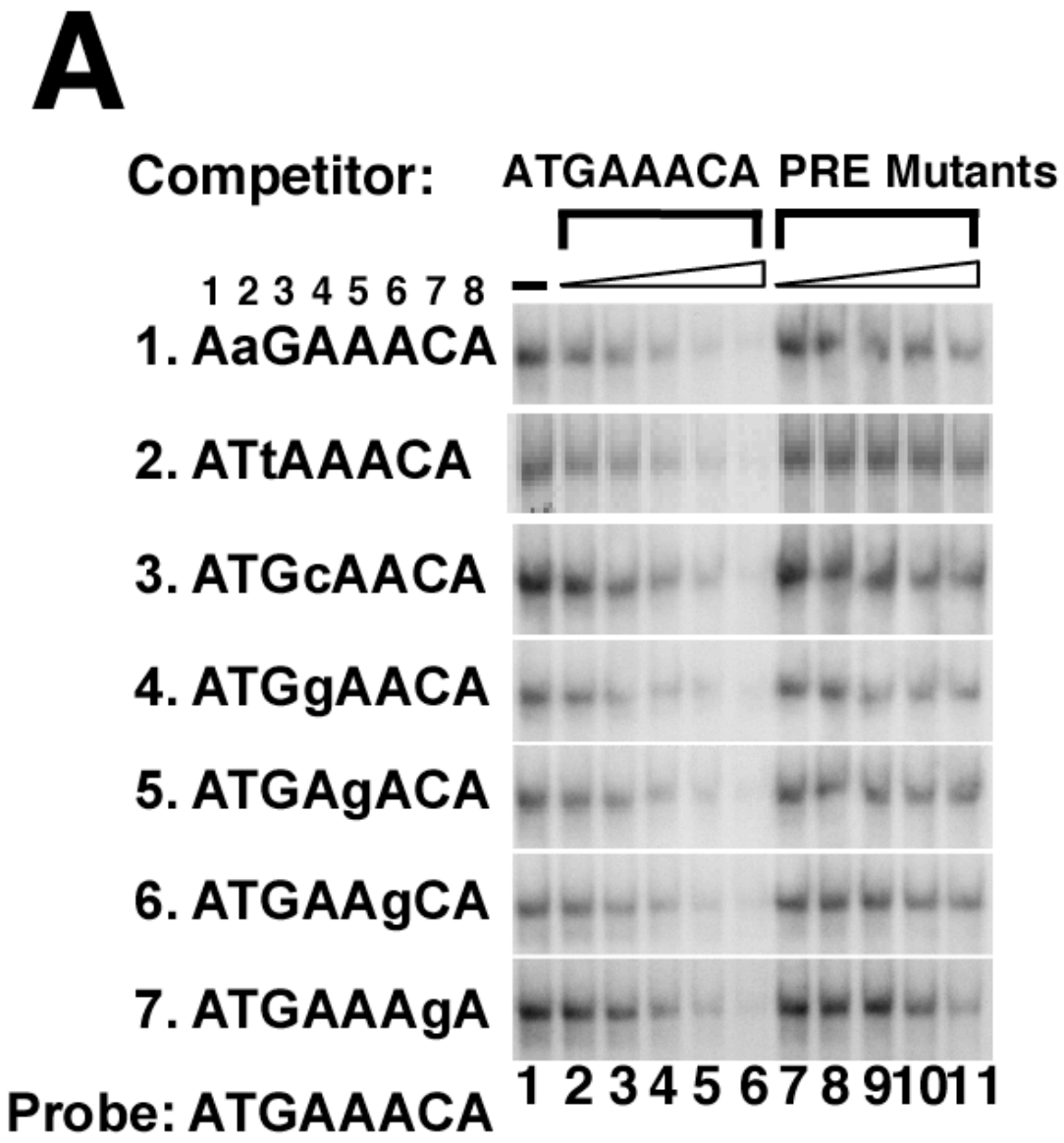
3.4 Analysis of individual nucleotide requirements of the PRE for Ste12 binding *in vitro*

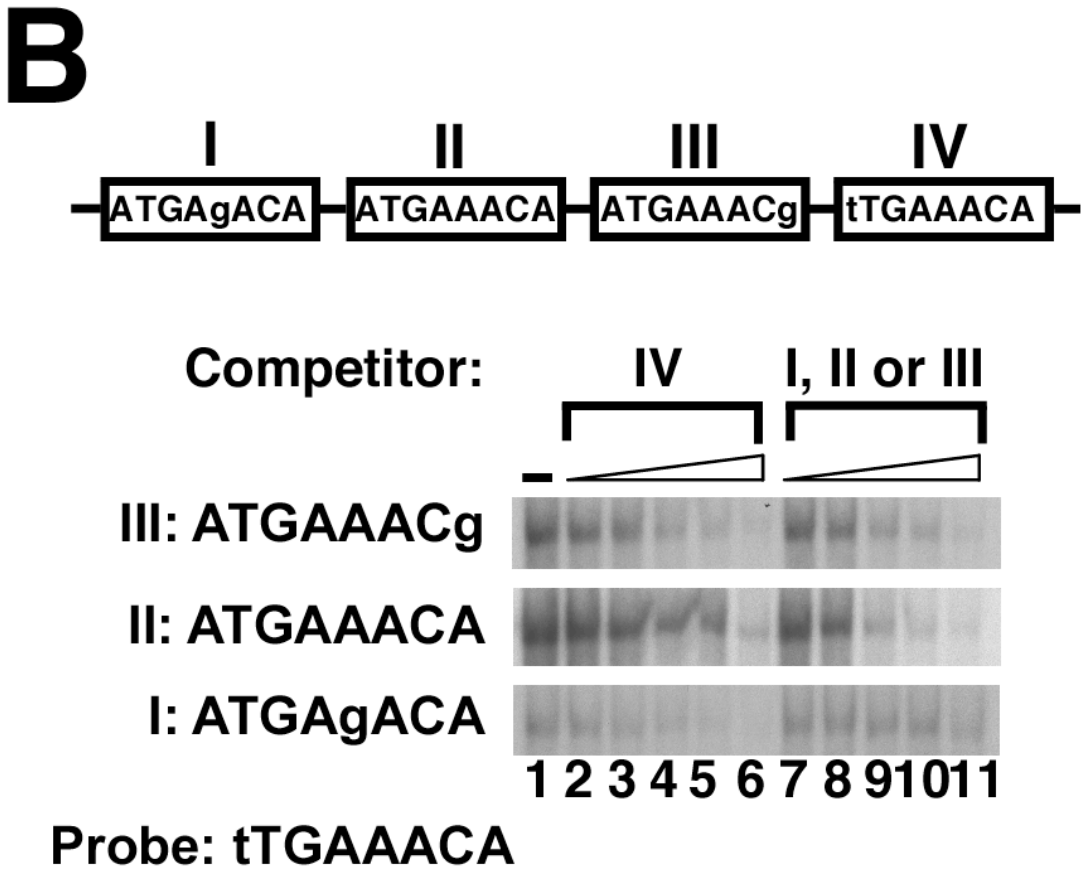
The typical sequence of the Ste12 binding site and pheromone response element was defined as ATGAAACA, based on genetic observations and global localization of Ste12 protein. However, potential PREs that differ from this consensus can be observed in promoter regions of various pheromone responsive genes. For example, there are four PREs in the *FUS1* promoter, only one of which completely matches the consensus. The other three variants have a single nucleotide substitution. To determine the extent that these differences affect Ste12 binding *in vitro*, I used a series of PRE mutants/variants in competitions for binding of Ste12 to the consensus PRE in EMSA. Each PRE mutant/variant bears a single nucleotide substitution from the consensus PRE. By comparing the differences that each nucleotide substitution causes in competition for binding with the consensus PRE, their individual contribution for binding Ste12 can be determined. EMSA reactions were performed using a consensus PRE probe, and competitions with increasing concentrations of unlabeled consensus (Fig. 3.4A, lanes 2-6) or the mutant/variant oligos (Fig. 3.4A, lanes 7-11). Complexes formed with Ste12 protein in the presence of the competitor oligonucleotides were quantified, and results relative to the control reactions are presented in Fig. 3.5. In these experiments, I found that substitutions of any of the nucleotides within the consensus PRE impairs Ste12 binding to at least some extent. In particular substitutions at nucleotides G3, A5 and A6 greatly reduced competition by these mutant/variant oligos. I also compared the relative affinity of the four PREs from the *FUS1* promoter (Fig. 3.4B, designated PRE I, II, III, IV, 5' to 3', top) for binding of Ste12 using competitions in EMSA. Among these, PRE

II represents the consensus sequence, while PRE III and PRE IV have substitutions at the 3' end (A to g) and 5' end (A to t) respectively. PRE I has a substitution in the central AAA tri-nucleotide, where A5 is substituted by G. In similar EMSA competition experiments, I found that that each of the *FUS1* PREs has a different relative affinity for Ste12, which can be ranked, from strongest to weakest, PRE II (the consensus), IV, III and I (Fig. 3.4B). To quantify the differences in relative affinity of each of the PRE mutants/variants and the four PREs of *FUS1* promoter, I calculated a relative competition strength (RCS) for each of the PRE sequences. The RCS value for each mutant/variant PRE sequence represents the amount of competitor oligonucleotide needed to compete 50% of Ste12 binding relative that of the consensus PRE oligo (Fig. 3.5A and Table 3.1). Based on the RCS values for the variant PREs from the *FUS1* promoter, I can represent the relative contribution for each nucleotide within the consensus PRE for binding of Ste12 *in vitro* (Fig. 3.5B).

Figure 3.4 Nucleotides required for binding of full-length Ste12 to the consensus PRE *in vitro*.

(A) EMSA reactions were performed with recombinant wild-type Ste12 and a labeled oligonucleotide bearing a single consensus PRE (RS010/011). Binding reactions contained no competitor (lane 1), or a 0.625- (lanes 2 and 7), 1.25- (lanes 3 and 8), 2.5- (lanes 4 and 9), 5- (lanes 5 and 10) or 10- (lanes 6 and 11) fold molar excess of unlabeled consensus oligo (lanes 2–6) or the indicated mutant oligos (lanes 7–11). Mutant oligos (lines 1–7) contained a single nucleotide substitution from the consensus PRE (Table 3.1). (B) The sequence of the *FUS1* promoter indicating the position of four PREs (designated sites I, II, III and IV, 5'–3'). EMSA reactions were performed as in (A) but using a labeled oligonucleotide bearing PRE IV (IS1428/1429), and with the unlabeled competitors as indicated.


A
Competitor:
ATGAAACA
PRE Mutants
1 2 3 4 5 6 7 8
1. AaGAAACA
2. ATtAAACA
3. ATGcAACA
4. ATGgAACA
5. ATGAgACA
6. ATGAAgCA
7. ATGAAAgA
Probe: ATGAAACA
1 2 3 4 5 6 7 8 9 10 11


B
I
II
III
IV
ATGAgACA
ATGAAACA
ATGAAACg
tTGAAACA
Competitor:
IV
I, II or III
-
III: ATGAAACg
II: ATGAAACA
I: ATGAgACA
1 2 3 4 5 6 7 8 9 10 11
Probe: tTGAAACA

3.5 The relative PRE strength for binding of Ste12 *in vitro* influences pheromone response

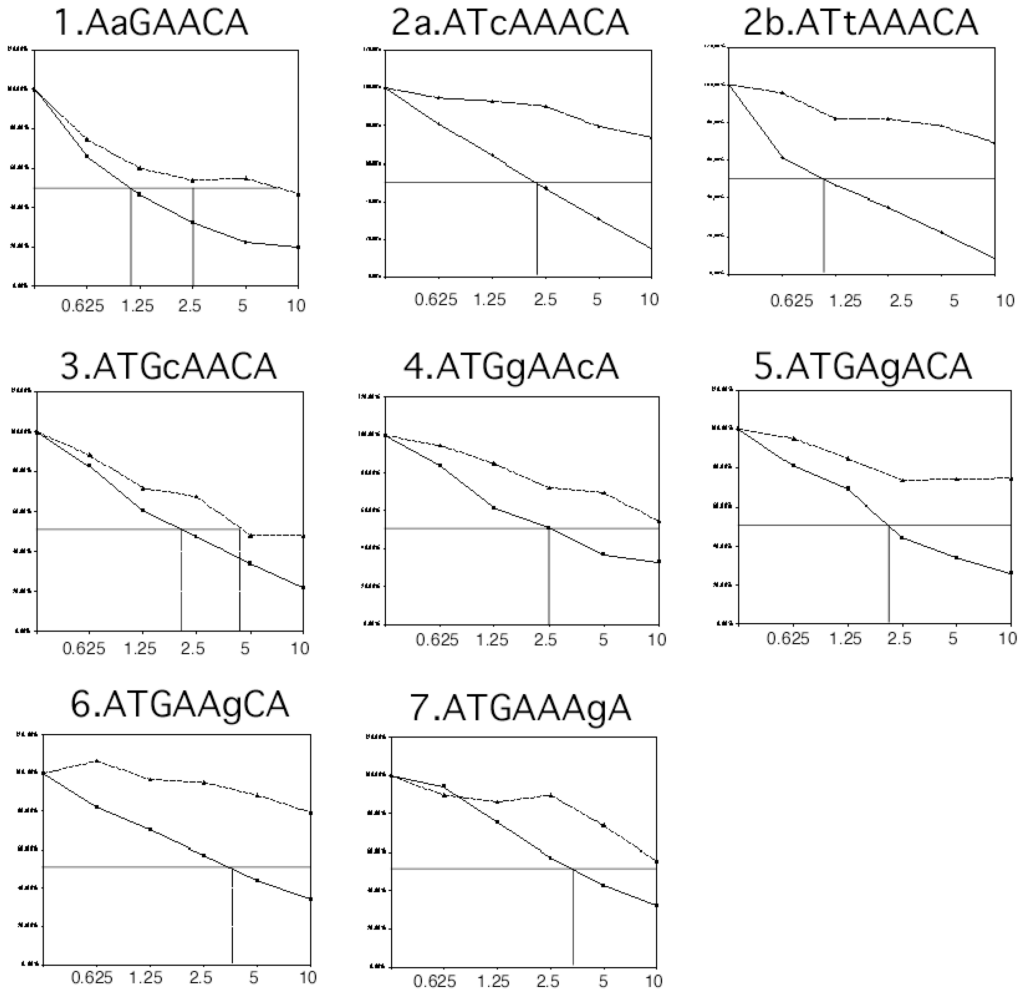
To examine how the relative strength of PREs for binding of Ste12 *in vitro* affects transcriptional response to pheromone *in vivo* I constructed a series of artificial reporter genes. The constructs contained two directly repeated PREs, upstream of a minimal *GAL1* core promoter fused to *LacZ*. The reporter genes were integrated at single copy into a disrupted *lys2* locus. Because the copy number and the location of integration is identical, the amount of β-galactosidase activity produced is directly proportional to the extent of the pheromone response. Consistent with previous results, I found that neither a single consensus PRE, nor any of the PRE mutants, caused induction in response to pheromone (Fig. 3.6A, lines 1-3), whereas in contrast two directly repeated PRE consensus elements produced ~2000 fold induction within 60 minutes of pheromone treatment (Fig. 3.6B, line 1). To examine how the RCS, which reflects the strength of Ste12 binding *in vitro*, affects induction by pheromone, I compared the response of reporters bearing two copies of the PRE variants. I found that each of the PREs caused a response to pheromone, and the extent of induction seemed proportional to the RCS values of the variants. Two PREs representing PRE II from the *FUS1* promoter produced a response only slightly less than the full *FUS1* promoter (Fig. 3.6B, compare lines 1 and 5). In contrast, two copies of the weakest PRE I from *FUS1*, showed the lowest level of pheromone responsiveness (Fig. 3.6B, line 4).

Figure 3.5 Calculation of the relative competition strength (RCS) values.

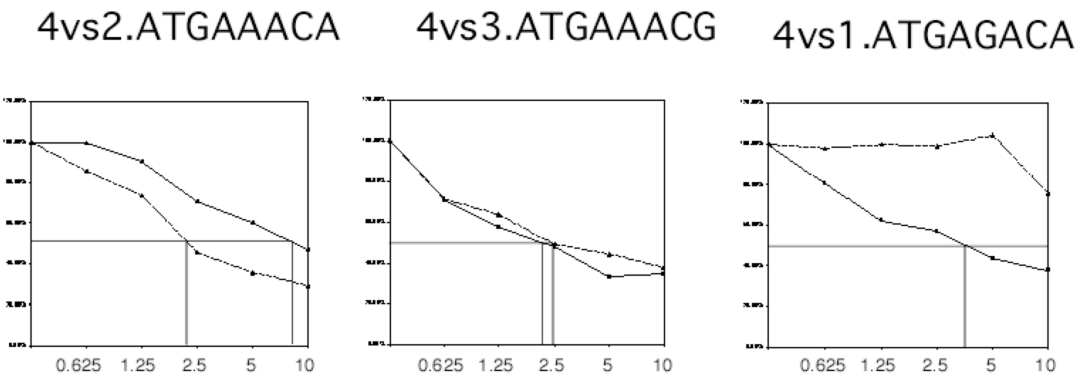
(A) Panel 1: Protein-DNA complexes produced in the EMSA reactions shown in Figure 4A were quantified using ImageQuant, and the % Ste12 protein bound relative to samples containing no competitor oligonucleotide was plotted vs. the fold molar excess unlabeled competitor. From each plot, a molar excess was calculated that produced a 50% decrease in binding of Ste12 to the labeled probe. The Relative Competition Strength (RCS) for each oligonucleotide was calculated as a ratio of molar excess required to produce 50% competition relative to the wild type oligo in a parallel experiment (Table3.1). Panel 2: As above, with results from the EMSA reactions shown in Fig 3.4B. (B) The RCS was calculated for each mutant oligo (Table 3.1). The effect that mutation of each nucleotide of the consensus PRE has on the binding of Ste12 *in vitro* is indicated proportional to the font size for each residue.

A

1 - Competitions Figure 3.5A (and not shown):



2 - Competitions Figure 3.5B:



B

Table 3.1 RCS of mutant PREs for binding of wild-type Ste12 to a PRE consensus (ATGAAACA) *in vitro*.

***FUS1* PRE**[a]	**Sequence**	**RCS**[b]
II	ATGAAACA	1.00
IV	tTGAAACA	0.27[C]
	AaGAAACA	0.45
	ATaAAACA	0.81
	ATcAAACA	0.03[C]
	ATtAAACA	0.01[C]
	ATGcAACA	0.69
	ATGgAACA	0.20[C]
I	ATGAgACA	0.02[C]
	ATGAAgCA	0.05[C]
	ATGAAAgA	0.30
III	ATGAAACg	0.26

[a] PREs represented in the *FUS1* promoter (Fig. 3.4B). [b] RCS for each oligo was calculated from the concentration of unlabeled competitor oligonucleotide required to compete 50% of total Ste12 protein bound to the consensus/variant PRE, relative to competition in the same experiment with a PRE mutant/variant (Fig. 3.5). [c] Concentrations of oligo required for 50% competition was calculated by extrapolation.

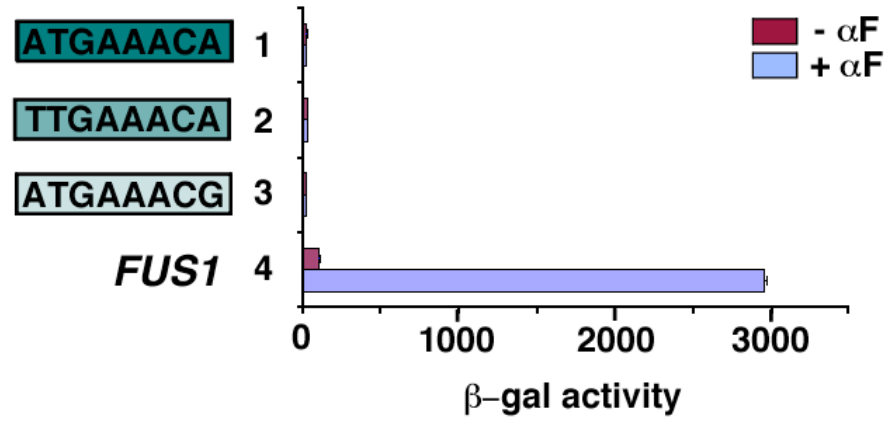
According to the data shown above, using the artificial reporter system, there is a positive correlation between the relative affinity for Ste12 *in vitro* and the response to pheromone *in vivo*. I examined this effect further to determine the extent that pheromone responsiveness is affected when only one of the two directly repeated PREs bears a substitution that affects Ste12 DNA binding *in vitro*. In the same *LacZ* reporter vector, a series of mutations were introduced into the 3' PRE (Fig. 3.6C, lines 1-5). I found that reporters bearing substitutions within the 3' PRE still produced a response to pheromone, but to a lesser extent (Fig. 3.6C, lines 1-2). For example, mutation of the central A5 residue of the AAA trinucleotide, causes an approximately two fold reduction in pheromone inducibility in combination with a consensus PRE (compare Fig. 3.6B, line 1, and Fig. 3.6C, line 1). More severe mutations of the 3' AAA trinucleotide caused a more severe loss of induction, as mutation of two of the central A residues inhibits response by approximately ten-fold (Fig. 3.6C, line 2), and a PRE bearing substitution of all three A residues completely prevents the response to pheromone. These results indicate that pheromone response can be mediated by a single consensus PRE in combination with non-consensus PREs, but to a lesser extent.

