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**Research Article**

# Constitutive activation of the *Saccharomyces cerevisiae* transcriptional regulator Ste12p by mutations at the amino-terminus

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## Abstract

**The transcriptional activator Ste12p is required for the expression of genes induced by mating pheromone in the yeast *Saccharomyces cerevisiae*. We identified mutations in the amino-terminal DNA-binding domain of Ste12p that lead to constitutively high-level transcription of pheromone-induced genes. The behaviour of these mutant proteins is consistent with an enhanced DNA-binding ability. Cells carrying these hyperactive proteins retain their sensitivity to pheromone treatment, and their phenotype is largely dependent on the presence of at least one of the MAP kinases (Fus3p or Kss1p) and the scaffold protein Ste5p. Deletion of either *FUS3* or *KSS1* leads to a marked increase in Ste12p activity, consistent with a negative regulatory role for Fus3p, similar to that described for Kss1p. The properties of the constitutive mutants support the idea that the pheromone response pathway plays a role in basal as well as pheromone-induced transcription. Copyright © 2000 John Wiley & Sons, Ltd.**

**Keywords: Ste12p; pheromone response; mating pathway; transcription; MAP kinases; cell-type-specific expression**

Ste12p is a transcriptional regulator essential for the initiation of different developmental programmes in *Saccharomyces cerevisiae*. In haploid cells, Ste12p regulates the expression of genes essential for mating (Fields and Herskowitz, 1985; Dolan *et al*., 1989; Errede and Ammerer, 1989). In diploid cells, Ste12p is necessary for the development of filamentous growth (Liu *et al*., 1993; Roberts and Fink, 1994). In the mating process, peptide pheromones bind to specific receptors on the cell surface (Ste2p in **a** cells or Ste3p in $\alpha$ cells), resulting in the activation of a signal transduction cascade composed of a set of protein kinases (Ste20p, Ste11p, Ste7p, Fus3p and Kss1p). The activity of the kinase cascade is regulated by a heterotrimeric G protein that is coupled to the pheromone receptor. Receptor activation produces dissociation of the G protein, with the $\beta\gamma$ dimer (Ste4p/Ste18p) relaying the signal to the downstream kinases (reviewed in Leberer*et al*., 1997). The protein kinases interact with the scaffold protein Ste5p, which appears to regulate the specificity and efficiency of the mating signal (Elion, 1995; Mahanty *et al*., 1999). The activity of the protein kinases may relieve the negative regulatory activity that the Dig1p and Dig2p proteins exert on Ste12p (Cook *et al*., 1996; Tedford *et al*., 1997; Bardwell *et al*., 1998). The MAP kinases, Fus3p and Kss1p, play an additional role in controlling the developmental fate of cells (Sprague, 1998). Kss1p acts as a negative regulator of filamentous growth, while Fus3p prevents the inappropriate activation of the filamentous growth pathway by pheromone-dependent signalling (Cook *et al*., 1997; Madhani *et al*., 1997; Bardwell *et al*., 1998).

The targets of Ste12p include three sets of cell type-specific genes: **a**-specific genes (expressed only in **a** cells), $\alpha$-specific genes (expressed only in $\alpha$

cells), and haploid-specific genes (expressed in both **a** and α-cells). Both haploid-specific and **a**-specific genes are regulated through binding of Ste12p to a regulatory sequence known as the pheromone response element (PRE). The prototypic haploid-specific gene is *FUS1*, whose regulatory region contains several tandem copies of the PRE which are sufficient for Ste12p-dependent expression. Ste12p regulates **a**-specific genes, such as *MFA2*, by binding to PREs in upstream sequences cooperatively with the general transcriptional regulator Mcm1p (Jarvis *et al*., 1988; Hagen *et al*., 1991). Regulation of α-specific genes, such as *MFα1*, is carried out by a complex composed of Ste12p, Mcm1p and the α cell-specific regulator α1. The *MFα1* gene contains binding sequences for both Mcm1p and α1; in this case the presence of a PRE is not required for the activity of Ste12p (Sengupta and Cochran, 1991; Yuan *et al*., 1993).

Ste12p is a nuclear protein whose amino-terminal 215 amino acid residues contain the sequence(s) necessary for nuclear localization (Hung *et al*., 1997) and the DNA-binding domain (Yuan and Fields, 1991). The central portion of the protein contains the domain required for transcriptional activation and induction (Song *et al*., 1991; Kirkman-Correia *et al*., 1993), and the carboxy-terminal region contains sequences required for the interaction with Mcm1p (Errede and Ammerer, 1989). Ste12p interacts with the MAP kinases Fus3p and Kss1p as well as with the negative regulators Dig1p and Dig2p (Cook *et al*., 1997; Tedford *et al*., 1997; Bardwell *et al*., 1998), with the Ste12p–Fus3p interaction being dependent on Dig1p and Dig2p (Tedford *et al*., 1997). Fus3p is also able to phosphorylate Ste12p (Elion *et al*., 1993). Although Ste12p is constitutively phosphorylated, pheromone treatment produces a modest increase in the phosphorylation state of the protein (Hung *et al*., 1997). However, it has not been formally demonstrated whether phosphorylation is the mechanism that causes Ste12p activation or is a consequence of the activation of the pheromone pathway.

The amino-terminus of Ste12p has been shown to contain a potential negative-regulatory domain (Song *et al*., 1991). We decided to examine this region of the protein through the identification of mutations that produce a constitutive phenotype (increased expression of cell type-specific genes in the absence of pheromone stimulation). The activity of the constitutive Ste12p mutants was found to depend, however, on upstream components of the mating pathway. These results support a role for the pheromone response pathway in both the basal and pheromone-induced activity of Ste12p.

## Materials and methods

### Yeast strains and methods

The yeast strains used in this study are listed in Table 1. Yeast transformations were carried out using the lithium acetate–polyethylene glycol method (Ito *et al*., 1983). Yeast media were prepared according to standard recipes (Sherman *et al*., 1986).

### Recombinant DNA procedures

Recombinant DNA manipulations were carried out according to published procedures (Berger and Kimmel, 1987). YCpSte12T-3 (wild-type *STE12 TRP1* CEN) as well as the mutants and their HA-tagged versions, and YEp12T-3 HA-tagged (wild-type *STE12 TRP1* 2μ) are based on the YCplac22 and YEplac112 vectors (Gietz and Sugino, 1988). The *STE12* alleles were expressed from the wild-type *STE12* promoter and carry artificial restriction sites flanking the *STE12* open reading frame, an *Mlu*I site at the 5′ end of the coding sequence and a *Sma*I site at the 3′ end. The Gal4p DNA-binding and activation domains used in the Ste12p–Gal4p hybrids are derived from the vectors pGBT9 and pGAD424, respectively (Bartel *et al*., 1993).

Gene deletions and integration of reporter constructs were carried out using the one-step gene disruption method (Rothstein, 1983). Deletions of *STE12* were constructed using either a 6.3 Kb *Sac*I–*Sph*I fragment from pSUL17 (*ste12::LEU2*) (Fields and Herskowitz, 1987), or a 4.2 Kb *Cla*I fragment from pNC163 (*ste12::URA3*) (Company *et al*., 1988). The *STE2* disruption in strain JC22 was generated using a 5.0 Kb *Bam*HI fragment from pUSTE202 (*ste2::URA3*) (Nakayama *et al*., 1985). The *FUS3* deletion in JC15 was generated using a 2.8 Kb *Eco*RI fragment from pGA1832 (*fus3::URA3*) (provided by Gustav Ammerer, Institute of Molecular Pathology, Vienna, Austria). The *KSS1* deletion in JC16 was generated using a 2.0 Kb *Eco*RI–*Sph*I fragment from pGA1848 (*kss1*::*URA3*) (Gartner *et al*., 1992). The reporter