I also tested the effect of mutations in both directly repeated PREs in the artificial promoter constructs for pheromone response. The results showed that inducibility was strongly reduced when both PREs have mutations that limit binding of Ste12 *in vitro*. For example, directly-repeated PREs with substitutions of residues A1 and A8 respectively, each of which has a relatively minor effect on binding Ste12 *in*

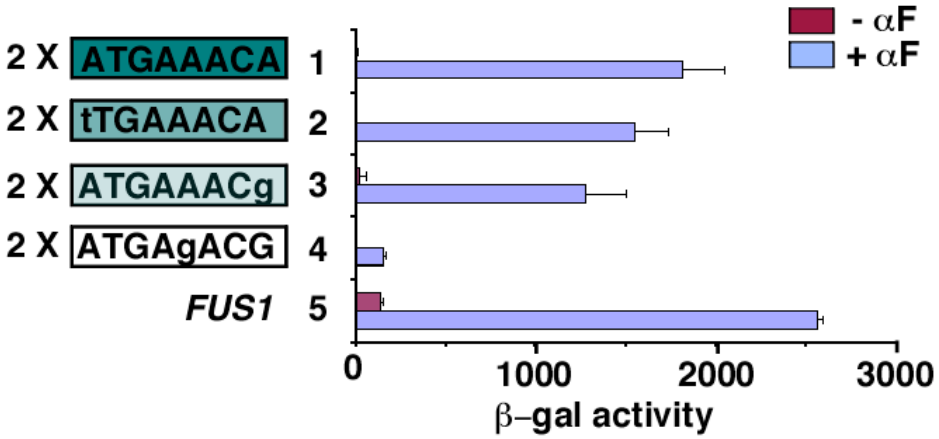
Figure 3.6 The pheromone response conferred by two directly-repeated PREs *in vivo* is proportional to their relative affinity for Ste12 *in vitro*.

(A) Strains bearing single-copy integrations of a minimal *GAL1-LacZ* reporter with one copy of the indicated PRE (lines 1–3) were left untreated (red bars) or treated with α-factor for 60 min (blue bars) prior to harvesting the cells and assaying β-galactosidase activity from three independent experiments. The shading of the boxes containing the PRE sequence indicates the relative competition strength for Ste12 *in vitro*, with the stronger PREs being shaded darker and the weaker PREs shaded lighter. Line 4 shows results from a strain bearing the full *FUS1-LacZ* promoter. (B) Strains bearing single-copy integrations of a minimal *GAL1-LacZ* reporter with two copies of the indicated PRE (lines 1–4). Cells were treated the same as in (A). (C) Reporter genes bearing a consensus PRE and PREs containing substitutions of the central AAA trinucleotide were assayed as in (A). (D) Combinations of consensus PREs and PREs bearing the indicated mutations were assayed in the same context as described above. The spacing between two PREs is 3 bp in B, C, and D.

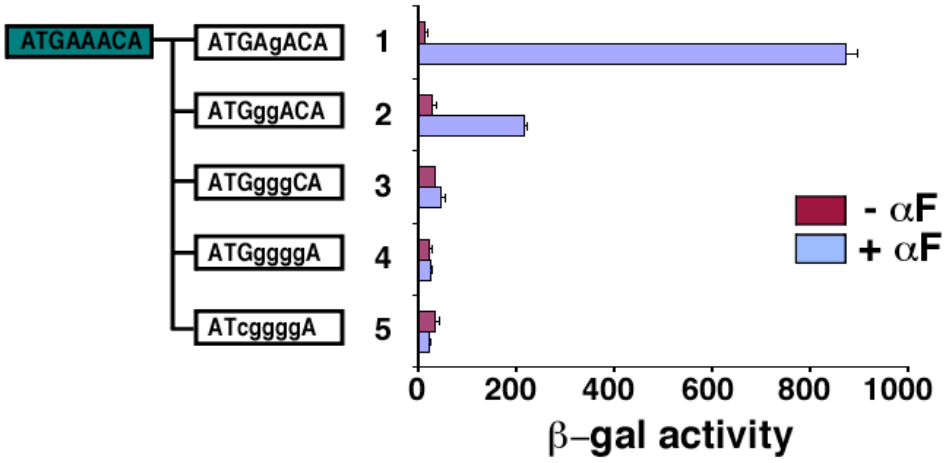
A


ATGAAACA 1
TTGAAACA 2
ATGAAACG 3
FUS1 4
- αF
+ αF
0
1000
2000
3000
β–gal activity

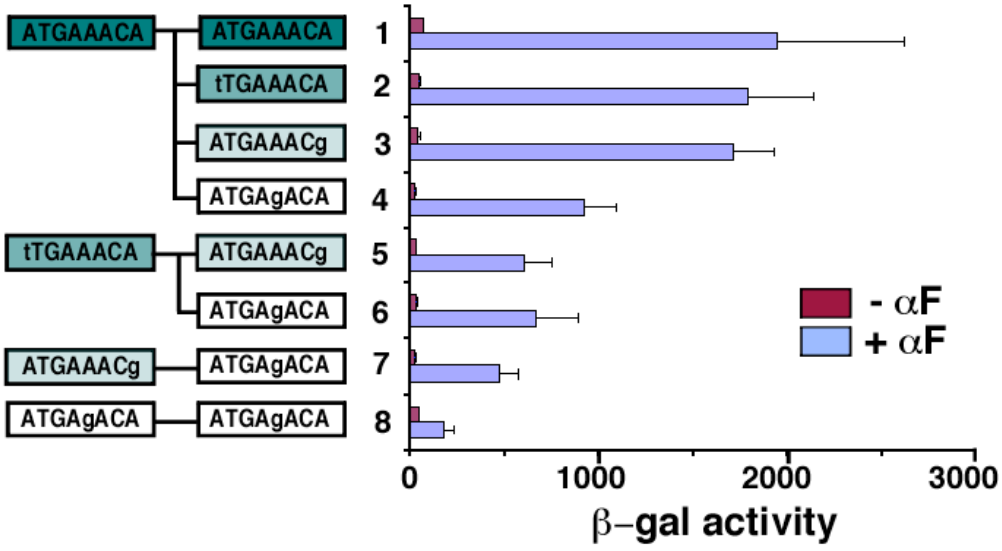
B


2 X ATGAAACA 1
2 X tTGAAACA 2
2 X ATGAAACg 3
2 X ATGAgACG 4
FUS1 5
- αF
+ αF
0
1000
2000
3000
β–gal activity

C


ATGAAACA
ATGAgACA
ATGggACA
ATGgggCA
ATGggggA
ATcggggA
1
2
3
4
5
- αF
+ αF
0
200
400
600
800
1000
β–gal activity

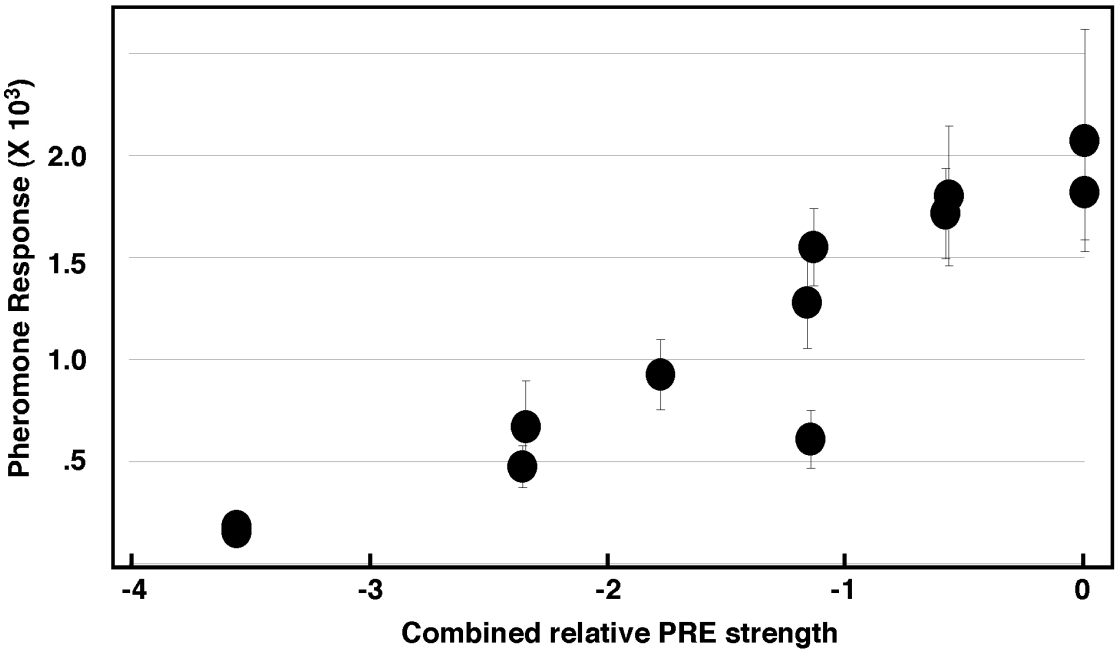
D


ATGAAACA
ATGAAACA
tTGAAACA
ATGAAACg
ATGAgACA
tTGAAACA
ATGAAACg
ATGAgACA
ATGAAACg
ATGAgACA
ATGAgACA
1
2
3
4
5
6
7
8
- αF
+ αF
0
1000
2000
3000
β–gal activity

vitro (Fig. 3.4), caused an approximately four-fold defect in inducibility relative to two consensus PREs (Fig. 3.6D, line 5). Combinations of PREs that have more serious defects in binding Ste12 produce proportionally less response (Fig. 3.6D, lines 6 and 7), although even two quite weak directly-repeated PREs retain a detectable level of inducibility (Fig. 3. 6D, line 8). These results demonstrated that a normal response to pheromone can be conferred by a single strong consensus PRE in combination with much weaker adjacent PREs, with a level of inducibility proportional to the relative strength of the second PRE. Interestingly, when we examined the effect of the combined RCS of two directly-repeated PREs on the response to pheromone, we observed a direct and simple linear relationship between the product of the RCS values and pheromone responsiveness (Fig. 3.7), which implies that binding of Ste12 to DNA is a limiting event for induction of pheromone-responsive transcription.

Figure 3.7 The combined relative strength of two directly-repeated PREs produces a proportionally linear response to pheromone.

A combined relative PRE strength for each of the reporter genes described in Fig. 3.6 was calculated as $\log(RCS_{PRE1} \text{ X } RCS_{PRE2})$ and plotted against the respective pheromone responsiveness for each reporter (β-galactosidase activity X 10^3).


Pheromone Response (X 10³)
2.0
1.5
1.0
.5
-4
-3
-2
-1
0
Combined relative PRE strength

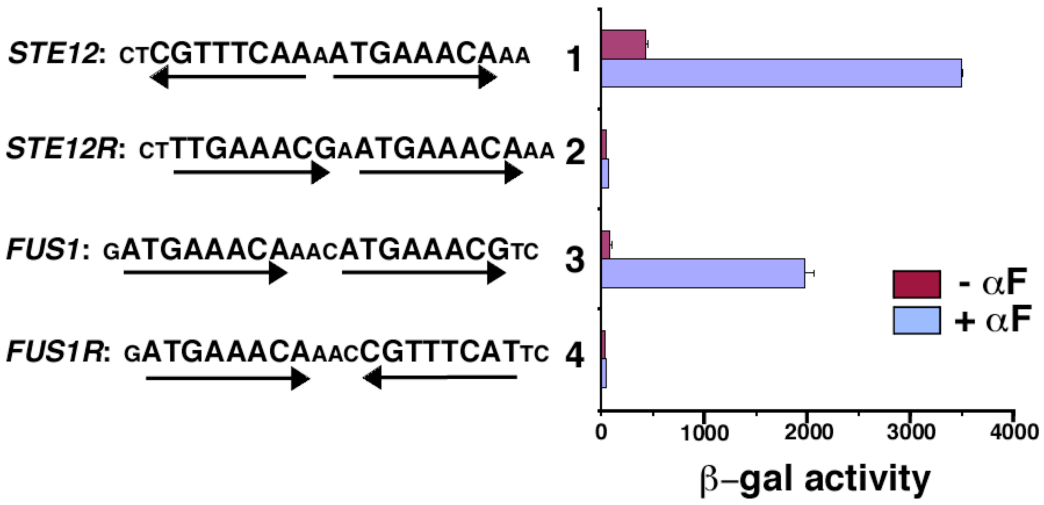
3.6 Organizational constraints on two PREs for pheromone response

With the previous results I addressed how nucleotide residues in the PRE affect the binding of Ste12 *in vitro* and pheromone responsiveness *in vivo*. In this section I examine how the orientation of two PREs and their intervening spacing contribute to the pheromone response. Amongst the strongly inducible pheromone-responsive promoters shown in Fig. 1, I can distinguish three different types of orientations between multiple PREs. These include two PREs with a head-to-tail (*FIG1* and *FUS1*), tail-to-tail (*PRM6*) and head-to-head orientation (*STE12*). Depending on the number of PREs, there might be different types of orientations observed within a single promoter, such as those in *FUS1* and *STE12*. To examine how these different PRE orientations affect pheromone response, I compared two different types of PRE orientations from partial promoter sequences of *STE12* and *FUS1*. Two of the PREs from the *STE12* promoter are closely positioned in a tail-to-tail orientation. On the other hand, two closely spaced PREs from the *FUS1* promoter are oriented head-to-tail. I found that when inserted upstream of the minimal *GAL1* promoter-LacZ fusion reporter, both of these orientations produced response to pheromone, with the tail-to-tail orientation from *STE12* promoter slightly stronger (Fig. 3.8A, lines 1 and 3). Additionally, I noted that the *STE12* tail-to-tail orientation produces a higher level of basal expression than does the head-to-tail orientation (Fig. 3.8A, lines 1 and 2). *STE12* is known to be expressed at a constitutively high basal level without pheromone treatment. This is necessary to maintain steady levels of Ste12 protein for response to pheromone. Consequently, this result suggests

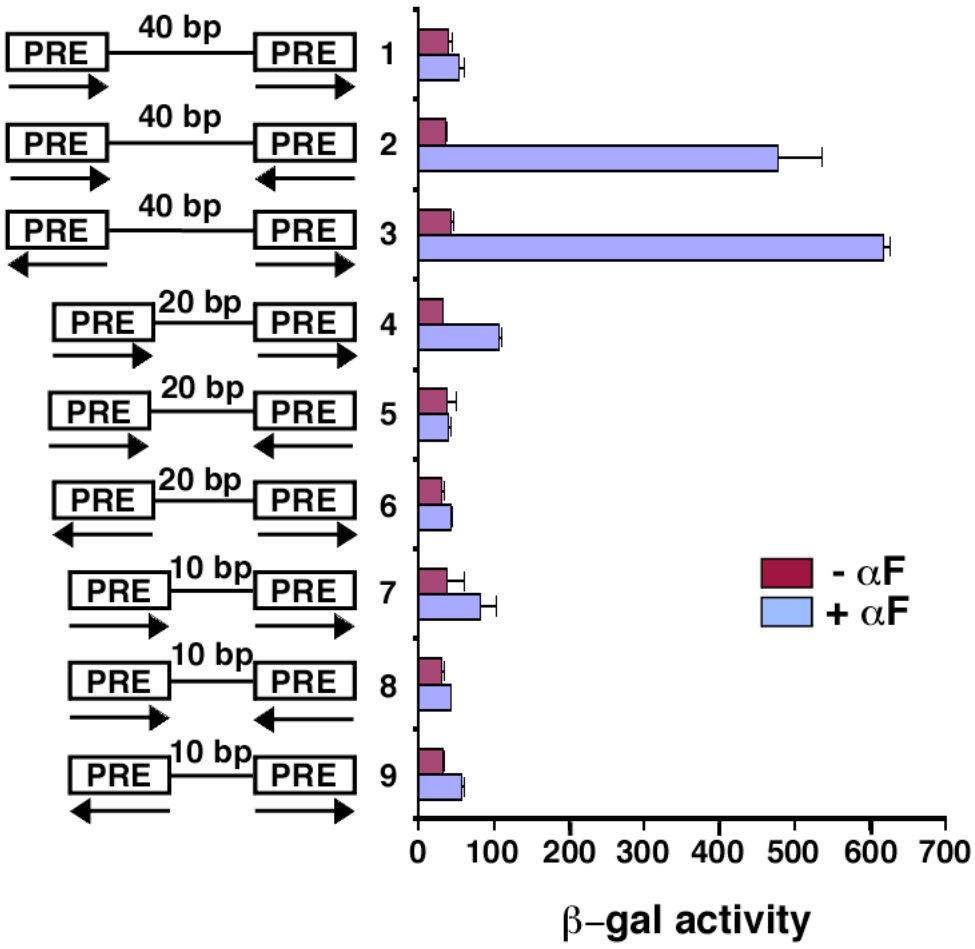
Figure 3.8 Organizational constraints on closely-spaced PREs for pheromone response *in vivo*.

(A) Pheromone responsiveness of minimal promoters containing PREs II and III from the *STE12* promoter in a tail-to-tail orientation (line 1), directly-repeated consensus PREs from the *FUS1* promoter (PRE II, line 2) or with the second consensus PRE inverted into a head-to-head orientation (line 3). (B) The consensus PREs from the *FUS1* promoter were moved apart to produce an intervening spacing of ten (lines 7–9), 20 (lines 4–6) or 40 (lines 1–3) nucleotides, with the orientation of the PREs as indicated.

A


STE12: CTCGTTTCAAAATGAAACAAA 1
STE12R: CTTTGAAACGAATGAAACAAA 2
FUS1: GATGAAACAAACATGAAACGTC 3
FUS1R: GATGAAACAAACCGTTTCATTC 4
- αF
+ αF
0
1000
2000
3000
4000
β–gal activity

B


PRE
40 bp
PRE
1
2
3
20 bp
4
5
6
10 bp
7
8
9
- αF
+ αF
0
100
200
300
400
500
600
700
β–gal activity

that constitutive *STE12* expression might result from the tail-to-tail orientation of the PREs. Importantly, not all orientations of closely spaced PREs are tolerated, because inverting the 5' PRE from the *STE12* promoter into a head-to-tail orientation and the 3' PRE from the *FUS1* promoter into a head-to-head orientation eliminate both the basal level and pheromone responsive expression (Fig. 3.8A, lines 2 and 4). This demonstrates that there are constraints on how two consensus PREs can be oriented for induction by Ste12 in the context of an artificial promoter.

Because most pheromone-responsive promoters have multiple PREs separated by more than several nucleotides, I further examined these constraints by determining the effect of the intervening nucleotide spacing on pheromone response. For this analysis, I altered the spacing between PREs in tail-to-tail, head-to-head, and tail-to-tail orientations (Fig. 3.8B). The results showed that with spacing of 10 and 20 bp, none of the PRE orientations produced a normal response to pheromone. However, with a 40 bp spacing, both the head-to-head and tail-to-tail orientations were induced by pheromone, but not the head-to-tail orientation. This result suggests that the PRE orientation and length of intervening sequence are both determinants for pheromone response, and together, they dictate constraints on PRE organization within the promoter. Pheromone responses for the head-to-tail orientation with spacings of five, seven, and nine bp were also examined and did not produce response (data not shown). Important to note, the two PREs oriented in a head-to-tail orientation from the *FUS1* promoter (Fig. 3.8A, line 3) produces a level of expression nearly as strong as the full *FUS1* promoter itself, which suggests that not every PRE in the promoter may be functional, at least simultaneously, and that a

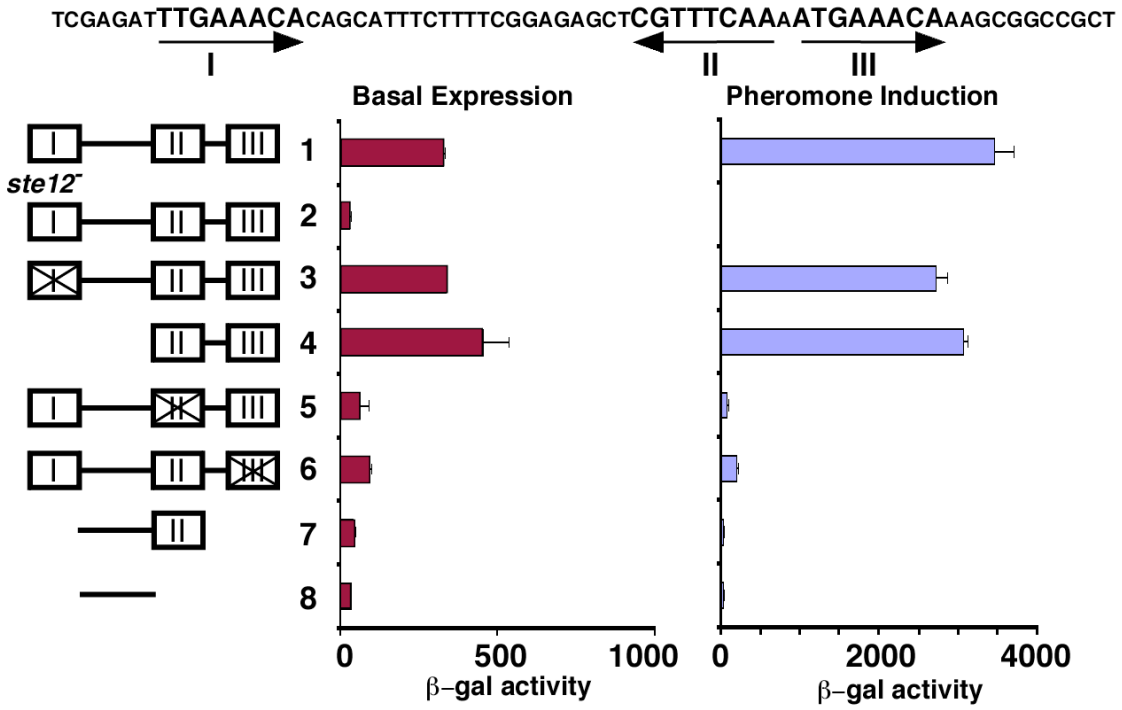
sufficiently strong response can be produced by only two correctly oriented elements (compare Fig. 3.6B, lines 1 and 5).