Table 1. Strains used in this study

| Strain | Relevant genotype | Reference |
|---|---|---|
| EG123 | *MAT***a** *trp1 leu2 ura3 his4 can1* | Siliciano and Tatchell, 1984 |
| AN42-2A | EG123 *fus3::URA3 kss1::URA3* | Neiman *et al.*, 1993 |
| SF437 | EG123 *ste5::LEU2* | S. Fields (unpublished) |
| SF458 | EG123 *ste12::LEU2 FUS1–lacZ::URA3* | Yuan *et al.*, 1993 |
| CK3-1b | EG123 *ste12::LEU2 MFA2–lacZ* | Kirkman-Correia *et al.*, 1991 |
| CK2-6b | *MAT***a** *ste12::LEU2 mfa1–lacZ* (isogenic to EG123) | Kirkman-Correia *et al.*, 1991 |
| JC14 | EG123 *ste12::URA3 ste5::LEU2 FUS1–lacZ::HIS4* | This work |
| JC15 | EG123 *ste12::LEU2 fus3::URA3 FUS1–lacZ::HIS4* | This work |
| JC16 | EG123 *ste12::LEU2 kss1::URA3 FUS1–lacZ::HIS4* | This work |
| JC17 | AN42-2A *ste12::LEU2 FUS1–lacZ::HIS4* | This work |
| JC22 | EG123 *ste12::LEU2 ste2::URA3 FUS1–lacZ::HIS4* | This work |
| YM709::171 | *MAT***a** Δ*gal14* Δ*gal80 his3 trp1 GAL1–lacZ::URA3* | Song *et al.*, 1991 |

construct *FUS1–lacZ–HIS4* was inserted as a *Pml*I digest of YIpH4-Z (Crosby, 1997).

HA epitope-tagged versions of Ste12p were constructed using the triple HA tag (Tyers *et al.*, 1992); the tag was introduced at the *Hin*dIII site in p650-lk668 (a plasmid that carries a *STE12* gene in which a *Sac*I restriction site at codon 668 has been converted to a *Hin*dIII site) (Kirkman-Correia, 1991). Finally, a 1.3 Kb *Pvu*II–*Ssp*I fragment from this construct was used to substitute a 1.1 Kb *Pvu*II–*Sma*I in plasmids carrying *STE12* sequences.

## Mutagenesis of *STE12*

Random mutagenesis of the *STE12* coding region was carried out according the PCR-based method described by Leung *et al.* (1989). The 5′ end PCR primer, which anneals to the nucleotides encoding the initiator ATG and downstream codons, contained a *Mlu*I restriction site (GGGCAACGCGT GTATGAAAGTCCAA); the 3′ end primer, which anneals to the nucleotides encoding the reverse complement of the last *STE12* codons, contained an *Eco*RV restriction site (GGGCCCGGGATATC AGGTTGCATCTGGA). The PCR product was cut with *Mlu*I and *Xba*I to obtain the mutagenized Ste12p DNA-binding domain (Figure 1A). The mutagenized DNA was used to produce an amino-terminal mutant pool by replacing the corresponding wild-type sequence in YCp12T-3.

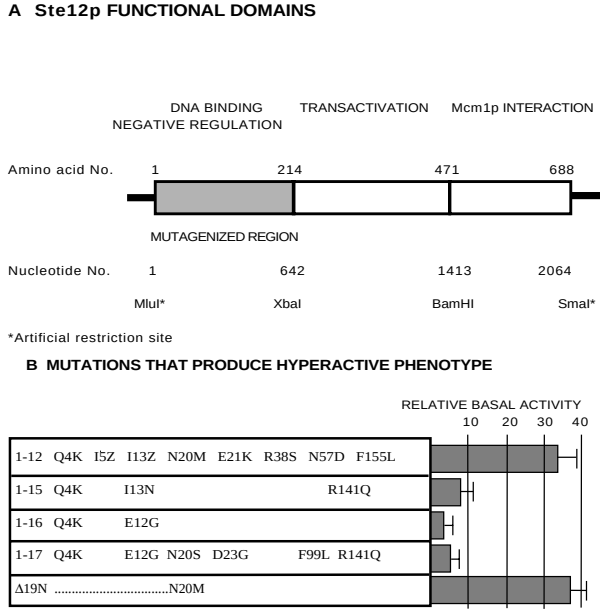


**Figure 1.** Domains of Ste12p and mutational changes identified in hyperactive mutants. (A) Diagram of Ste12p illustrating the different domains of the protein and the restriction sites in the *STE12* open reading frame that were used to construct the mutagenized pools. (B) Positions and sequence changes in Ste12p that produce hyperactive proteins ('Z', termination codon), and quantitation of the transcriptional activity of the mutants. Activities were measured in the absence of pheromone and are shown relative to that of the wild-type (0.11 ± 0.2 Miller units)

## Screen for constitutive *STE12* alleles

Strain SF458 (*ste12 FUS1–lacZ*) was transformed with the pool of mutagenized *STE12* described above. Transformants were plated on SD-trp medium and constitutive mutants identified as β-galactosidase-positive colonies in a filter assay (see below). The phenotype was confirmed by isolating the plasmids and reintroducing them into the reporter strain, after a round of amplification in

*E. coli*. The DNA sequence of the mutants was determined using the Sanger dideoxy chain-termination method (Sanger *et al*., 1977), using Sequenase (Amersham).

### β-galactosidase assays

Qualitative assays were performed on filter lifts of yeast colonies using X-gal (5-dibromo 4-chloro 3-indolylgalactoside) as substrate; quantitative assays were performed using chloroform-permeabilized cells and either ONPG (*o*-nitrophenyl-β-D-galacto-pyranoside) or CPRG (chlorophenol red β-D-galacto-pyranoside) as substrates, as described previously (Bartel *et al*., 1993). The reporter gene activity in pheromone-treated cells was measured after a 2 h incubation at 30°C; **a**-factor was obtained as the filtrate of a saturated culture of *MAT***a**-type cells; synthetic α-factor was purchased from Sigma Chemical Co.

### Western blot analysis

Ste12p extracts were prepared according to a previously published procedure (Company *et al*., 1988). Immunodetection of HA-tagged Ste12p proteins was carried out using the monoclonal antibody 12CA5 (Boehringer-Mannheim) and alkaline phosphatase-conjugated goat anti-mouse antibodies and the chromogenic reagent BCIP/NBT (bromo-4-chloro-3-indolylphosphate/4-nitroblue tetrazolium) (Bio-Rad Laboratories). Protein expression levels were analysed using a fluorescent light densitometer (GS-700, Bio-Rad Laboratories).

## Results

### Identification of constitutive Ste12p mutants

To identify mutations in the DNA-binding domain of Ste12p that resulted in constitutively high-level expression of cell type-specific genes, we mutagenized the sequence encoding this domain (Figure 1A) by an error-prone PCR procedure. The mutagenized DNA was used to replace the wild-type sequence in a centromeric plasmid-borne copy of the *STE12* gene. Approximately 12 000 independent clones were introduced into a *MAT***a** *ste12* yeast strain carrying the Ste12p–dependent reporter gene *FUS1–lacZ* (Trueheart *et al*., 1987). Transformants were screened for expression of the reporter gene in the absence of pheromone.

The screen identified four constitutive mutants, and sequence analysis showed that all have multiple mutations at the amino-terminus of the protein (Figure 1B). In each case, mutations in the region encoding the first 20 amino acid residues were associated with the hyperactive phenotype. The strongest mutant (1–12) was about 30 times more active than the wild-type and about four-fold more active than any of the other mutants. 1–12 carries two in-frame stop codons at positions 5 and 13, a new potential initiation codon at position 20, and additional codon changes; the other mutants contain multiple codon changes (Figure 1B).

The observation that 1–12 would encode a protein missing the amino-terminal 19 residues, and that 1–15, 1–16 and 1–17 contain changes within this same region, suggested that this short segment of the protein is involved in the regulation of the activity of Ste12p. To test whether loss of the first 19 residues alone was responsible for the constitutive phenotype of 1–12, we generated a deletion of these residues by PCR and assayed it for activity. The activity of this mutant protein, Δ19N, on the *FUS1–lacZ* reporter gene was also more than 30-fold higher than that of wild-type Ste12p (Figure 1B).