3.7 Organizational constraints on the *STE12* promoter for pheromone response

As a key regulator of pheromone response, regulation of *STE12* basal level and pheromone-induced expression is critical for coordination of the mating response. Based on the results shown above, I examined a segment of the *STE12* promoter to determine how these constraints on PRE organization may dictate *STE12* basal and pheromone-induced expression. I found that a segment spanning -475 to -428 from the transcriptional start site in the *STE12* promoter, spanning three PREs (designated PRE I, PRE II and PRE III) was sufficient to confer response to pheromone (Fig. 3.9, top). A reporter gene bearing this segment upstream of a minimal *GAL1* core promoter - *LacZ* fusion conferred a much higher level of basal expression and was induced ~10 fold in response to pheromone (Fig. 3.9, line 1). Basal and induced expression are both Ste12-dependent, because no response was observed in a *ste12* null mutant strain (line 2). A mutation of PRE I, either alone or in combination with deletion of the intervening sequence between PRE I and II, did not have a obvious effect on either the basal or pheromone responsive expression (lines 3 and 4). This suggests that PRE II and III on their own confer the majority of *STE12* expression. This is further supported by the finding that deletion or mutation of either PRE II or PRE III prevents expression (lines 5-8). Important to note, PRE I and II are spaced 22 nucleotides apart in a head-to-head orientation, and I and III 31 nucleotides apart in a tail-to-tail orientation. Based on the results shown above, neither of these combinations of PREs on their own would be predicted to confer pheromone induction.

Figure 3.9 Orientation and spacing of PREs contributing to response of the *STE12* promoter.

The sequence of the *STE12* promoter region containing the three most distal PREs (designated I, II, and III, 5'–3') is indicated. An oligonucleotide representing this sequence, or bearing mutations or deletions as indicated, was inserted upstream of the minimal *GAL1* core promoter-*LacZ* reporter gene. The expression of the reporter was measured in untreated cells (basal expression, left) or cells treated with α-factor for 60 min (pheromone induction).


TCGAGATTTGAAACACAGCATTTCTTTTCGGAGAGCTCGTTTCAAAATGAAACAAAGCGGCCGCT
I
II
III
Basal Expression
Pheromone Induction
ste12-
1
2
3
4
5
6
7
8
0
500
1000
β–gal activity
0
2000
4000
β–gal activity

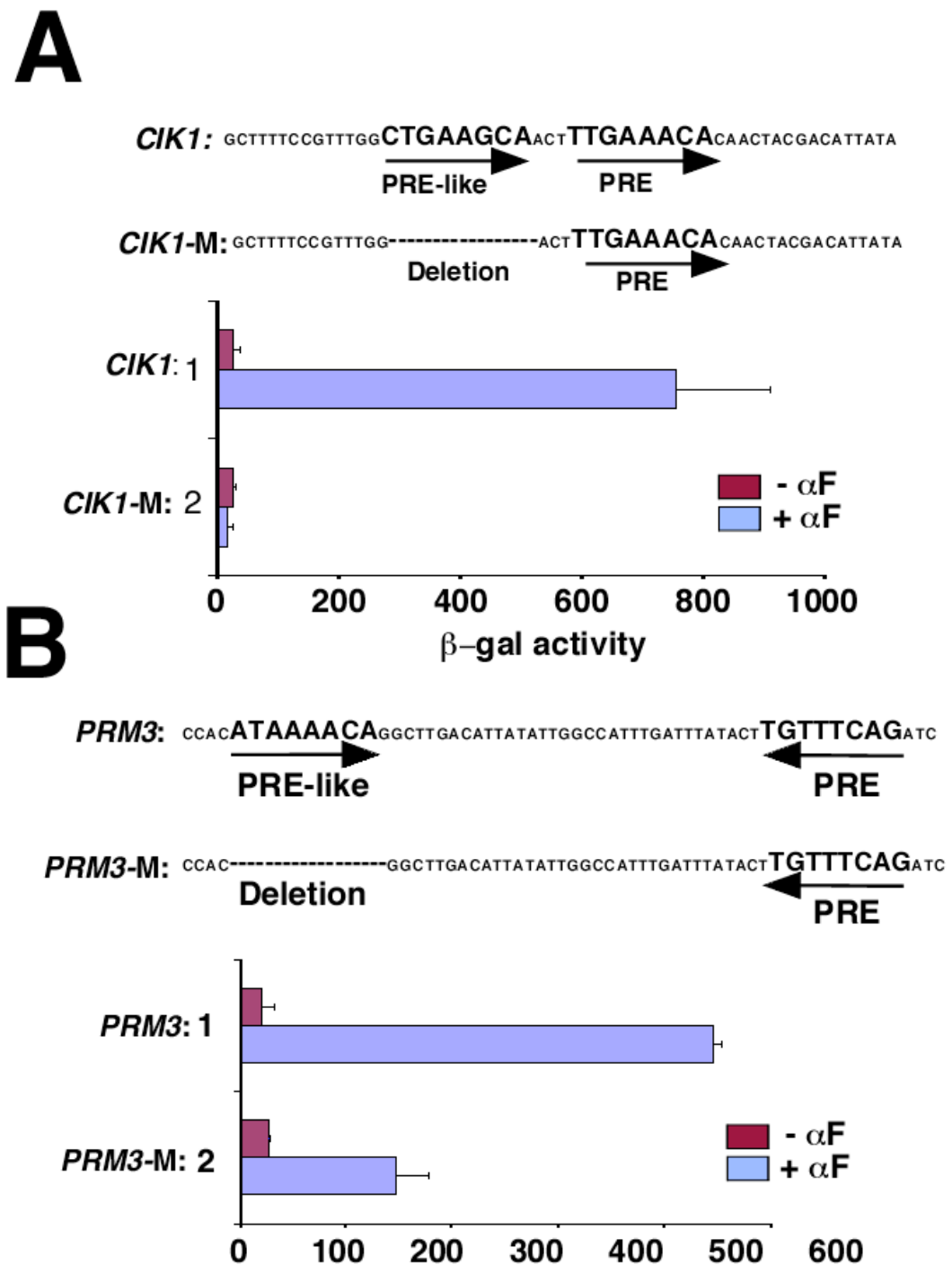
3.8 The role of PRE-like elements on pheromone responsiveness of promoters with one consensus PRE

Considering the above results, the question remains how genes like *PRM3* and *CIK1*, which appear to have only a single PRE, can respond to pheromone as this contradicts the previously defined requirement of at least two PREs for pheromone response. My results shown above indicate that a strong consensus PRE can confer at least some inducibility in combination with a much weaker PRE with proper orientation and spacing. Therefore I wondered whether some pheromone-responsive promoters with only a single PRE depend upon weaker non-consensus, or PRE-like sequences positioned within the constraints defined above. Consistent with this, I found candidate PRE-like elements in the promoters of two genes. In the *CIK1* promoter, a PRE-like sequence **c**TGAA**g**CA was found 3 bp upstream of the consensus PRE in a head-to-tail orientation. I found that a minimal *GAL1* promoter-*LacZ* reporter gene containing this segment responded to pheromone induction. Deletion of the PRE-like sequence completely abolished pheromone responsiveness (Fig. 3.10A, lines 1 and 2), indicating that this PRE-like element is functional for conferring pheromone response. A similar result was obtained with the *PRM3* promoter. A PRE-like candidate sequence AT**a**AAACA was found 36 bp upstream of the single consensus PRE in a head-to-head orientation. A subfragment of the *PRM3* promoter representing this region inserted into the minimal *GAL1-LacZ* reporter also showed pheromone responsiveness. Furthermore, deletion of the PRE-like element greatly reduced responsiveness (Fig. 3.10B, lines 1 and 2). These results suggest that non-consensus PRE-like elements in combination with a single

consensus PRE with proper orientation and spacing can confer normal response to pheromone.

Figure 3.10 A single consensus PRE can confer pheromone responsiveness in conjunction with PRE-like sequences.

(A) Sequence of the *CIK1* promoter region, indicating the consensus PRE and a PRE-like sequence. An oligonucleotide representing this sequence, or bearing a deletion of the PRE-like sequence, was inserted upstream of the minimal *GAL1* core promoter-*LacZ* reporter, and expression was measured in untreated and pheromone-treated cells. (B) Sequence of the *PRM3* promoter indicating the location of a consensus PRE and PRE-like sequence. The pheromone responsiveness of the minimal promoter bearing oligonucleotides representing the wild-type or mutant promoter sequences was measured in untreated and pheromone-treated cells.


A
CIK1: GCTTTTCCGTTTGGCTGAAGCAACTTTGAAACACAACTACGACATTATA
PRE-like
PRE
CIK1-M: GCTTTTCCGTTTGG----------ACTTTGAAACACAACTACGACATTATA
Deletion
PRE
CIK1: 1
CIK1-M: 2
- αF
+ αF
0
200
400
600
800
1000
β–gal activity
B
PRM3: CCACATAAAACAGGCTTGACATTATATTGGCCATTTGATTTATACTTGTTTCAGATC
PRE-like
PRE
PRM3-M: CCAC----------GGCTTGACATTATATTGGCCATTTGATTTATACTTGTTTCAGATC
Deletion
PRE
PRM3: 1
PRM3-M: 2
- αF
+ αF
0
100
200
300
400
500
600

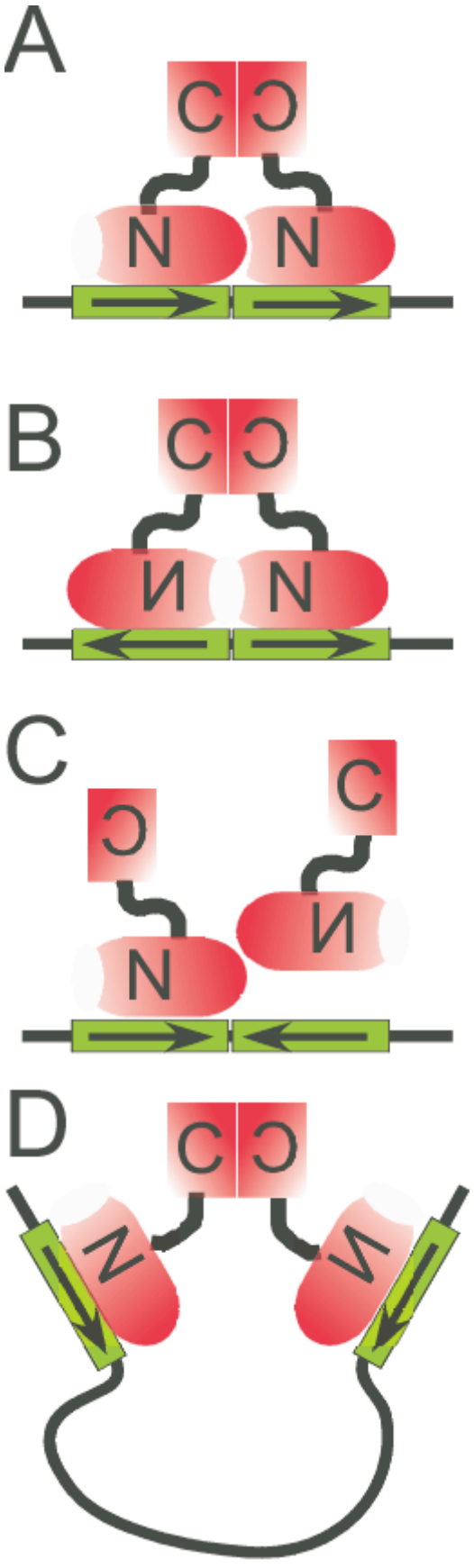
3.9 Features of Ste12 *cis*-elements that confer pheromone-response

The results in this chapter have examined how nucleotide sequences within the Ste12 *cis*-element (PRE) affect binding of Ste12 *in vitro*, how the relative affinity of PREs affects pheromone response, and how organization of two PREs is constrained to produce induction. My results are surprising in that they indicate a much more stringent requirement for positioning of two PREs for pheromone induction than could be predicted by examining elements within the promoters of responsive genes. For genes that are solely dependent upon Ste12, as is the case with my artificial promoters, the intervening spacing between two PREs must be either several nucleotides in the head-to-tail or head-to-head orientations, or has to be extended to as long as 40 base pairs. I can make several predictions from these results. First, there must be structural constraints on the Ste12 protein that limit its interaction to form multimers to bind closely spaced sequences. Interaction between Ste12 multimers bound to sequences with longer intervening sequences could be achieved by bending or twisting the DNA, which may be accommodated by interaction of two Ste12 molecules. A possible model for interaction of Ste12 on multiple sequences is depicted in Fig. 3.11. Secondly, although I have found two cases where non-consensus PREs cooperate with a PRE consensus sequence to produce pheromone response, many pheromone-responsive genes do not seem to have multiple PREs with an appropriate arrangement that could produce response to Ste12 on its own. Therefore, I suggest that Ste12 must have to act cooperatively with other transcription factors to produce a response on a significant number of its target genes. Finally, the finding that the orientation of two PREs can influence basal level expression (tail-to-tail vs. head-to-tail) indicates that the orientation of two Ste12 proteins bound to

DNA can govern transcriptional activation independently of the upstream pheromone response pathway. Implications of these observations are discussed in chapter 5.

Figure 3.11 Possible structural constraints on Ste12 for binding closely-positioned PREs.

Schematic representation of a possible mechanism for recognition of closely-spaced PREs in different conformations by Ste12 multimers. Interaction with directly-repeated PREs, positioned three nucleotides apart (A) or in a tail-to-tail orientation (B) may involve an interaction with C-terminal sequences separated from the N-terminal DNA binding domain by a flexible linker region. Some closely-spaced configurations appear to be excluded from binding Ste12 multimers, as in a closely-spaced head-to-head orientation (C). Head-to-head and tail-to-tail orientations may be accommodated providing that the sites are separated sufficiently to allow bending or twisting of the intervening DNA to enable binding of Ste12 multimers (D).


A
C
C
N
N
B
C
C
И
N
C
C
C
И
N
D
C
C
N
И

Chapter 4 Regulation of pheromone response by Cdc55

The role of the two MAP kinases Fus3 and Kss1, and the pheromone response pathway for regulation of Ste12 is well recognized, and it is presumed that this pathway affects the phosphorylation status of Ste12 and its function in pheromone response. Ste12 is known to be a heavily phosphorylated protein even in untreated haploid yeast (78). The results shown in chapter 3, in combination with previous results from our lab (Tamarkina and Sadowski, 183) indicate that wild type Ste12 protein produced in insect cells is incapable of binding both of two closely spaced PREs *in vitro* (Fig 3.3A), even though the identical configuration of PREs are capable of causing pheromone response *in vivo* (Fig. 3.6D, line 3), indicating that Ste12 expressed in yeast must undergo additional interactions or modifications to allow binding to these sites *in vivo*. Previous results from our lab have shown that Ste12 produced in insect cells becomes phosphorylated on all of the sites that are observed in yeast (Hung and Sadowski, unpublished). Consequently, I wondered whether the ability of Ste12 to interact with closely spaced PREs might require dephosphorylation. The effect of phosphatase(s) for direct regulation of Ste12 activity has not previously been addressed. However, complete dephosphorylation of our recombinant Ste12 *in vitro* using calf intestinal alkaline phosphatase (CIAP) or shrimp alkaline phosphatase (SAP) was found to prevent binding to DNA (Tamarkina and Sadowski, unpublished). Additionally, recombinant full length Ste12 protein expressed in *E. coli* is also incapable of binding DNA *in vitro* (Hung and Sadowski, unpublished).

This chapter is based on work conducted in UBC's Dr. Sadowski laboratory by Ting-Cheng Su.

Several reports have demonstrated the involvement of calcineurin, a nuclear phosphatase, in controlling cell survival during pheromone response (127, 197). However, it is unknown whether calcineurin has direct effects on Ste12. Based on this, I repeated the EMSA experiment shown in Fig. 3.3A, but pre-treated Ste12 with bovine calcineurin before addition of the probe oligo bearing two copies of PRE. As in previous experiments, untreated Ste12 does not form multiple complexes on the 2 PRE oligo. However, I found that upon calcineurin treatment, a slower migrating subpopulation of Ste12 complexes appeared in the EMSA, perhaps representing formation of multimers (Fig. 4.1). These complexes were confirmed to represent Ste12, because they could be eliminated by treatment with anti-Ste12 antisera (not shown). In contrast, I found that a truncated form of Ste12, deleted of the C-terminal 93 residues (Ste12(595)), forms a slower migrating complex with the same probe, which migrates similarly to species produced by treatment of wild type Ste12 with calcineurin. Although preliminary, these results suggest that dephosphorylation of Ste12 may be required for multimerization and binding to closely spaced PREs. Given these results, I directly examined whether calcineurin affects Ste12 activity *in vivo* by measuring the effect of *cna1* and *cnb1* mutations, encoding the calcineurin subunits, on pheromone response of a reporter bearing the 2 PREs and found no differences relative the wild type yeast strain (not shown). This indicates that although mild treatment with bovine calcineurin seems to affect binding of wild type Ste12 *in vitro*, it does not appear that pheromone signaling is directly affected by this phosphatase *in vivo*. Nevertheless, based on results discussed above, I propose that dephosphorylation on Ste12 by one or more phospatase(s) must be involved in regulating Ste12 activity and yeast pheromone response (Fig. 4.2)

Another phosphatase subunit that was previously reported to possibly affect Ste12 activity is Cdc55, which is a regulatory subunit of yeast PP2A. Mutations of *cdc55* were shown to prevent filamentous growth of yeast by two different groups, although neither of these studies had shown direct involvement of Ste12 (82, 126). Based on these results, I examined the possible effect of Cdc55 for regulation of pheromone response through Ste12 activity.

Figure 4.1 Treatment of Ste12 with calcineurin may promote Ste12 multimerization. EMSA reactions were performed with a labeled oligo containing two copies of a PRE (ET1/2) and full-length Ste12 from insect cells (lane 1). Ste12 protein was treated with increasing concentrations (0.2, 0.4, 0.6 and 0.8 U) of bovine calcineurin for 30 minutes prior to addition to the EMSA reactions (lanes 2 to 5). A C-terminal truncated Ste12 derivative, Ste12(595), produced in insect cells using baculovirus was added to the reaction shown in lane 6.