### Activity of the mutants on cell-type-specific gene expression

We compared the activity of three cell type-specific genes in cells carrying the mutant Ste12p proteins, in either the absence or presence of the relevant mating pheromone. For the *FUS1–lacZ* gene, used in the primary screen, the two strongest mutants as well as the Δ19N deletion were all fully capable of mediating transcriptional induction in the presence of α-factor (Figure 2A). The induced levels were two to four fold higher than that produced by the wild-type cells treated with pheromone.

At the **a**-specific *MFA2–lacZ* gene, Ste12p binds to DNA in cooperation with the DNA-bound Mcm1p. Neither of the Ste12p constitutive point mutants resulted in significantly elevated basal activity, while the Δ19N mutant led to a 1.5-fold increase in basal activity (Figure 2B). All three mutants could mediate increased transcription in response to α-factor; however, the induced levels were not greater than that produced by the wild-type protein.

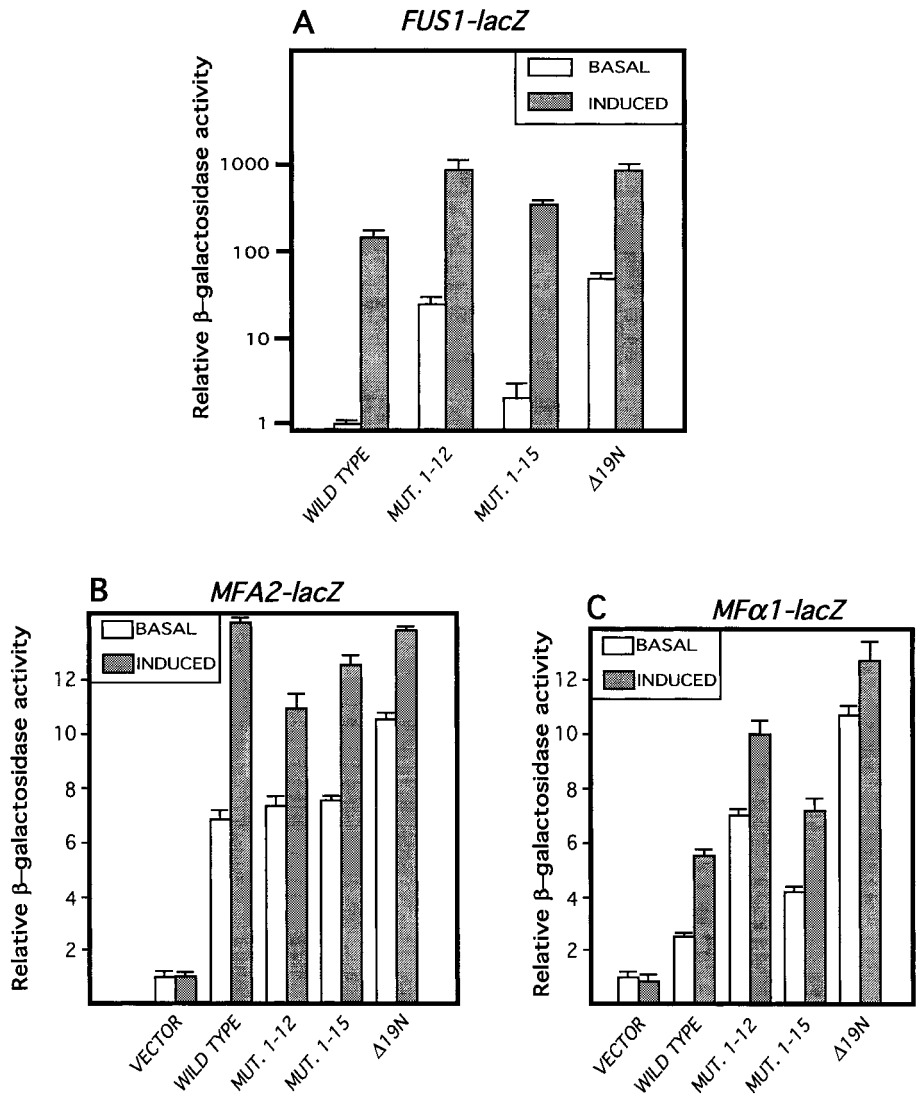


**Figure 2.** Transcription from mating-specific promoters directed by the Ste12p mutants. (A) Basal and pheromone-induced transcriptional activity of the haploid-specific reporter gene *FUS1–lacZ*. Wild-type or mutant forms of *STE12* on the plasmid YCplac22 were transformed into strain SF458. Values are normalized to that of cells expressing wild-type Ste12p. (B) Transcriptional activity of the mutants at the cell type specific reporter gene *MFA2–lacZ*. (C) Transcriptional activity of the mutants at the cell type specific reporter gene *MFα1–lacZ*. *ste12* strains (CK3-1b and CK2-6b) were transformed with the different alleles of *STE12* and the transcriptional activity of the reporter genes measured in exponentially growing cells. Values are normalized to those of cells carrying the empty vector YCplac22. The activity of the reporter genes was measured as β-galactosidase activity before or after treatment of exponentially growing cells with either 1 μM synthetic α-factor or the supernatant of a saturated culture of an **a**-type strain

At the *MFα1–lacZ* reporter gene, no PRE is present and Ste12p acts cooperatively with the DNA-bound α1 and Mcm1p. The two point mutants and the Δ19N mutant all resulted in constitutively high basal expression of the reporter, in two cases to a level beyond that produced by the wild-type protein in cells exposed to **a**-factor treatment (Figure 2C). The three mutants were inducible in cells treated with **a**-factor and the activity of the deletion mutants (1–12 and Δ19N) was on average twice of that produced by the wild-type protein.

## DNA-binding and transactivation by hybrids containing Ste12p and Gal4p domains

We sought to determine whether the change in activity displayed by the Δ19N mutant was reflected in changes in the DNA-binding properties of the protein or to changes in its transcriptional activity. To address these possibilities, we first generated hybrids between the DNA-binding domain of Ste12p and the activation domain of the yeast transcriptional activator Gal4p. Our logic was that assaying these fusions on a Ste12p-dependent reporter gene should measure only differences in the DNA binding properties of the Ste12p domain, in that the activity of the strong Gal4p activation domain should override any effects on Ste12p activation potential resulting from the mutations. Remarkably, the activity of a hybrid containing the Δ19N DNA-binding domain fused to the Gal4p activation domain was more than three-fold higher than that of the comparable hybrid carrying the wild-type Ste12p DNA-binding domain (Table 2). Therefore, this assay suggests that the Δ19N deletion increases the ability of Ste12p to bind the PRE. In this hybrid experiment, even given the high transactivation associated with the Gal4p activation domain, the different contribution of the wild-type vs. mutant Ste12p DNA-binding domains was still observed.

We also constructed the reciprocal hybrids which contain the Gal4p DNA-binding domain fused to Ste12p sequences. These hybrids, when assayed on a Gal4p-dependent reporter gene, should measure only the activation potential of the Ste12p sequences, in that binding to DNA by Gal4p should not be affected by mutations in the Ste12p moiety. Fusion of either the entire Ste12p, or the protein missing its amino-terminal 19 residues, to the Gal4p DNA-binding domain led to hybrid proteins with similar low transcriptional activities (Table 3). This result supports the idea that the amino-terminal deletion principally affects DNA-binding by Ste12p.