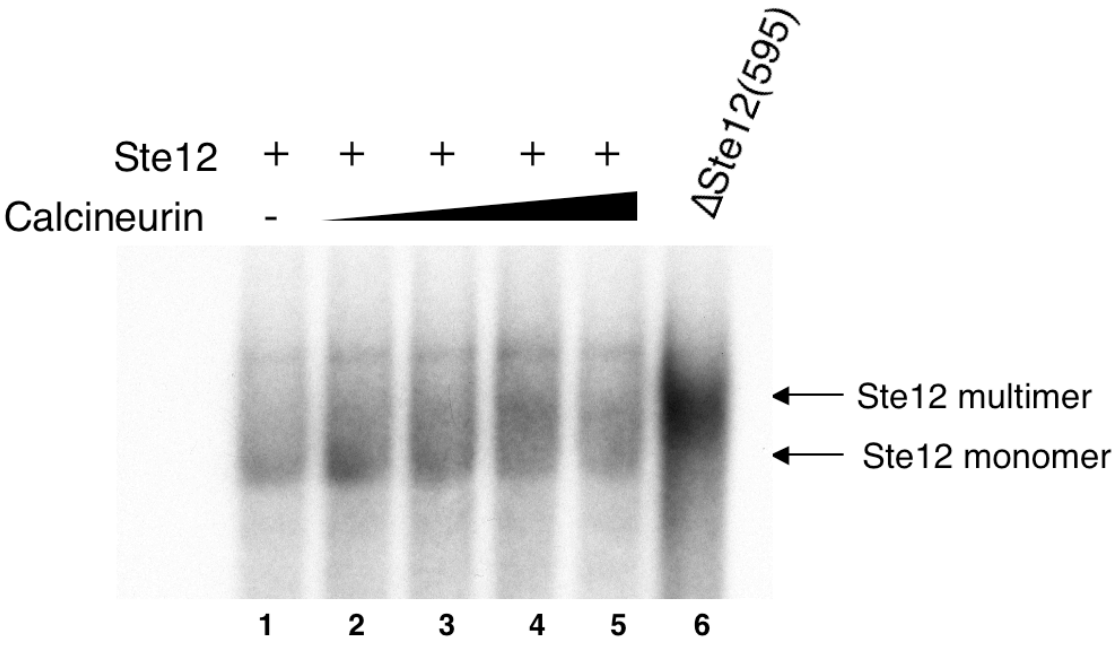
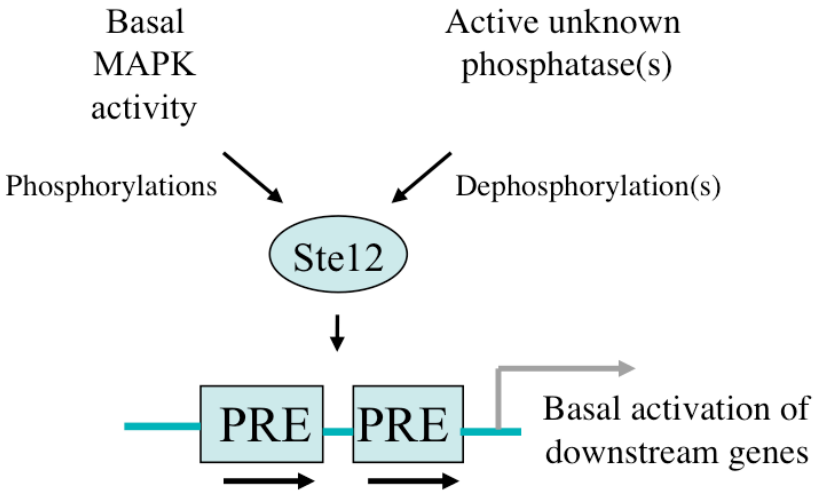

Ste12 + + + + +
Calcineurin -
ΔSte12(595)
Ste12 multimer
Ste12 monomer
1 2 3 4 5 6

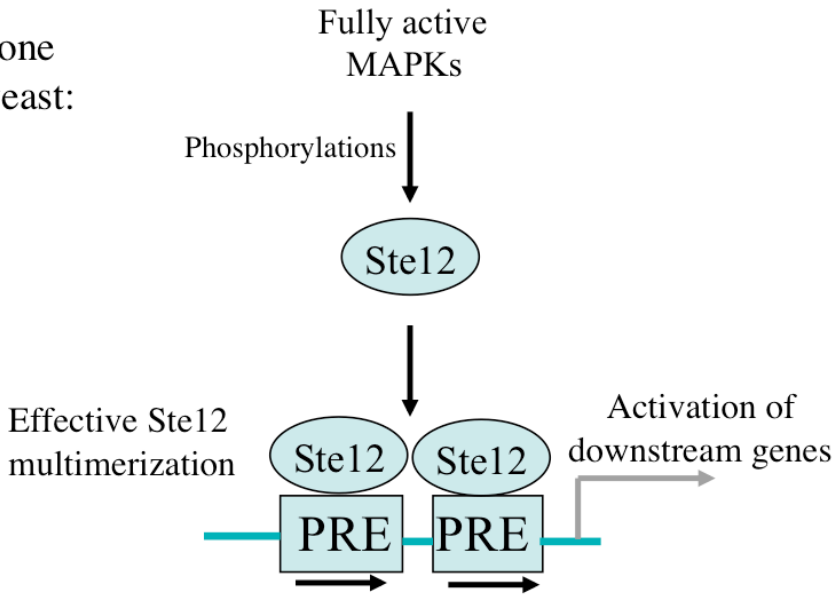
Figure 4.2 Model for regulation of Ste12 multimerization by dephosphorylation.

In untreated yeast, one or more unknown phosphatase(s) may dephosphorylate Ste12 at specific phosphorylation site(s). Dephosphorylation allows multimerization of Ste12 and binding to adjacent PREs. Basal MAPK activity allows Ste12 to activate target genes at a basal level in untreated yeast. In pheromone treated yeast, stimulation of the MAP kinases allows full activation by Ste12 to produce pheromone response.

Untreated yeast:



Pheromone
treated yeast:



4.1 Pheromone response is greatly reduced in a *cdc55* null mutant strain

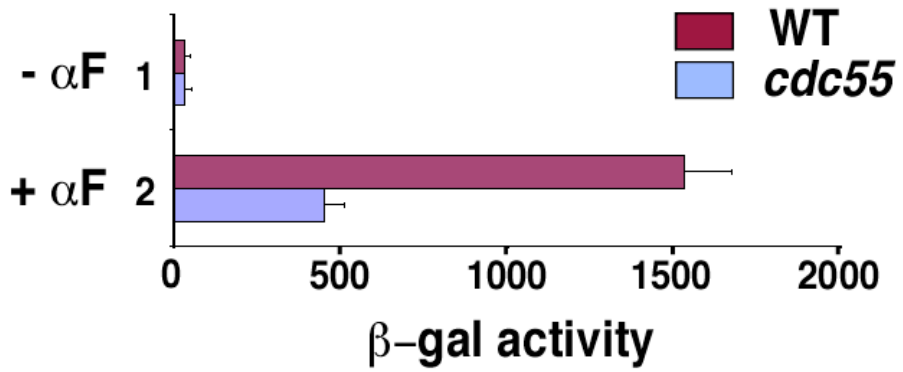
To examine the effect of Cdc55 on pheromone response, I integrated plasmid pTS126, containing two directly repeated PREs, representing PRE II and III from the *FUS1* promoter into congenic wild type and *cdc55* null mutant strains. The reporter plasmids were integrated into a *lys2* disruption at single copy. Pheromone response of strains bearing the reporters was examined following treatment with α-factor for 60 min. Basal level expression of the reporter was roughly equivalent in both strains (Fig. 4.3A, line 1) but expression was decreased by approximately 70% in the *cdc55* mutant treated with pheromone (Fig. 4.3A, line 2). This suggested that pheromone response of the reporter was at least partially dependent upon Cdc55.

Cdc55 is one of the regulatory subunits of protein phosphatase 2A, and so I also tested whether another regulatory subunit of this phosphatase, Rts1, has the same effect on pheromone response. For this experiment I constructed strains bearing disruptions of *rts1*, alone and in combination with *cdc55*. These strains, bearing the integrating reporter plasmid pTS126, were examined for pheromone response as described above. I found that the *rts1* mutation had little effect on response, and did not produce a further defect in a *cdc55* double mutant (Fig. 4.3B, lines 2 and 3) compared to wild type (Fig. 4.3A, line 1). This result indicates that the requirement for PP2A function in pheromone response is specific for Cdc55-dependent activity. Based on these results, it appears that the protein phosphatase PP2A regulates pheromone response, specifically through the regulatory subunit Cdc55.

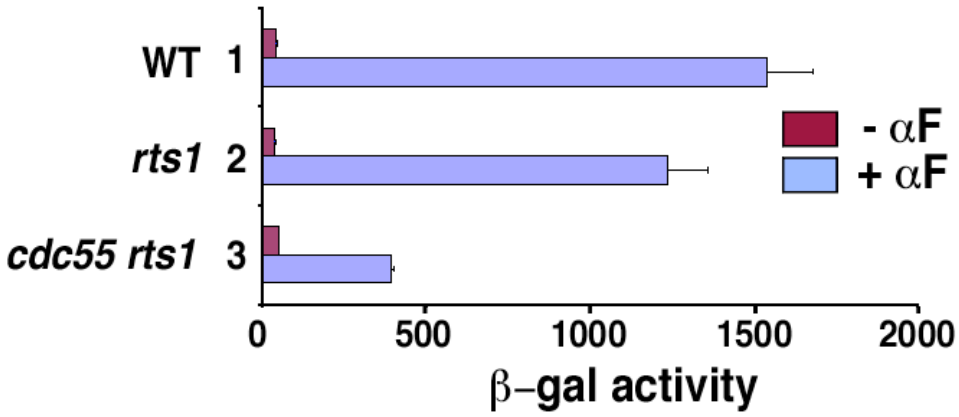
Figure 4.3 A null mutation of the protein phosphatase 2A regulatory subunit, Cdc55, greatly reduces the pheromone responsiveness.

(A) A minimal *GAL1-LacZ* reporter bearing two copies of a PRE was integrated into the wildtype or the *cdc55* null mutant strain in single copy. Cells were left untreated (line1) or treated with α-factor for 60 min (line 2) before harvesting and assaying β-galactosidase activity. (B) Wildtype yeast cells, *rts1*, and *cdc55 rts1* mutant strains bearing the same reporter gene integration as in (A) were examined for pheromone responsiveness.

A


WT
cdc55
- αF 1
+ αF 2
0
500
1000
1500
2000
β–gal activity

B

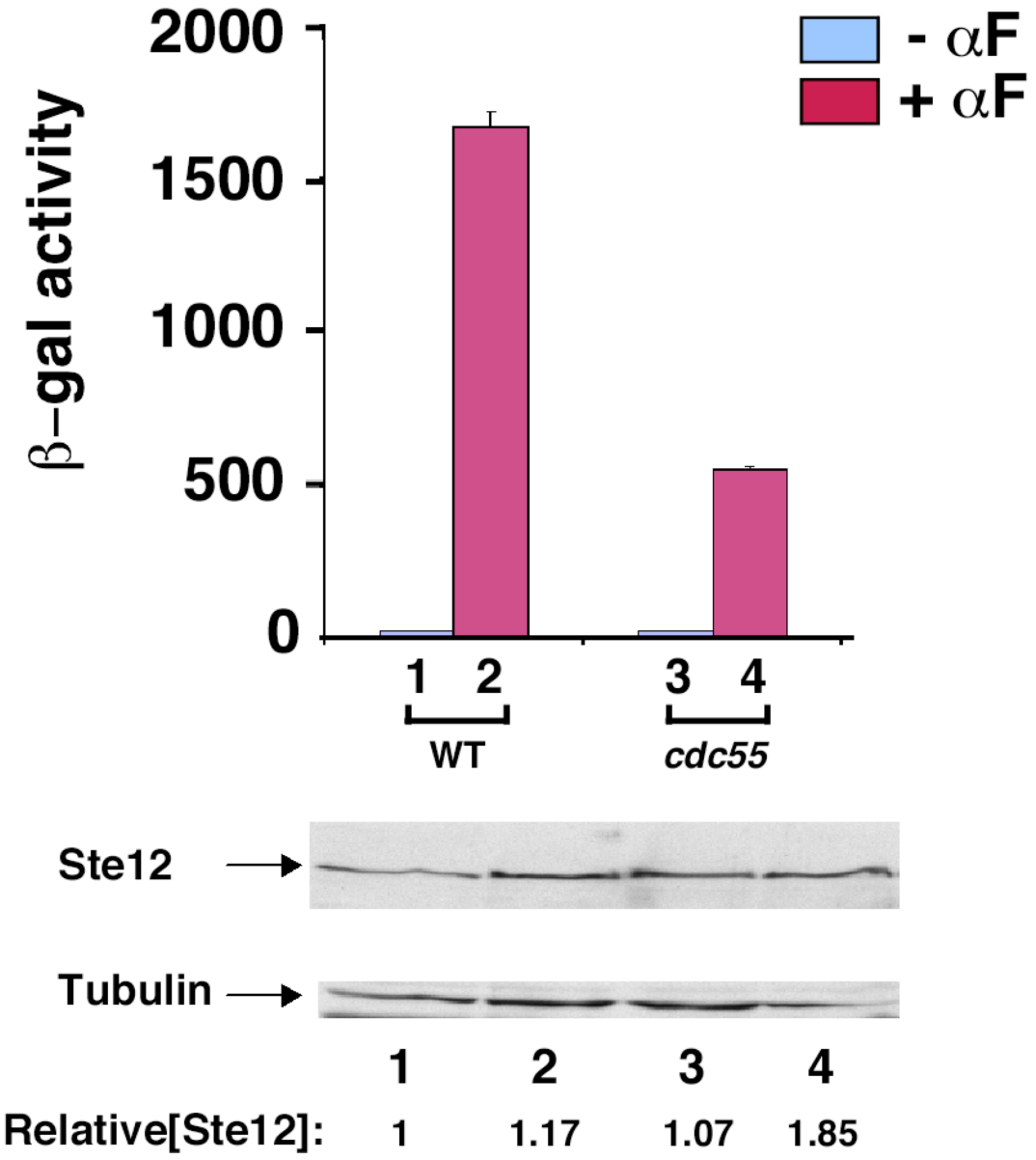

WT 1
rts1 2
cdc55 rts1 3
- αF
+ αF
0
500
1000
1500
2000
β–gal activity

4.2 The level of Ste12 expression is unaffected by the *cdc55* mutation

Ste12 is the key transcriptional activator which up-regulates expression of pheromone-responsive genes through binding to the PRE consensus sequence. To determine whether the decrease in pheromone responsiveness caused by the *cdc55* disruption resulted from altered Ste12 expression levels, I performed immunoblots on wildtype and the *cdc55* mutant strains that were untreated or treated with pheromone for 60 minutes. Consistent with previous experiments, pheromone responsiveness of the 2-PRE reporter gene (pTS126) was reduced in the *cdc55* strain relative to wild type (Fig. 4.4, upper panel). However, immunoblots of protein extracts from the same samples using Ste12 polyclonal antibodies showed that there was no obvious difference in Ste12 levels between the wildtype and *cdc55* mutant cells (Fig. 4.4, lanes 2 and 4 lower panel). This result indicates that the effect of *cdc55* on pheromone response cannot be simply explained by alterations in Ste12 protein levels. Interestingly, in comparing Ste12 protein from the untreated wildtype and *cdc55* mutant strain (lanes 1 and 3), I noticed a slightly thicker band in the *cdc55* mutant (lane 3). The same pattern is observed in both pheromone-treated wildtype and the *cdc55* strain (lanes 2 and 4). Ste12 is known to be a heavily phosphorylated protein in the absence of pheromone and becomes hyperphosphorylated after pheromone induction. Consequently, the thicker band observed in lanes 2 to 4, suggesting differences in mobility, could represent differential phosphorylation in the *cdc55* mutant relative to wild type prior to pheromone induction. This would be consistent with a possible direct role of Cdc55 as a subunit of PP2A on Ste12 function. These preliminary data indicating that Cdc55 may affect Ste12 phosphorylation are supported by further results shown below.

Figure 4.4 Expression levels of the key regulatory protein Ste12 are not affected by the *cdc55* mutation.

A single copy of a pheromone-responsive *GAL1-LacZ* reporter was integrated into both the wild type and cdc55 mutant strains. Cells were left untreated (lanes 1 and 3) or treated with α-factor for 60 min (lanes 2 and 4). Collected cells were assayed for β-galactosidase activity (upper panel) or subjected to immuno-blotting using Ste12 and α-tubulin antibodies (lower panel). The relative levels of Ste12 is indicated below, relative to tubulin, and normalized to the pheromone-untreated wild type strain (lower panel, lane 1).


2000
1500
1000
500
0
β–gal activity
- αF
+ αF
1 2
3 4
WT
cdc55
Ste12
Tubulin
1 2 3 4
Relative[Ste12]: 1 1.17 1.07 1.85

4.3 The *cdc55* mutation does not alter pheromone-responsive cell cycle arrest

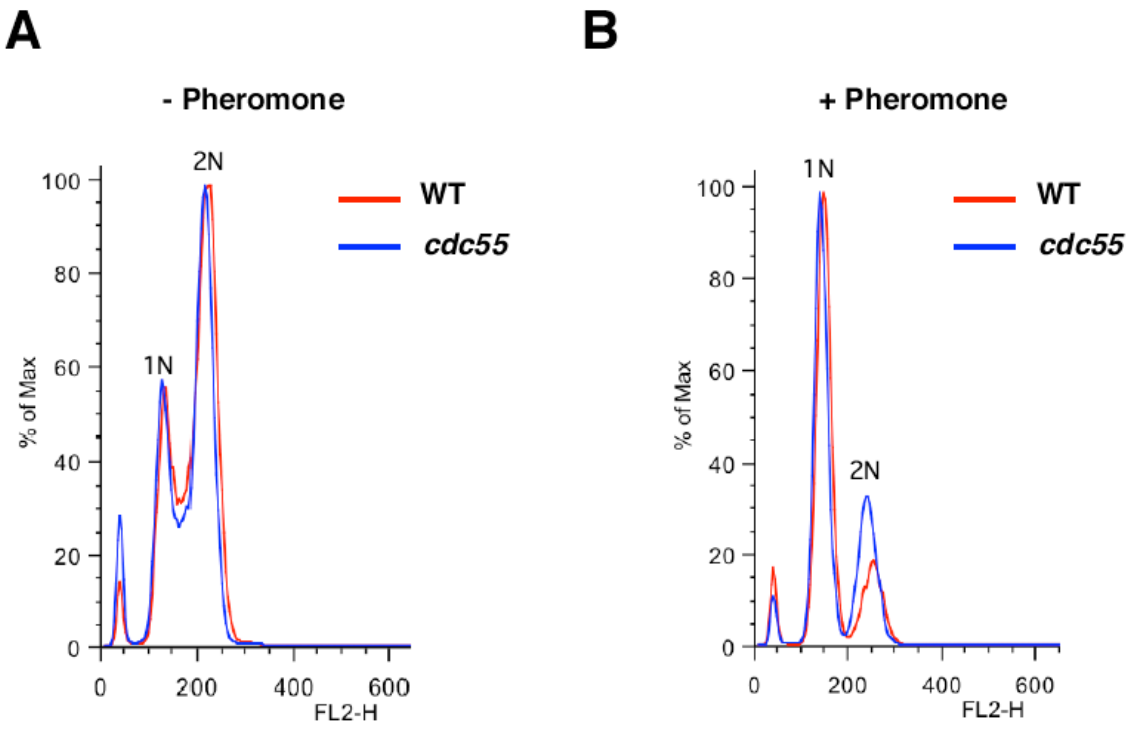
Haploid yeast undergoing mating in response to pheromone arrest in G1 phase of the cell cycle in order to prevent aneuploidy of the resulting diploid yeast. Considering that Cdc55 was previously shown to regulate G2/M progression, I considered it possible that the effect of the *cdc55* disruption on pheromone response might be an indirect effect of differential distribution of cell populations in the cell cycle. To examine this, I compared exponentially growing wildtype and *cdc55* mutant strains by flow cytometry, in both untreated and pheromone-treated populations. The results showed that the *cdc55* mutant displayed a similar pattern of cell cycle distribution as the wildtype, in both treated and untreated cells (Fig. 4.5). This suggests that lower pheromone responsiveness of the *cdc55* mutant does not result from a disproportionate accumulation of cells outside of G1 phase prior to pheromone treatment. Additionally, cell cycle arrest in G1 phase upon pheromone treatment is also not differentially affected in the *cdc55* mutant, indicating that signaling for G1 arrest is functional in this mutant strain.

4.4 Decreased mating efficiency is observed in the *cdc55* null mutant strain

Up to this point, the effect of Cdc55 on pheromone response was demonstrated using an artificial pheromone-responsive reporter with only two directly repeated PREs. In itself this doesn't represent the overall effect of pheromone response. To address this issue in more detail, I directly examined whether the mating efficiency of haploid cells is affected by the *cdc55* mutation. Wildtype and *cdc55* mutant *MATa* haploid strains were mated with a *MATα* wildtype strain, and the relative efficiency of mating was measured

Figure 4.5 The cell cycle distribution is not altered by *cdc55* null mutation in populations of untreated and pheromone treated yeast.

The wildtype and *cdc55* mutant cells were grown to mid-log phase in YPD and left untreated (left panel), or treated with pheromone for 60 minutes (right panel), and then were harvested and analyzed by flow cytometry for determination of cell cycle distribution.


A
- Pheromone
2N
1N
WT
cdc55
% of Max
100
80
60
40
20
0
0
200
400
600
FL2-H
B
+ Pheromone
1N
2N
WT
cdc55
% of Max
100
80
60
40
20
0
0
200
400
600
FL2-H

as the number of diploid yeast formed as a proportion of the total *MATa* cells in the reaction. When the mating reaction was carried out in a rich media environment (YEPD), I found that mating efficiency of the *cdc55* mutant was only 56% that of the wildtype yeast (Fig. 4.6A, lanes 1 and 2). This was reduced to 35% for the *cdc55* mutant strain when the mating assay was carried out in synthetic complete media (SC, Fig. 4.6A, lanes 3 and 4). I also examined the effect on mating efficiency when both haploid mating partners carried the *cdc55* null mutant in a rich media environment, and found that efficiency was reduced to 10% compared to wildtype mating populations (Fig. 4.6B, lanes 1 and 2). These results demonstrate that the effect of Cdc55 on Ste12-dependent pheromone responsiveness as observed for the 2 PRE reporter gene translates into a biological effect on mating efficiency.