## The constitutive phenotype is not due to increased Ste12p protein level

Overproduction of Ste12p produces an increase in the basal transcription mediated by this protein (Dolan and Fields, 1990). In order to examine whether the constitutive mutants we isolated led to an increase in the level of Ste12p protein, we added the triple-HA epitope tag (Tyers *et al*., 1992) to the wild-type and mutant Ste12p proteins and examined their expression level by Western immunoblot using the anti-HA antibody (Figure 3). The 1–12 mutant, whose DNA sequence contains two in-frame stop codons, accumulated to only about two-thirds of the level of the wild-type protein. The other mutants showed a steady-state protein level equivalent to that of the wild-type. These results demonstrate that the amino-terminal mutations do not produce an increase in Ste12p levels, and thus the constitutive activation of pheromone-responsive genes mediated by the mutants is due to a change in Ste12p properties.

## The constitutive mutants do not result in increased sensitivity to pheromone

Another possibility that could account for the constitutive phenotype is that the Ste12p mutants are more sensitive to the signal generated by the pheromone response pathway. To examine this possibility, we assayed the activities of the mutants in cells treated with increasing concentrations of synthetic α-factor. Although the individual absolute activities were different in magnitude, the half-maximal ($EC_{50}$) values were similar ($10^{-7}$–$10^{-8}$ M)

Table 2. Transcriptional Activity of Ste12p-Gal4p Hybrids

| DNA-binding domain | Activation domain | Activity (β-gal Units) | Pheromone induction |
|---|---|---|---|
| Ste12p (1–252 | – | 0.3 + 0.2[a] | – |
| Δ19N (20–252) | – | 0.2 + 0.1[a] | – |
| Ste12p (1–252) | Gal4p (768–881) | 5.5 + 0.4[a] | – |
| Δ19N (20–252) | Gal4p (768–881) | 18.6 + 1.3[a] | – |

[a]*FUS1–lacZ* activity. Constructs carrying either the wild-type Ste12p DNA-binding domain, residues 1–252, or the amino-terminal deletion Δ19N, residues 20–252, were fused to the Gal4p activation domain, residues 768–881. The transcriptional activity of these hybrids was assayed on the *FUS1–lacZ* reporter gene in strain SF458. Activities are expressed as Miller units.

Table 3. Transcriptional Activity of Gal4p-Ste12p Hybrids

| DNA-binding domain | Activation domain | Activity ($\beta$-gal Units) | Pheromone induction |
|---|---|---|---|
| Gal4p (1–147) | – | 0.1 + 0.1[a] | – |
| Gal4p (1–147 | Ste12p (1–688) | 0.7 + 0.1[a] | + |
| Gal4p (1–147) | Δ19N (20–688) | 0.8 + 0.1[a] | + |

[a]*GAL1–lacZ* Activity.

The wild-type Ste12p and the amino terminal mutant Δ19N were fused to the Gal4p DNA binding domain and assayed for activity in strain YM709::171, which carries the *GAL1–lacZ* reporter gene. Activities are expressed as Miller units.

(Figure 4). Overall, the activities of the mutants paralleled that of the wild-type protein. We interpret this result to indicate that the mutants are not more sensitive than the wild-type protein to signalling generated by the response pathway. Furthermore, when the amino-terminal Ste12p mutants are expressed in diploid cells, their activity is not sufficient to induce filamentous growth in the absence of nitrogen deprivation (data not shown). Therefore, the regulation that the MAP kinases and the Dig proteins exert on Ste12p (Cook *et al*., 1996; Tedford *et al*., 1997; Bardwell *et al*., 1998) does not appear to be bypassed.