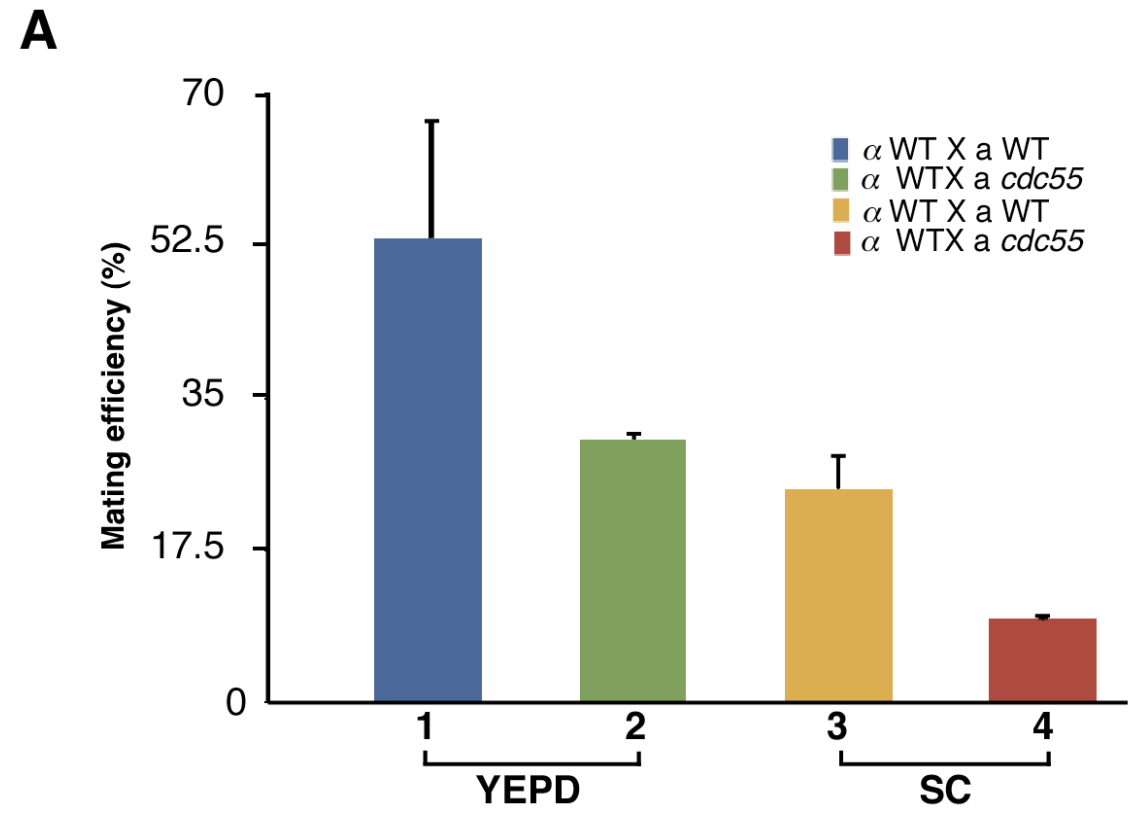
4.5 Cdc55 does not directly regulate signaling through the pheromone response MAPK pathway

Given the fact Cdc55 regulates activation by Ste12 of the 2 PRE reporter gene, I examined whether this effect might represent an alteration in signaling through the pheromone response MAP kinase pathway. For these experiments I introduced deletions of the *FUS3* and *KSS1* genes, which encode the pheromone-response MAP kinases, into the wild type and *cdc55* mutant strains bearing an integration of reporter plasmid pTS126, with the two directly repeated PREs upstream of the minimal *GAL1-LacZ* reporter fusion. The strains were assayed for response to pheromone by Northern blotting for *LacZ* RNA and by measuring β-galactosidase activity (Fig. 4.7A and B respectively). In the wild type strain, the *fus3* deletion greatly reduced pheromone

responsiveness but not the *kss1* mutant (Fig. 4.7A lanes 2, 4, and 6), consistent with previous observations (42). By Northern blotting, I also observe an ~5 fold reduction of *LacZ* expression in *cdc55* mutant cells compared to wild type (compare lanes 2 to 8), consistent with the results shown above. Additionally, deletion of *fus3* in the *cdc55* mutant strain caused a much larger decrease in pheromone-induced expression, whereas the *kss1* deletion did not produce an additional effect. This indicates that the effects of Cdc55 and the MAP kinases are non-epistatic, and must regulate Ste12 function through separate mechanisms. If Cdc55 and Fus3 acted through a common pathway it would be expected that mutation of *cdc55* in combination with *fus3* would produce a similar effect as *cdc55* alone (Fig. 4.7A lanes 8, 10, and 12). A comparable result was observed by measuring β–galactosidase activity produced by the reporter, where disruption of *fus3* in a *cdc55* mutant strain causes a reduction in pheromone response, proportional to the effect seen in the wild type strain (Fig. 4.7B). These results indicate that Cdc55 may regulate Ste12 activity and pheromone response through a pathway parallel to the pheromone MAP kinase pathway.

Figure 4.6 The *cdc55* null mutant showed decreased mating efficiency.

(A) Wildtype and *cdc55* mutant *MATa* strains were examined for mating efficiency by mating with a *MATα* wild type strain for 2 hours at 30°C. Mating efficiency was calculated as the number of resulting diploid colonies produced divided by the total of the *MATa* wild type (lanes 1 and 3) or *MATa cdc55* mutant (lanes 2 and 4) in the mating reaction. Mating was carried out on YEPD or SC plates. (B) Mating efficiency of wild type haploid strains (*MATα* wildtype mating with *MATa* wildtype, lane 1) was compared to that of two *cdc55* mutant strains (*MATα cdc55* mutant mating with *MATa cdc55* mutant, lane 2). The mating reactions were carried out on YEPD.


A
70
52.5
35
17.5
0
Mating efficiency (%)
α WT X a WT
α WTX a cdc55
α WT X a WT
α WTX a cdc55
1
2
3
4
YEPD
SC

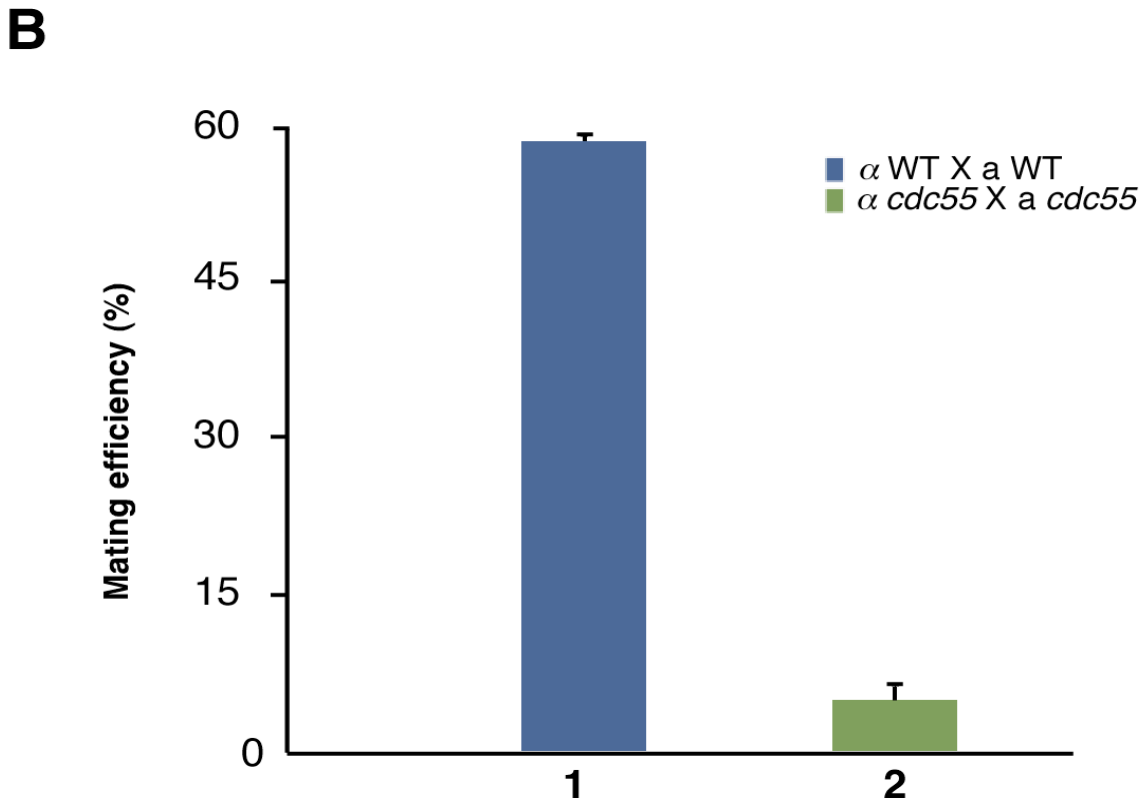
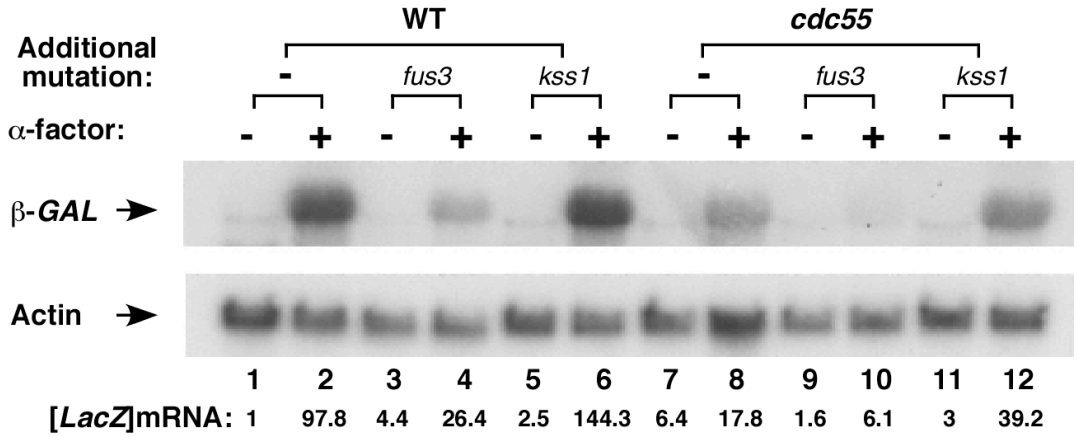

B
60
45
30
15
0
Mating efficiency (%)
α WT X a WT
α cdc55 X a cdc55
1
2

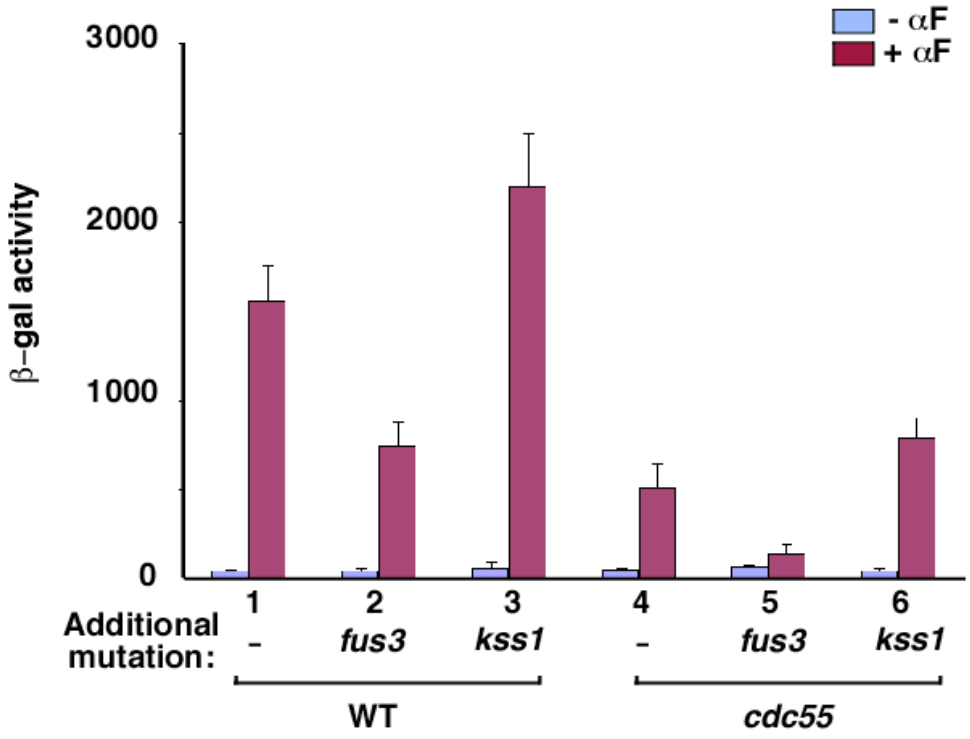
Figure 4.7 The effect of Cdc55 on pheromone response does not involve the pheromone MAP kinase pathway.

(A) The *fus3* and *kss1* genes were disrupted in wildtype and *cdc55* null mutant strains bearing an integrated 2PRE *GAL1-LacZ* pheromone-responsive reporter. The strains were left untreated, or treated with pheromone for 30 min prior to harvesting. RNA was analyzed by northern blotting for *β-GAL* and actin (*ACT1*) mRNAs. RNA expression was quantified by densitometry, and the intensity of *β-GAL* mRNA was normalized relative to actin RNA. The relative amounts of [mRNA] were normalized to the untreated sample from wild type cells (lane 1, 1). (B) The yeast strains as described above were assayed for the β-galactosesidase activity from untreated cells and cells treated with α-factor for 60 min.

A


WT
cdc55
Additional mutation:
-
fus3
kss1
-
fus3
kss1
α-factor:
- + - + - + - + - + - +
β-GAL
Actin
1 2 3 4 5 6 7 8 9 10 11 12
[LacZ]mRNA: 1 97.8 4.4 26.4 2.5 144.3 6.4 17.8 1.6 6.1 3 39.2

B

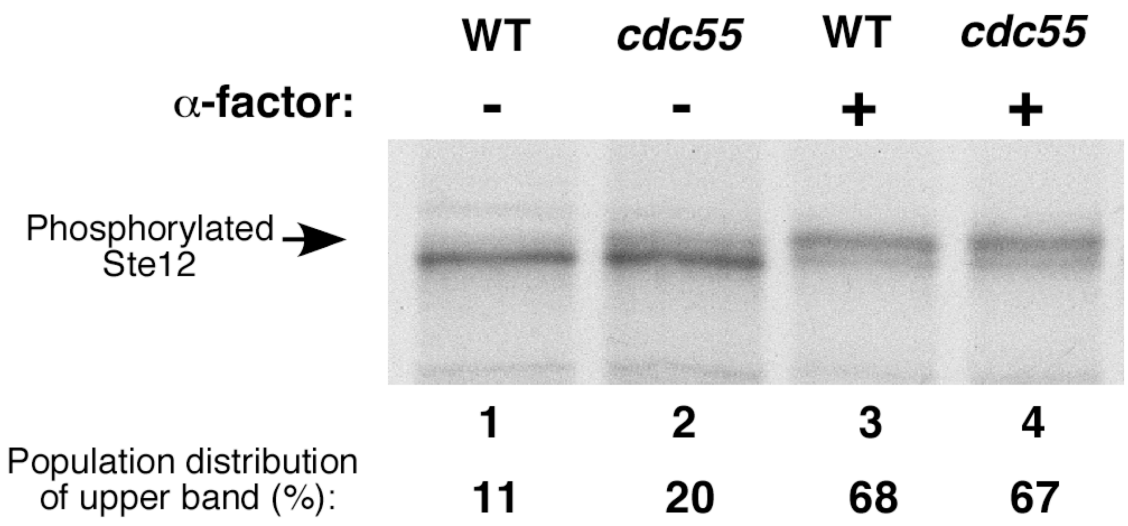

- αF
+ αF
β-gal activity
3000
2000
1000
0
1 2 3 4 5 6
Additional mutation:
- fus3 kss1 - fus3 kss1
WT
cdc55

4.6 Ste12 phosphorylation status is altered in the *cdc55* null mutant

Based on results shown above that there may be a difference in mobility of Ste12 protein expressed in the wildtype and the *cdc55* mutant by immunoblotting, I examined this in more detail using metabolic labeling. For this experiment, I used a plasmid from which Ste12 was produced from the *GAL1* promoter in order to induce synthesis during the labeling reaction. *In vivo* ^{35}S-labelling was carried out in both a wild type and *cdc55* mutant strain. The cells were first incubated in ^{35}S-labelling mix and induced by galactose, and were left untreated or treated with pheromone for 1 hour during the labeling reaction. The cells were harvested and Ste12 was immunoprecipitated and resolved on a 7.5% SDS-PAGE gel. In these experiments I observed that Ste12 expressed in the *cdc55* mutant produced a more distinct slower migrating band as compared to the wild type strain in untreated cells (Fig. 4.8 lanes 1, and 2). Slower migrating forms of Ste12 were previously shown to represent hyperphosphorylated species (78). Upon pheromone treatment a fraction of Ste12 becomes shifted to the slower migrating species in both the wild type and *cdc55* mutant strains. This result indicates that Cdc55 alters the Ste12 phosphorylation status by dephosphorylation prior to pheromone treatment. However, these results cannot distinguish whether the effect of Cdc55 on Ste12 phosphorylation is direct or indirect.

Figure 4.8 An altered phosphorylation pattern of Ste12 was observed in the *cdc55* mutant strain prior to pheromone induction.

A plasmid expressing Ste12 under control of the *GAL1* promoter was transformed into the wildtype and *cdc55* null mutant strains. Ste12 expression was induced with 2% galactose while labeling with ^{35}S protein labeling mix. The cells were left untreated (lanes 1 and 2) or treated with α-factor for 60 min (lanes 3 and 4) before harvesting. The cells were lysed, and Ste12 was recovered by immunoprecipition with Ste12 antibodies and the proteins resolved on a 7.5% SDS-PAGE gel, and analyzed by autoradiography. To quantify the proportion of Ste12 protein in the slower migrating fraction, the image was scanned and analyzed using ImageJ software. The proportion of slower migrating Ste12 protein, relative to total, is indicated below the lanes.


WT
cdc55
WT
cdc55
α-factor:
-
-
+
+
Phosphorylated Ste12
1
2
3
4
Population distribution of upper band (%):
11
20
68
67

4.7 The TOR signaling pathway functions upstream of Cdc55 for regulation of pheromone response

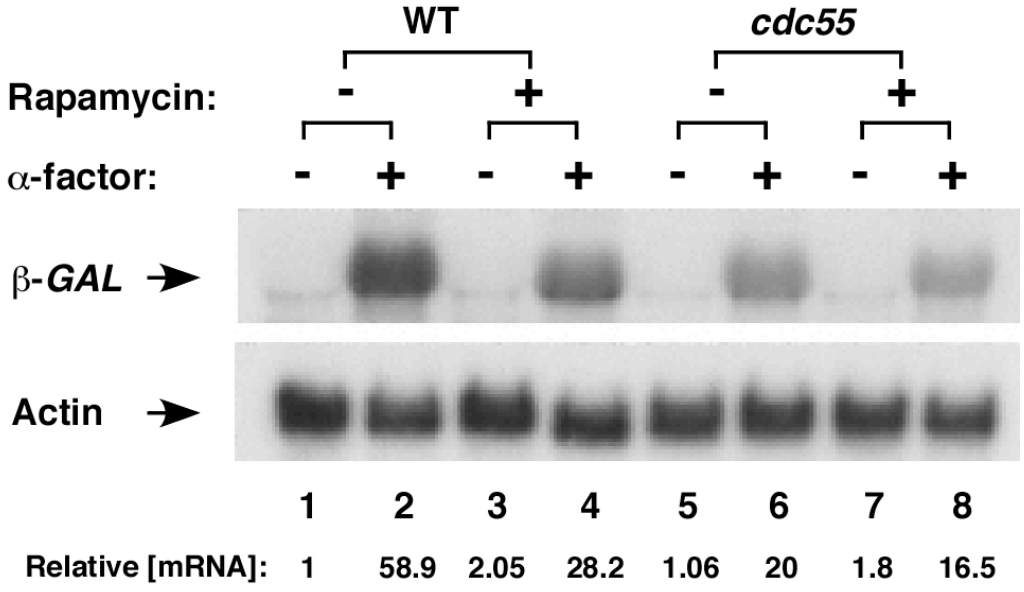
Cdc55 has been shown to be involved in regulating cell morphology, mitosis, and nutrition sensing downstream of the TOR signaling pathway (68, 81, 190). To examine whether the effect of Cdc55 on pheromone response reflects its role in the TOR signaling pathway, I used rapamycin, a highly specific small molecule inhibitor of TOR, to examine the epistatic relationship between Cdc55 and TOR. For these experiments I examined the effect of rapamycin treatment on pheromone responsive expression of the 2 PRE minimal *GAL-LacZ* reporter gene in wildtype yeast and *cdc55* mutant strains by Northern blotting to monitor *LacZ* expression levels. In wild type cells, I found that *LacZ* mRNA expression in pheromone treated cells was reduced in the presence of rapamycin (Fig. 4.9A, lanes 2 and 4). However, in contrast, treatment of the *cdc55* mutant strain with rapamycin did not cause the same extent of decrease in pheromone-induced expression, suggesting that inhibition of TOR does not have an additional effect in the absence of Cdc55 (Fig. 4.9A, lanes 6 and 8). This suggests that Cdc55 and TOR are part of a common signaling pathway that regulates pheromone response. This is further supported by the finding that inhibition of TOR with rapamycin in wildtype yeast causes a roughly equivalent effect on expression as does the *cdc55* mutant (Fig. 4.9A, compare lanes 4 and 6). I also examined the effect of rapamycin on β-galactosidase activity produced by the reporters. In wild type cells, rapamycin completely inhibited expression of the reporter in pheromone treated cells (Fig. 4.9B, lane 2). Consistent with the results shown above, in this experiment the *cdc55* mutation largely inhibited pheromone-induced

expression of the reporter in otherwise untreated cells (lane 3). But, interestingly, treatment of *cdc55* yeast with rapamycin only reduced reporter gene expression by another several fold in this background (lane 4). In fact, expression of the reporter in the rapamycin-treated *cdc55* strain was found to be ~ 4 fold higher than in the rapamycin-treated wild type strain (compare lanes 2 and 4). TOR signaling is known to regulate translation and consequently, these results indicate that Cdc55 may also be involved in rapamycin-mediated translation inhibition of pheromone-responsive expression.

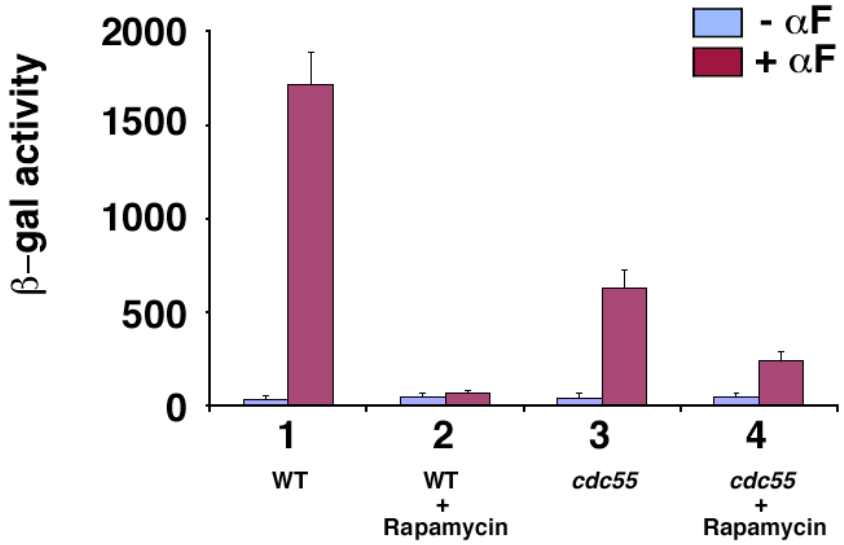
Figure 4.9 The TOR signal transduction pathway is upstream of Cdc55-regulated pheromone response.

(A) Wildtype and the *cdc55* null mutant strain bearing integrated copies of the 2-PRE *GAL1-LacZ* pheromone reporter were left untreated (lanes 1-2 and 5-6), or treated with rapamycin (lanes 3-4, and 7-8) for 60 min. The cells were then left unstimulated (lanes 1, 3, 5 and 7) or induced with pheromone 30 min (lanes 2, 4, 6 and 8) before harvesting and extracting RNA for measurement of *β-GAL* and actin RNA expression by Northern blotting. The relative levels of *β-GAL* RNA is indicated below, relative to actin levels, and normalized to the pheromone-untreated wild type strain (lane 1, 1). (B) The yeast strains described above were assayed for expression of β-galactosidase activity after 60 minutes pheromone treatment. (C) Mating efficiencies of the wildtype and the *cdc55* mutant strains were measured in the presence of rapamycin (lanes 2 and 4) and without rapamycin treatment (Lanes 1 and 3). Mating was carried out on YEPD.

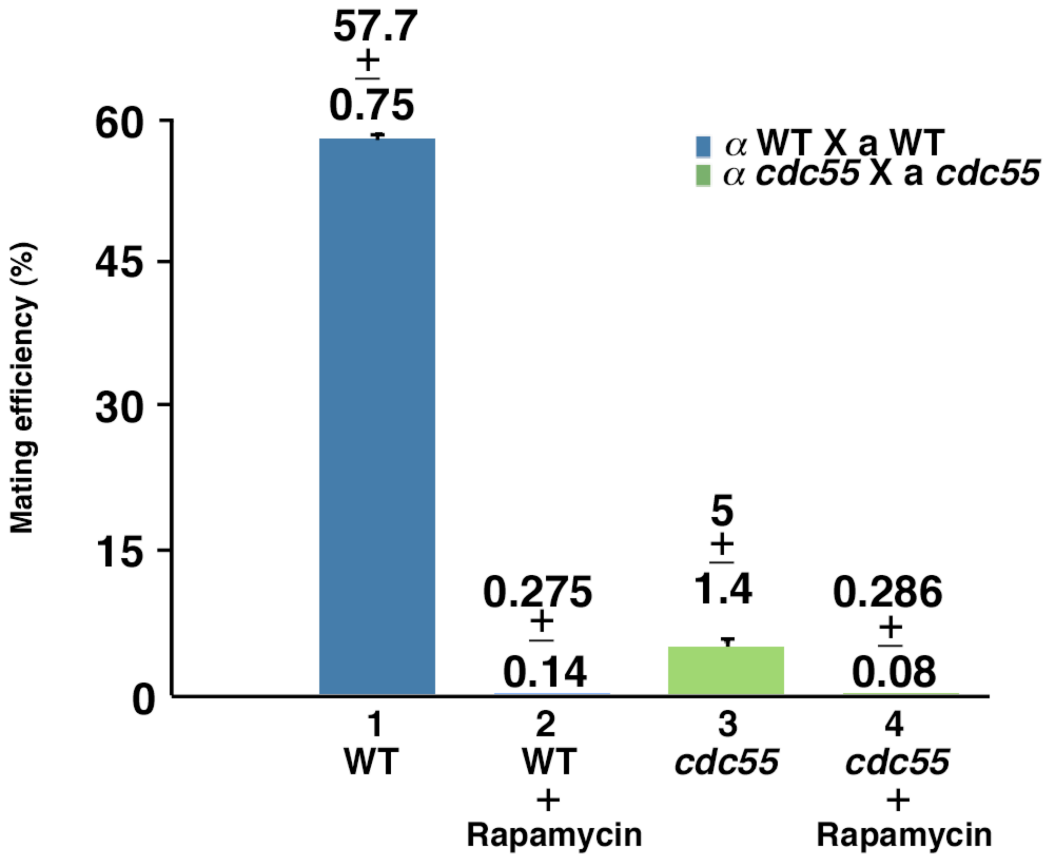
A


WT
cdc55
Rapamycin:
- + - + - + - +
α-factor:
- + - + - + - +
β-GAL
Actin
1 2 3 4 5 6 7 8
Relative [mRNA]: 1 58.9 2.05 28.2 1.06 20 1.8 16.5

B


β–gal activity
2000
1500
1000
500
0
- αF
+ αF
1
WT
2
WT + Rapamycin
3
cdc55
4
cdc55 + Rapamycin

C


57.7
±
0.75
60
45
30
15
0
Mating efficiency (%)
α WT X a WT
α cdc55 X a cdc55
0.275
±
0.14
5
±
1.4
0.286
±
0.08
1
WT
2
WT
+
Rapamycin
3
cdc55
4
cdc55
+
Rapamycin

To examine the effect of TOR signaling on pheromone response further, I examined how mating efficiency is affected by rapamycin. Wild type and *cdc55* mutant haploid strains were mated in the presence of sublethal concentrations of rapamycin in a rich media environment, and the mating efficiency was measured by assaying production of diploids. I found that rapamycin has a severe effect on mating efficiency of wild type yeast (Fig. 4.9C, lanes 1 and 2), and reduces production of diploids by nearly 200 fold. Consistent with the results shown above, a *cdc55* mutant haploid mates at much lower efficiency than wild type (Fig. 4.9C, lanes 1 and 3), and treatment with rapamycin further reduces the mating efficiency of *cdc55* haploids by around 20 fold (Fig. 4.9C, lanes 3 and 4). However, important to note, wild type and *cdc55* haploids, when treated with rapamycin, mate at approximately the same efficiency (Fig. 4.9C, lanes 2 and 4). This result indicates that the *cdc55* deletion does not produce an additional inhibitory effect on mating efficiency along with rapamycin, implying that Cdc55 is a downstream effector of TOR for regulation of mating. Since TOR also controls other cellular functions like translation and ribosome biogenesis, the reduced mating phenotype observed in strains treated with rapamycin may result from a global inhibitory effect on other cellular aspects in addition to the Cdc55 branch of signaling downstream of TOR.

4.8 Cdc55 does not affect expression of the *FUS1* or *STE12* genes

FUS1 and *STE12* are two important genes in regulating pheromone response. To investigate whether Cdc55 and TOR signaling has an effect on their expression, I performed Northern blots on RNA from wildtype and *cdc55* mutant strains treated with

rapamycin and pheromone. In contrast to what I observed with the artificial 2 PRE reporter gene, I found that neither rapamycin treatment, or the *cdc55* mutation, had an obvious effect on pheromone-induced *FUS1* expression (Fig. 4.10A, lanes 2 and 4, and lanes 6 and 8, respectively). A similar result was also observed for *STE12* expression, where induction was as efficient in rapamycin-treated cells or *cdc55* mutant yeast (Fig. 4.10B, Lanes 2, 4, 6, and 8). Based on these results it seems that not all pheromone responsive genes, including *FUS1* and *STE12* are under control of the TOR signaling pathway.

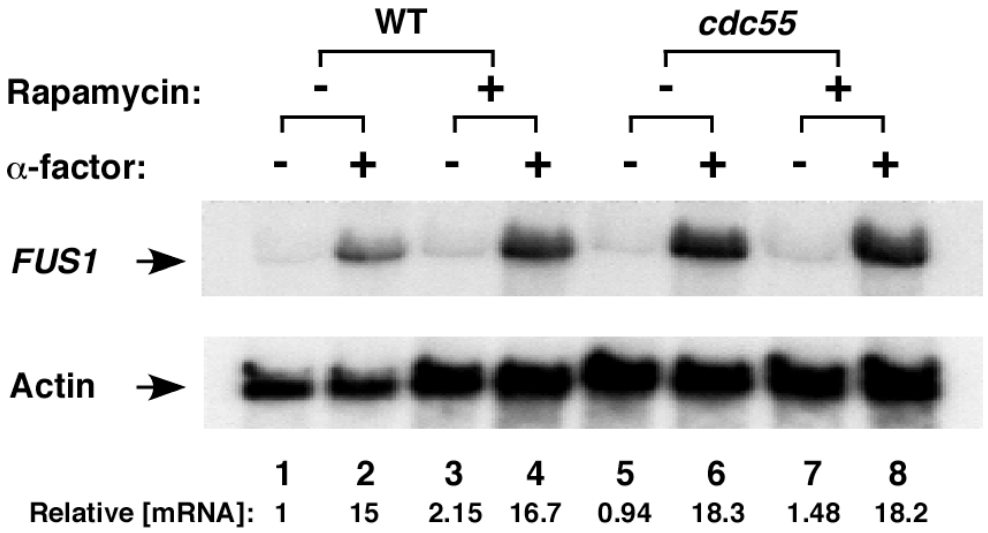
4.9 The effect of Cdc55 on pheromone response may be limited to specific arrangements of Ste12 binding sites

The results shown above with the *FUS1* and *STE12* genes indicate that Cdc55 must only regulate a subset of pheromone-responsive promoters. Additionally, the results shown in Chapter 3 indicate that the orientation of two PREs can confer different effects on basal and pheromone-induced expression of Ste12-dependent genes. Therefore, I examined how Cdc55 affects the response of artificial reporter genes with two consensus PREs in different orientations. For these experiments I integrated reporters bearing two consensus PREs in various orientations upstream of a minimal *GAL1-LacZ* reporter gene into congenic wildtype and *cdc55* mutant strains, and examined expression in untreated cells and cells treated with pheromone for 60 minutes. I found that expression of a promoter bearing PREs configured into the tail-to-tail arrangement, from the *STE12* promoter, was affected proportionally less by the *cdc55*

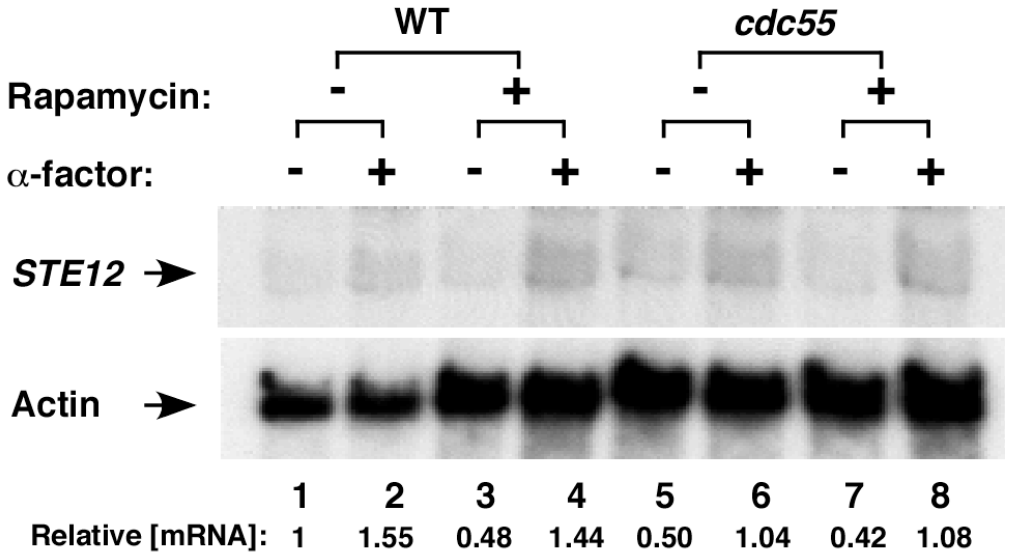
Figure 4.10 Expression of *FUS1* and *STE12* are not greatly affected by the TOR signaling pathway.

(A) Wildtype and *cdc55* null mutant strains were left untreated (lanes 1-2, and 5-6) or treated with rapamycin (lanes 3-4, and 7-8) for 60 min. The cells were either left uninduced (lanes 1, 3, 5, 7) or induced with α-factor for 30 min (lanes 2, 4, 6, 8) prior to harvesting RNA for analysis of *FUS1* and actin RNA by northern blotting. Relative levels of *FUS1* expression normalized to actin mRNA are indicated below, and are expressed relative to the wild type untreated sample (lane 1, 1). (B) Expression of *STE12* was measured in wild type and *cdc55* mutant strains as described in panel (A).

A


WT
cdc55
Rapamycin:
- + - + - + - +
α-factor:
- + - + - + - +
FUS1
Actin
1 2 3 4 5 6 7 8
Relative [mRNA]: 1 15 2.15 16.7 0.94 18.3 1.48 18.2

B

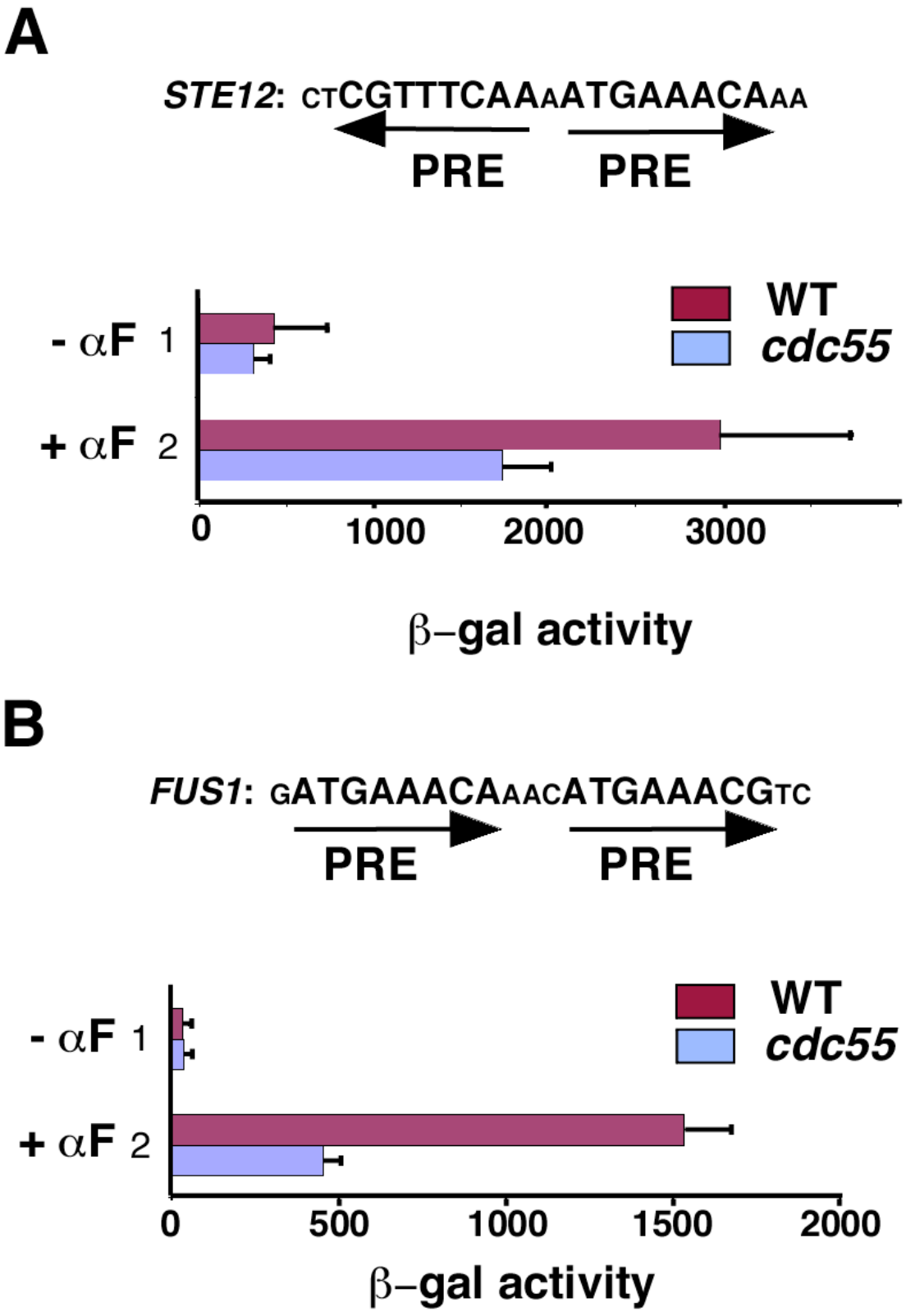

WT
cdc55
Rapamycin:
- + - + - + - +
α-factor:
- + - + - + - +
STE12
Actin
1 2 3 4 5 6 7 8
Relative [mRNA]: 1 1.55 0.48 1.44 0.50 1.04 0.42 1.08

mutant (Fig. 4.11A, B, line 2), than was a reporter with the head-to-tail arrangement of PREs, from the *FUS1* promoter. Expression of the tail-to-tail reporter was reduced by ~ 40% (Fig. 4.11A, line 2), whereas the head-to-tail arrangement of PREs was reduced by about 70% in the *cdc55* mutant (Fig. 4.11B, line 2).

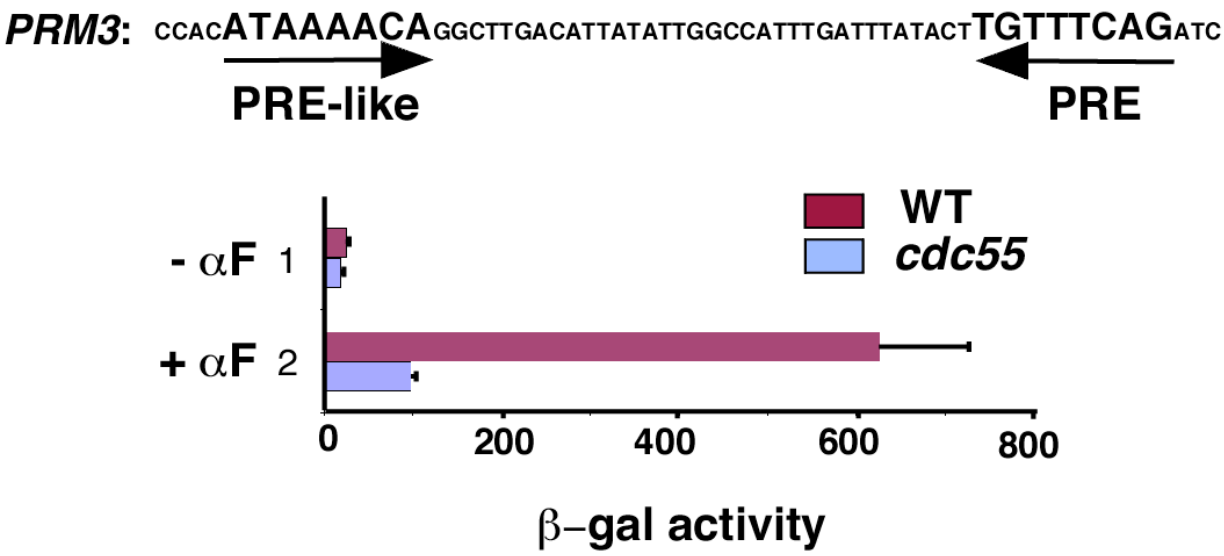
I also examined the effect of Cdc55 on pheromone response produced by promoter fragments that were shown to depend upon a single consensus PRE in combination with a non-consensus PRE-like sequence. In these experiments I found that pheromone-induced expression of the *PRM3* promoter fragment, which has the PRE-like sequence and consensus PRE separated by 31 nucleotides, was reduced by about 85% in the *cdc55* mutant compared to wild type (Fig. 4.11C, line 2). Similarly pheromone response of the *CIK1* promoter fragment, which has a closely spaced PRE and PRE-like sequences in a head-to-tail orientation, was reduced by about 70%in the *cdc55* mutant (Fig. 4.11D, line 2). These observations indicate that Cdc55 has an effect on Ste12 function that is dependent upon the orientation of the PREs within the promoter of target genes. Some orientations of PREs, such as the tail-to-tail seen in the *STE12* promoter seem to be less sensitive than are two PREs in the head-to-head orientation. Because the orientation between PREs strongly dictate responsiveness to pheromone, the results of this section imply that Cdc55 may regulate interaction between Ste12 monomers to multimerize for binding of adjacent PREs *in vivo*.

Figure 4.11 The spatial arrangement of PREs determines the extent of Cdc55-regulated pheromone response.

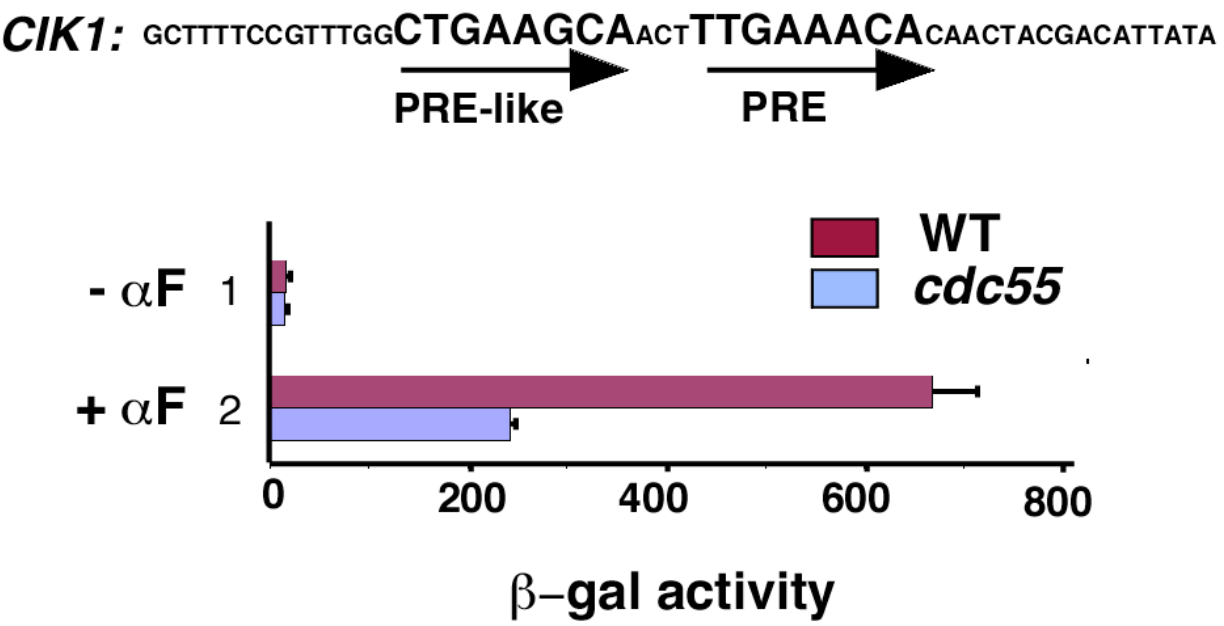
Wildtype and *cdc55* null mutant strains bearing integrated copies of *GAL1-LacZ* reporter genes with 2 upstream PREs arranged in various orientations, or upstream promoter regions from four pheromone-responsive genes, were examined for pheromone response. The strains were untreated (line1) or treated with α-factor for 60 min (line 2). β-galactosidase activity was then measured after 60 minutes. (A) The effect of *cdc55* on a tail-to-tail orientation of PREs from the *STE12* promoter. (B) The effect of *cdc55* on the head-to-tail orientation of PREs from the *FUS1* promoter. (C) Response of a reporter with a head-to-head orientation of PREs from *PRM3* promoter. (D) The effect of *cdc55* on the head-to-tail orientation of PREs from the *CIK1* promoter.


A
STE12: CTCGTTTCAAAATGAAACAAA
PRE
PRE
WT
cdc55
- αF 1
+ αF 2
0
1000
2000
3000
β–gal activity
B
FUS1: GATGAAACAAACATGAAACGTC
PRE
PRE
WT
cdc55
- αF 1
+ αF 2
0
500
1000
1500
2000
β–gal activity

C


PRM3: CCACATAAAACAGGCTTGACATTATATTGGCCATTTGATTTATACTTGTTTCAGATC
PRE-like
PRE
WT
cdc55
- αF 1
+ αF 2
0
200
400
600
800
β–gal activity

D


CIK1: GCTTTTCCGTTTGGCTGAAGCAACTTTGAAACACAACTACGACATTATA
PRE-like
PRE
WT
cdc55
- αF 1
+ αF 2
0
200
400
600
800
β–gal activity

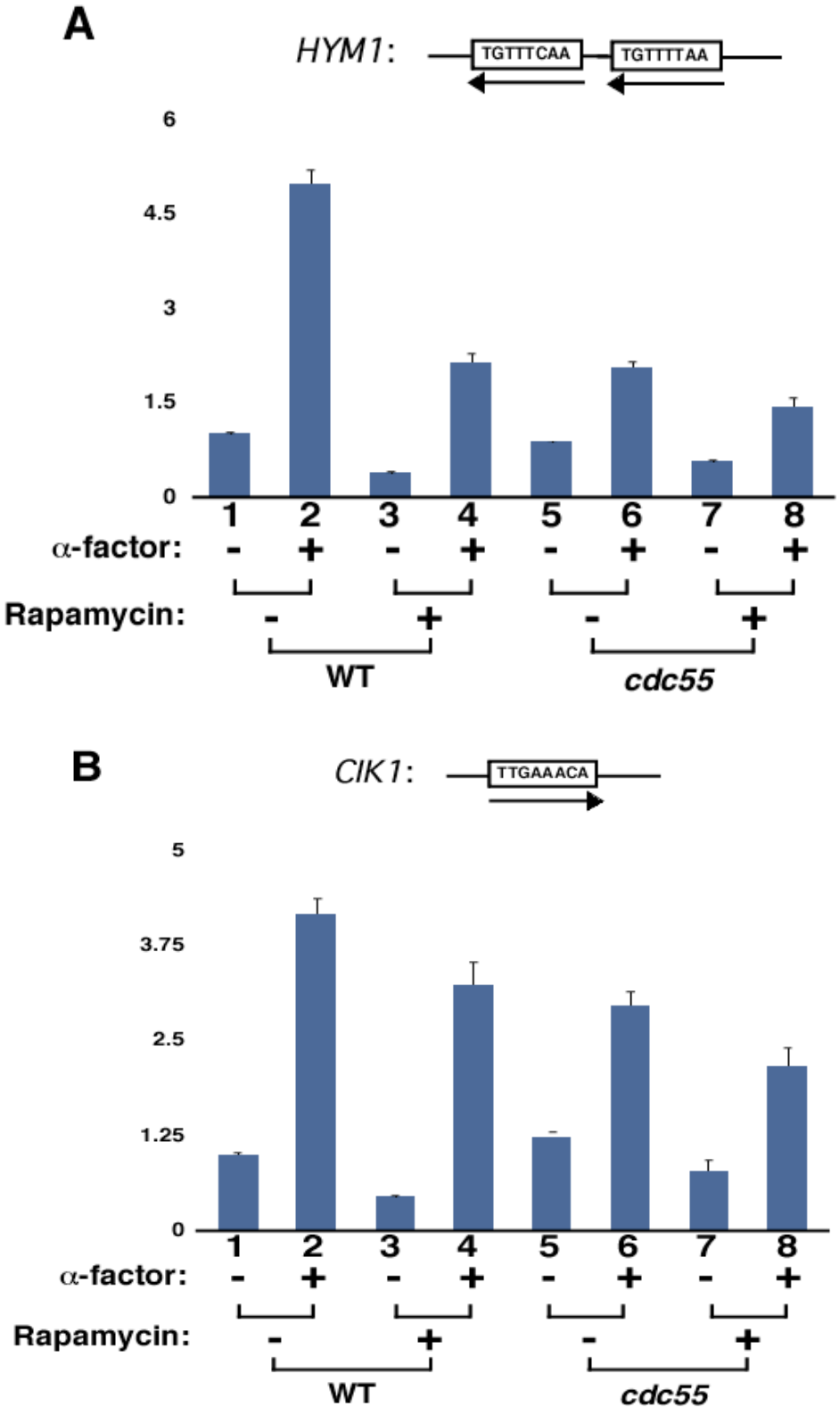
4.10 The effect of Cdc55 on pheromone response is restricted to a subset of genes

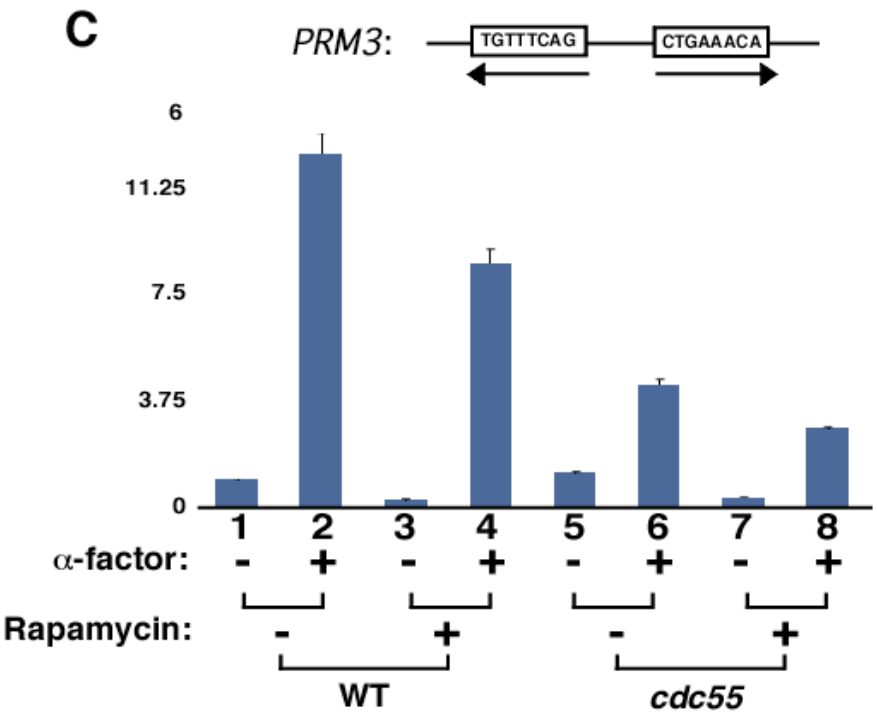
My results indicate that mutation of *cdc55* inhibits pheromone response of artificial promoters bearing only 2 PREs in specific conformations, causes an alteration in Ste12 phosphorylation, and inhibits mating of yeast haploids. However, expression of two relatively well characterized pheromone-responsive genes, *FUS1* and *STE12* seem to be unaffected by *cdc55* deletion or rapamycin treatment. These observations suggest that TOR signaling through Cdc55 must regulate mating response by regulation of Ste12 function on only a subset of target genes. Based on the results above, it might be predicted that only those pheromone responsive genes which are solely dependent upon Ste12 bound to properly oriented PREs might be subject to regulation by this pathway. To investigate this, I examined expression of seven known pheromone responsive genes that potentially match this criteria. For this experiment I treated both wildtype and *cdc55* mutant strains with rapamycin and then stimulated the cells with pheromone. RNA was extracted, and then analyzed by quantitative RT-PCR with primers specific for candidate genes of interest. I focused on seven genes as detailed in Figure 4.12. Amongst these, I observed various dependencies on TOR and *CDC55*. *HYM1* seemed to be the only gene examined where Cdc55 and TOR were epistatically linked for full response to pheromone (Fig. 4.12A). The *CIK1* gene is subject to similar regulation but to a lesser extent. Induction of some of the genes tested seemed to be inhibited by rapamycin but were not affected by the *cdc55* deletion, including *PRM4* and *SCW1*. Based on these results I can conclude that there are pheromone responsive genes that are both TOR and *CDC55*-dependent for full expression, but that such dependence is difficult to predict with my current data. Nevertheless, these results demonstrate that Cdc55 does regulate a subset of

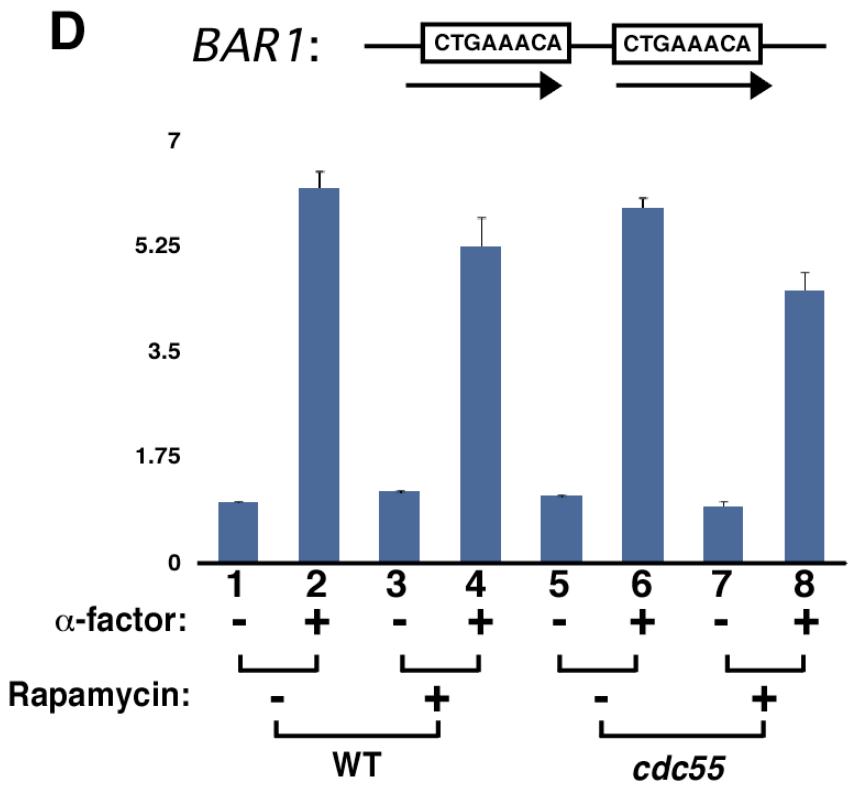
Ste12-dependent pheromone responsive genes, which must contribute to mating efficiency.

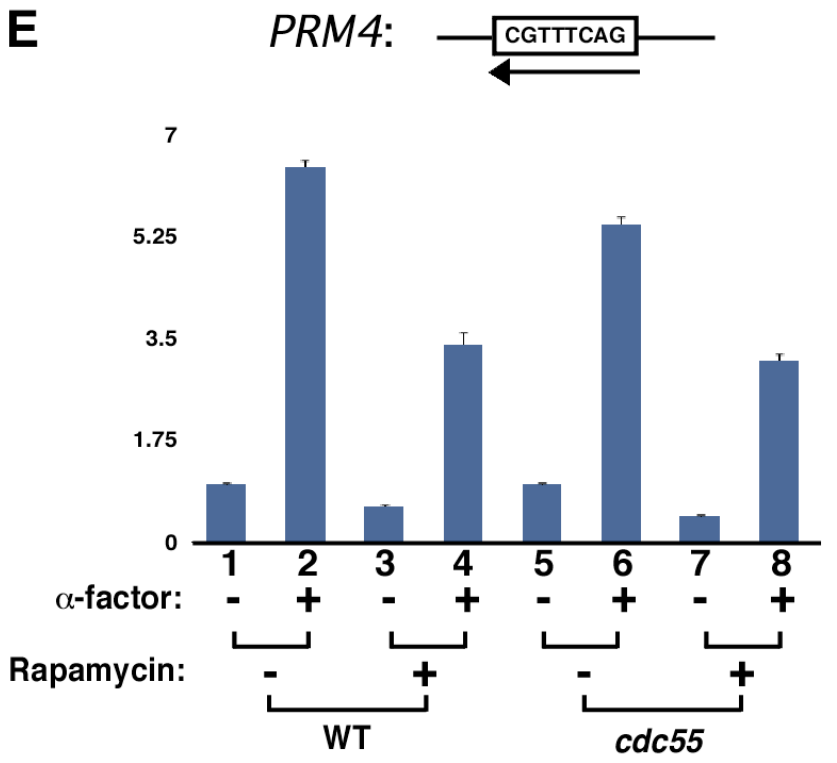
Figure 4.12 Effect of Cdc55 on expression of a selected set of pheromone-regulated genes.

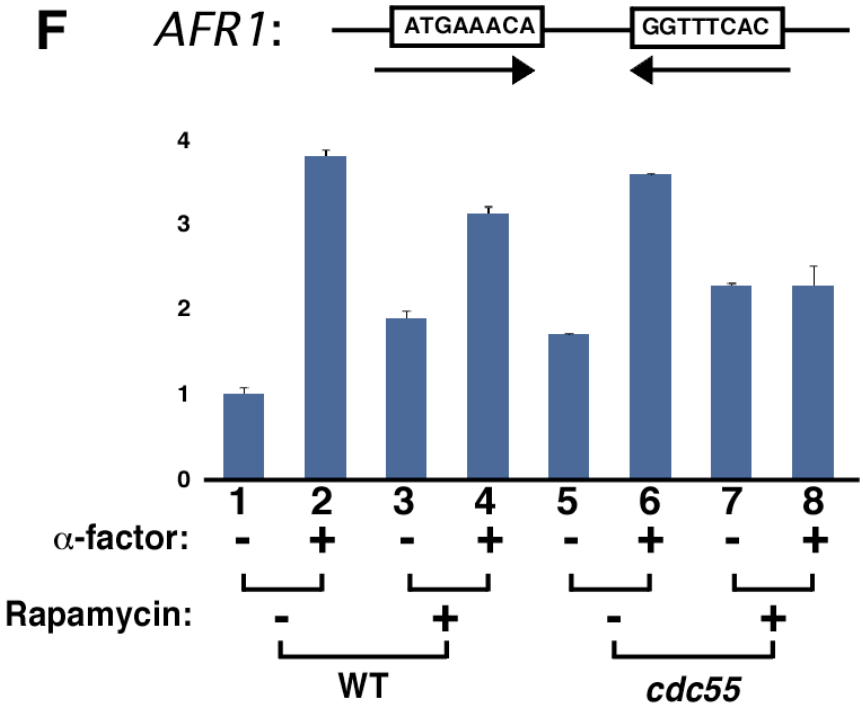
RNA was extracted from wild type and *cdc55* null mutant strains and analyzed by quantitative RT-PCR from triplicate samples of untreated and pheromone-treated cells, as indicated.

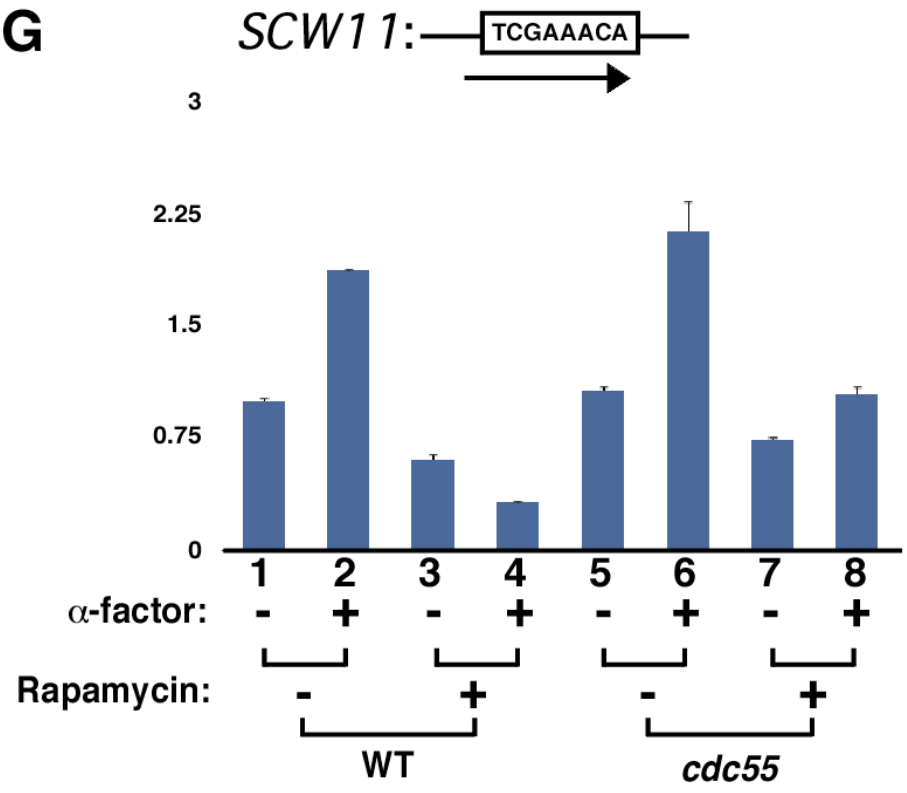

A
HYM1:
TGTTTCAA
TGTTTTAA
6
4.5
3
1.5
0
1 2 3 4 5 6 7 8
α-factor: - + - + - + - +
Rapamycin: - + - +
WT
cdc55
B
CIK1:
TTGAAACA
5
3.75
2.5
1.25
0
1 2 3 4 5 6 7 8
α-factor: - + - + - + - +
Rapamycin: - + - +
WT
cdc55


C
PRM3:
TGTTTCAG
CTGAAACA
6
11.25
7.5
3.75
0
1 2 3 4 5 6 7 8
α-factor: - + - + - + - +
Rapamycin: - + - +
WT
cdc55


D
BAR1:
CTGAAACA
CTGAAACA
7
5.25
3.5
1.75
0
1 2 3 4 5 6 7 8
α-factor: - + - + - + - +
Rapamycin: - + - +
WT
cdc55


E
PRM4:
CGTTTCAG
7
5.25
3.5
1.75
0
1 2 3 4 5 6 7 8
α-factor: - + - + - + - +
Rapamycin: - + - +
WT
cdc55


F
AFR1:
ATGAAACA
GGTTTCAC
4
3
2
1
0
1 2 3 4 5 6 7 8
α-factor: - + - + - + - +
Rapamycin: - + - +
WT
cdc55


G
SCW11:
TCGAAACA
3
2.25
1.5
0.75
0
1 2 3 4 5 6 7 8
α-factor: - + - + - + - +
Rapamycin: - + - +
WT
cdc55

Chapter 5 Discussion

5.1 Ste12 DNA-binding and transcription factor partners

The pheromone-response pathway in yeast has been studied for many years, and although it has provided an important model for understanding gene regulation by MAP kinase cascades, there remain many unanswered questions regarding the function of Ste12, its interaction with the pheromone-response element on DNA, and the mechanisms governing activity for response to pheromone signaling.

Prior to my research, it had been established that pheromone responsive transcriptional activation by Ste12 *in vivo* required at least two PREs (64), and several well-characterized target promoters, including *FUS1* were known to contain two closely spaced PREs capable of conferring pheromone response on their own. Therefore, it was surprising to find that wild type Ste12 protein expressed in insect cells is incapable of effectively binding as multimers to oligonucleotide probes containing two identically spaced PREs *in vitro* (183). Using EMSA experiments I showed that wild type Ste12 forms only a single complex with oligos containing 2 PREs, and binding is not affected by mutations in either of the two PREs (183). This indicates that DNA binding of Ste12 to multiple PREs *in vivo* must be subject to additional previously unrecognized regulation.

To examine this in more detail, I demonstrated using *in vitro* binding assays with combinations of truncated derivatives, that Ste12 likely binds as a monomer to a single

PRE *in vitro*. I also confirmed, using completely artificial promoter constructs, that a single PRE is incapable of conferring pheromone response *in vivo*, no matter how strong the interaction of Ste12 with the PRE *in vitro*. This data suggests that a Ste12 monomer is not sufficient to cause response to pheromone, or that Ste12 is somehow prevented from binding a single PRE *in vivo*. It was previously determined that pheromone response, at least in the context of the *FUS1* promoter, requires at least two copies of a PRE (64). I also confirmed this observation using artificial promoter constructs (Fig. 3.6B). These results suggest that multimerization of Ste12 is required for activation of genes whose expression is solely dependent upon Ste12.

5.2 PRE sequence variation and the effect on *in vivo* pheromone responsiveness

Using EMSA experiments, I systematically examined the contribution of nucleotides within the PRE for binding of Ste12 *in vitro*, and demonstrated that the extent of pheromone response *in vivo* is proportional to the combined relative strength of two PREs. For example, the typical PRE ATGAAACA has the highest relative affinity for Ste12 *in vitro*, and also produces the highest level of pheromone response *in vivo* in reporter constructs. Substitution of various residues of the consensus PRE all decrease the relative affinity for Ste12, and interestingly, these substitutions cause a proportional decrease in pheromone responsiveness. In fact, with these artificial reporter constructs bearing two directly repeated PREs, I observed a direct linear correlation between the relative binding strength of Ste12 for its *cis*-elements, arbitrarily compared using EMSA competition experiments, and the extent of pheromone response. This tight correlation between the *in vitro* PRE relative affinity for Ste12 and the resulting pheromone

responsiveness *in vivo*, suggests that with the same spacing and location in the genome, pheromone responsiveness can be solely determined by the relative strength of the PREs for binding Ste12 and this implies that the Ste12-PRE interaction *in vivo* must be the limiting factor to cause activation of transcription.

5.3 PRE orientations and the effect on activation by Ste12

Upon examination of pheromone-responsive promoter sequences it seemed that PREs are randomly arranged on most of the strongly inducible pheromone-responsive genes. Many of these genes have multiple PREs, consistent with my data and results from other labs, indicating that at least two PREs are necessary for pheromone response. But, considering the diverse arrangements of PREs in pheromone responsive genes, it seemed that there should not be restrictions on how PREs are arranged to produce a response to pheromone signaling. However, using the artificial reporter analysis, I found that different orientations of two PREs produced very distinct expression properties. For example, two PREs in a head-to-tail orientation (direct repeats), appears to cause reporter gene expression in the presence of pheromone but produces a low basal level of expression in the absence of pheromone. In contrast, two closely spaced PREs in a tail-to-tail orientation, such as in the *STE12* promoter cause response to pheromone but also are associated with higher basal level expression in the absence of pheromone.

I also found, surprisingly, that there are serious limitations on how two PREs can be positioned relative to one another to enable pheromone response of an artificial promoter. Two directly-repeated PREs cause activation only when located within three nucleotides of each other, separation of the sites by longer spacing with this orientation

completely prevents a response. In contrast, consensus PREs placed 3 nucleotides apart in a head-to-head orientation do not allow pheromone response, but a reporter where two PREs in this orientation are positioned 40 nucleotides apart was found to be inducible. Similarly, as mentioned above, PREs in a tail-to-tail configuration separated by a single nucleotide, as in the *STE12* promoter, causes high basal level expression and pheromone inducibility, but this effect is lost when the sites are moved further apart until the sites are separated by 40 nucleotides. These results indicate that Ste12 must have structural features that can accommodate multimerization for binding of closely-spaced sites oriented in several different conformations, but which must exclude binding to closely spaced sites in a head-to-head orientation. The capability to bind the head-to-head or tail-to-tail conformation positioned 40 nucleotides apart may suggest that Ste12 is capable of forming multimers that can bind these configurations, provided that the intervening DNA is able to bend or twist into a conformation that can accommodate binding.

5.4 Regulation of *STE12* expression

The mechanisms regulating *STE12* expression have not been adequately determined but are important, considering that Ste12 regulates both pheromone and filamentous growth. Ste12 protein is maintained at a low basal expression in the absence of pheromone but is strongly induced in response to pheromone. This is important because most of the genes encoding pheromone response pathway proteins are regulated by Ste12. Consequently basal Ste12 activity confers an ability of haploids to immediately respond to pheromone. Basal expression of *STE12* provides a sufficient Ste12 pool to maintain expression of the pheromone response pathway components,

without causing G1 arrest. In examining the sequence of the *STE12* promoter, there are four consensus PREs and no obvious binding sites for Mcm1, indicating that expression of Ste12 may be primarily dependent upon its own activity. This is in contrast to genes encoding the mating pheromones and receptors, *MFa1*, *MFα1*, *STE2* and *STE3*, whose basal expression is achieved with the participation of Mcm1. For the *STE12* promoter, the orientation of two PREs in a tail-to-tail orientation produces high basal level expression, and also causes induction in response to pheromone. This orientation of PREs may accommodate binding of a Ste12 multimer in a conformation that must partially bypass the inhibitory effect of Dig1 and/ or Dig2 in unstimulated cells. Because Dig2 interacts directly with the DNA binding domain, such a conformation may exclude binding of this inhibitor, leaving regulation subject solely to Dig1.

5.5 Role of non-consensus PRE-like elements for pheromone responses

I also noted a second curious feature of highly induced pheromone-responsive promoters, in that some appear to have only a single, or in some cases no, consensus PREs. This contradicts results, including my own, demonstrating a requirement of at least two PREs for pheromone response. However, my results indicate that, in combination with a strong PRE like the typical ATGAAACA, a second, properly oriented PRE with variations in sequence can still produce a response to pheromone. For example, as shown in Fig. 3.6C, a PRE with two point mutations produces pheromone response in combination with a consensus PRE. Based on results like this, I hypothesize that for those genes with only one PRE in their promoter regions, pheromone response could be conferred by the existence of a second properly oriented PRE-like sequence.

This is supported by the identification of functional PRE-like elements in the *CIK1* and *PRM3* promoters, which importantly have orientations that would be predicted to confer pheromone response based on my analysis of artificial promoters.

An additional possibility is that on pheromone-responsive promoters without a consensus PRE, or with only a single PRE, Ste12 may activate transcription through cooperative interaction on weaker elements with additional DNA binding proteins, such as Mcm1 and Kar4 (101), and perhaps with previously unrecognized additional factors. Consistent with this possibility, it was shown that a significant number of pheromone-responsive promoters also have potential binding sites for Flo8 (13), suggesting that pheromone response for many genes may involve an association between Ste12 and Flo8.

5.6 Organization of PREs in the promoters of pheromone responsive genes

My results using artificial reporter genes have shown that there are stringent requirements for the orientation of PREs necessary to cause pheromone-responsive gene expression. I have also compared these requirements to the arrangement of PREs in natural pheromone responsive promoters and found some interesting features. For example, it seems that at least some promoters contain PREs that may be nonfunctional or are redundant for pheromone response. A good example of this is represented by the *STE12* promoter. This promoter has three consensus PREs, I have designated PRE I, II and III. In Fig 3.9, I show that the majority of the basal level, and pheromone-induced expression is produced from PRE II and III. Mutation or deletion of the region containing PRE I has only a minor effect on pheromone-responsive expression. However, because this experiment was performed by introducing partial *STE12* promoter

fragments into an artificial promoter, I cannot exclude the possibility that PRE I is functional in the full *STE12* promoter where it may contribute to response in combination with other factors. The relationship between multiple PREs for pheromone responsiveness and regulation by the TOR-Cdc55 pathway is discussed in more detail below.

5.7 The potential role of Ste12 dephosphorylation for regulation of pheromone response by Cdc55 - a possible effect on Ste12 multimerization

We have expressed full length Ste12 protein in insect cells using baculovirus, but strangely, it was found to be incapable of binding both PRE sites in an oligonucleotide containing two closely spaced PREs *in vitro*, even though the same arrangement of PREs causes pheromone-responsive expression *in vivo*. However, in preliminary experiments, I found that mild treatment of Ste12 from insect cells with phosphatase (bovine calcineurin) causes the formation of slower migrating complexes in EMSA using a probe with two PREs. Additionally, I noticed that a truncated form of Ste12, deleted of the C-terminal 93 amino acids, likely does form multimers on the same oligo probe even without phosphatase treatment. These observations suggest that multimerization of Ste12 for binding DNA might be regulated by a C-terminal region whose function is controlled by dephosphorylation. This hypothesis lead to the discovery that Ste12 activity and pheromone response is modulated by the PP2A regulatory subunit Cdc55. Consistent with this possibility, I found that Cdc55 affects the Ste12 phosphorylation status in the absence of pheromone, in that I observe a subpopulation of what may be hyperphosphorylated Ste12 species in *cdc55* mutant yeast, compared to wildtype.

Furthermore, I observed decreased pheromone responsiveness of a reporter gene bearing two PREs, and lowered mating efficiency in a *cdc55* mutant strain relative to wild type. This supports the hypothesis that hyperphosphorylation somehow affects Ste12 activity in responding to pheromone induction. Based on my preliminary EMSA results, I propose that Ste12 phosphorylation status affects multimerization by a mechanism involving the C-terminus.

Cdc55 may directly or indirectly modify the C-terminal domain of Ste12, which is involved in mediating Ste12-Ste12 interactions (141). In addition, the C-terminus may also be involved in conformational changes that enable Ste12 to bind PREs with different orientations (Fig. 4.11). Consistent with these possibilities, I have examined effects of the *cdc55* mutant on induction of reporter genes bearing different PRE orientations. Reporters with PREs oriented in a tail-to-tail fashion, seem to show the least spatial hindrance for induction by Ste12 and the highest basal level expression. Accordingly, I also found that basal level expression and pheromone responsiveness of this arrangement of PREs are the least affected in the *cdc55* mutant, where I observed an approximately 45% reduction. In contrast, reporters bearing PREs in a head-to-tail orientation, either with two consensus PREs or a non-consensus and consensus PRE (Fig. 4.11 B, D) show an approximately 70% decrease in response in the *cdc55* mutant. This result further supports the possibility that Cdc55 affects Ste12-Ste12 interaction, through the C-terminal domain. Finally, a reporter with PREs in a head-to-head format, which requires a spacing of at least 40 nucleotides and possibly twisting or bending of the intervening DNA, is more severely affected by the *cdc55* mutation, which causes a greater than 80% reduction in response (Fig. 4.11C).

5.8 Role of Cdc55 in filamentous growth

Cdc55 was previously shown to modulate a Ste12-dependent function in a screen for mutants with defects in filamentous growth (126). However, the role of Cdc55 in this process has not been identified. My experiments *in vitro* suggest that recombinant Ste12 is capable of forming complexes with Tec1 on an FRE-containing oligo in EMSA, whereas formation of Ste12 multimers on 2 PREs requires dephosphorylation. In a simplistic view, this might indicate that activation of Ste12/Tec1-dependent genes may not directly require dephosphorylation of Ste12 by Cdc55, but I have yet to examine this directly using reporter genes or by northern blotting. Several genes encoding transcription factors necessary for filamentous growth are dependent upon Ste12 for expression, including *TEC1* and *PHD1* (32, 150) and therefore it is possible that *cdc55* mutations indirectly inhibit filamentous growth because expression of these factors is reduced. Alternatively, Cdc55 may be required for one or more additional factors involved in filamentous growth, because at least 12 different DNA binding proteins seem to be involved in this process (150). Elm1 is a Ser/Thr protein kinase that was identified in the same study as Cdc55 for effects on filamentous growth. In contrast however, *elm1* null mutations cause constitutive filamentous growth (90). This suggests that Elm1 plays an inhibitory role for filamentous growth, and there is some speculation that Elm1 and Cdc55 act on common substrates to regulate this process (90).

5.9 Linkage of pheromone response to the nutrient sensing TOR pathway

My genetic analysis shows that the TOR pathway functions upstream of the Cdc55-mediated effect on pheromone regulation. Inhibition of the TORC1 pathway with rapamycin does not cause an obvious decrease in reporter gene expression in a *cdc55* mutant strain (Fig. 4.9A). Also, I found that there is no difference in the mating efficiency between the wildtype and *cdc55* mutant strains when the TOR pathway is inhibited by rapamycin (Fig. 4.9 C). Examination of the effect of *cdc55* in combination with *fus3* and *kss1* mutants indicates that TOR acts in parallel to the MAP kinase pathway, and that pheromone-response is modulated independently of pheromone signaling by nutrient availability. Importantly, the effect of *cdc55* may represent a direct effect on Ste12 phosphorylation. Several phosphorylations have been identified on Ste12, including S400, T525 and T585 for which a function has yet to be established (60, 77, 78). A previous student in the lab had found that a Ste12 T525A mutant produced a subtle hyper-responsive phenotype in reporter and mating assays, and similar results were observed for Ste12 S400A and T585A (Hung and Sadowski, unpublished) (77). However, I was not able to link those phenotypes to the TOR-Cdc55 pathway (not shown). It is very likely that the effect of Cdc55 on Ste12 may involve multiple phosphorylation sites and this will require analysis of multiple mutations on Ste12. Regulation of pheromone response by nutrient availability is poorly understood in *Saccharomyces*. Mating reactions between wild type haploid yeast strains performed on rich media (YPD) show higher mating efficiency compared to mating on minimal synthetic complete (SC) media (Fig. 4.6A). This supports the commonly held notion that mating reactions should be performed on rich medium for the greatest efficiency.

However, I have not found a published systematic analysis of the effects of nutrient limitation on mating efficiency. Consequently, my results demonstrate a previously unrecognized aspect for pheromone-responsive regulation that promotes mating of *S. cerevisiae* haploids under ideal growth conditions. Interestingly, this effect is opposite that observed for other unicellular fungi, including *S. pombe*, and *K. lactis* which require nutrient starvation to undergo efficient mating response (12, 80). For *Saccharomyces*, regulation of mating by the availability of nutrients allows haploid yeast to save, rather than waste, energy involved in the complicated mating response, to ensure that the newly form diploid cell will not be immediately forced to produce spores in a nutrient poor environment.

5.10 The Significance for regulation of a subset of pheromone-responsive genes by Cdc55

My comparison of expression of a select subset of pheromone-responsive genes in wildtype and *cdc55* mutant yeast showed that not every pheromone-induced gene is affected by the *cdc55* mutation (Fig. 4.10, 4.12). This demonstrates that Cdc55 regulates only a subset of pheromone-related genes. This regulation may play an important role in balancing pheromone response and mating with nutrient availability. Importantly, expression of Ste12 is not affected by *cdc55* mutations, and this may be important to maintain a consistent Ste12 pool in order to respond to changes in the environment. With consistent levels of Ste12, yeast cells can rapidly respond to pheromone and restore the diploid state once environmental stress is relieved.

Both *FUS1* and *STE12* expression are not affected in the *cdc55* mutant (Fig. 4.10), even though, as shown in Fig. 4.11A and B, two closely spaced PREs from these promoters inserted into the artificial promoter are sensitive to the *cdc55* mutant and rapamycin. A possible explanation for this difference in phenotypes might be the presence of multiple PREs in the promoter regions of these genes. The additional PREs might buffer the effects of nutrient limitation by allowing alternative combination(s) of PRE arrangements. For example in the *FUS1* promoter, the combination of PRE I-III or II-IV may be used under conditions where activation by Ste12 from the PRE II-III format is affected in the *cdc55* mutant, or under conditions of nutrient limitation.

5.11 Future directions

Although *cdc55* mutations cause decreased mating efficiency, the mechanism of how Cdc55 regulates Ste12 is still unknown, especially relating to the potential effect of dephosphorylation and Ste12 multimerization. Importantly, future studies should include footprinting assays to further examine the effect of phosphatase treatment on binding of Ste12 to closely spaced PREs *in vitro*. In these types of assays it would be expected that, if dephosphorylation allows multimerization, treatment with phosphatase would cause filling of the two PREs at lower concentrations of Ste12 protein than untreated samples. An alternative way to examine the effect of phosphatase in promoting multimerization of Ste12 would be to use a biotinylated oligo with two PREs, in binding reactions with untreated or phosphatase-treated Ste12. DNA-bound Ste12 complexes could be recovered by avidin magnetic beads. The addition of a protein crosslinker would then be used to stabilize Ste12 multimers. The Ste12 complexes can be resolved by SDS-PAGE

and multimers detected by immunoblotting with Ste12 antibody. Secondly, it will also be important to identify the relevant site(s) of phosphorylation that are affected by Cdc55 on Ste12, and whether these phosphorylations affect Ste12 multimerization. As mentioned above, there is the likely possibility that multiple phosphorylations are involved. It will be important to identify these sites and demonstrate regulation of phosphorylation by TOR and Cdc55. However, identification of phosphorylations regulated by specific protein phosphatases is a difficult task. In a current update of PhosphoGRID (Sadowski et al., submitted), a database of experimentally defined phosphorylation sites in yeast, and a project that I have been involved in (176), only 122 phosphorylated residues on 19 proteins have been linked to specific phosphatase subunits. In contrast over 1800 specific phosphorylation sites have been identified for protein kinases. It will also be important to determine the effect of Cdc55 on global expression of pheromone-responsive genes. This could be done using global expression analysis with microarrays. Identification of a specific subset of Cdc55-regulated pheromone-responsive genes would allow a more comprehensive analysis of their promoter structures to establish the relationship for specific regulation by Cdc55 through Ste12 and interaction with the PREs.

Because I have yet to identify specific sites for regulation of Ste12 multimerization by Cdc55, it is possible that additional unknown transcriptional activators, or other proteins, may be required to assist Ste12 binding to multiple PREs *in vivo* under the control of Cdc55. To test this model, a candidate gene approach could be used with the yeast deletion set, where mutant genes that encode such potential factors should produce similar effects as the *cdc55* mutation with a reporter bearing 2 PREs. Chromatin immunoprecipitation (ChIP) assays with the null mutant candidate strains

could also be used to measure Ste12 binding to different PRE orientations *in vivo* as shown in Fig. 4.11.

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