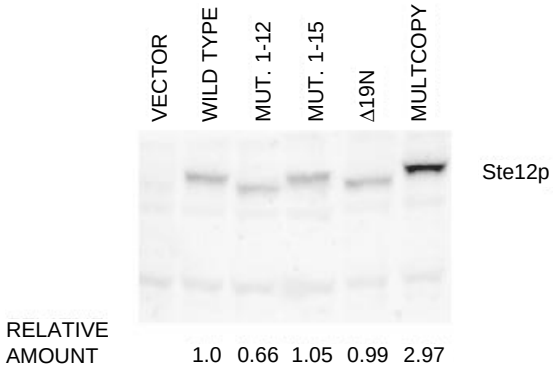


**Figure 3.** Western blot analysis of wild-type and mutant Ste12p proteins. Partially purified Ste12p extracts were prepared from SF458 cells expressing the different *STE12* forms. Aliquots containing 40 μg of total protein were analysed using the anti-HA tag monoclonal antibody 12CA5 and alkaline phosphatase-conjugated rabbit anti-mouse antibodies. The control lane corresponds to extract from cells carrying the empty vector YCplac22. Aliquots of an extract from exponentially growing SF458 cells carrying the multicopy plasmid YEp12T-3 (2μ *STE12*-HA) were used as standard. The relative amounts of tagged Ste12p, determined by densitometry, are shown relative to the wild-type level

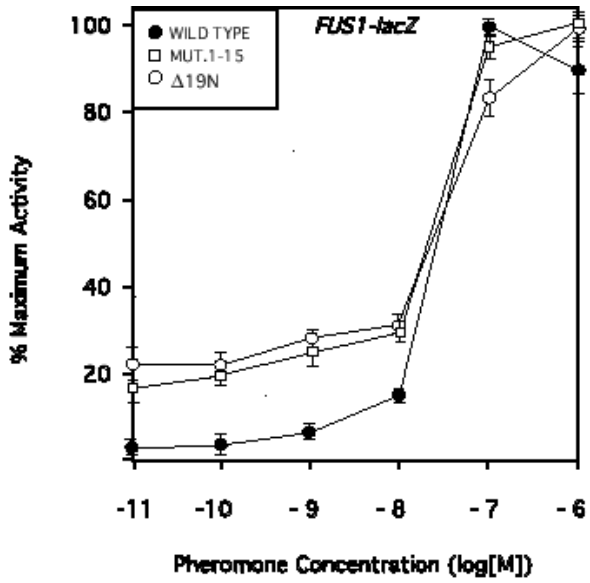


**Figure 4.** Transcriptional activity generated by the Ste12p mutants in response to pheromone treatment. Plasmids carrying wild-type *STE12* or mutants forms of the gene were transformed into the reporter strain SF458, and $\beta$-galactosidase activity was measured after treatment with different concentrations of synthetic **a** factor ($10^{-11}$–$10^{-6}$ M) for 2 h at 30°C. To facilitate comparisons, the activity generated by each *STE12* allele at each point is presented as a fraction percentage of its own maximum. The maximum activities generated by the different proteins (in Miller units) were: wild-type, 22.2 ± 0.8; mutant 1–15, 40.25 ± 0.41; Δ19N, 130.5 ± 8.8

## Requirement for components of the pheromone response pathway in the constitutive Ste12p phenotype

To further characterize the activity of the mutants, we examined the genetic relationships between *STE12* and several components of the response pathway by assaying the 1–15 and Δ19N mutant proteins in strains in which components of the

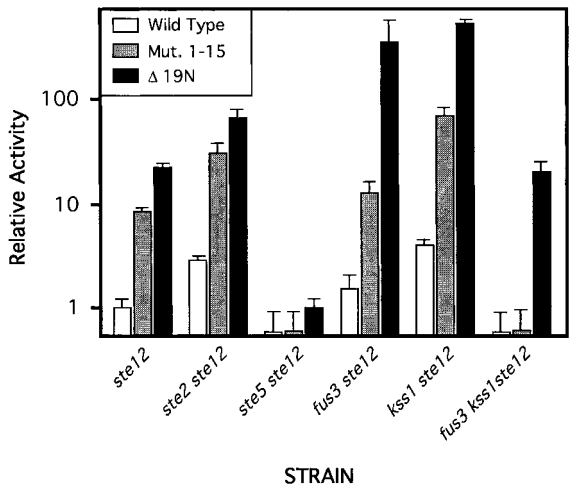


**Figure 5.** Transcriptional activity of the Ste12p mutants in strains deleted for components of the pheromone response pathway. The mutants were transformed into strains defective in mating-related functions as indicated. The activity of the reporter gene *FUS1–lacZ* is shown relative to that generated by wild-type Ste12p in a *ste12* strain

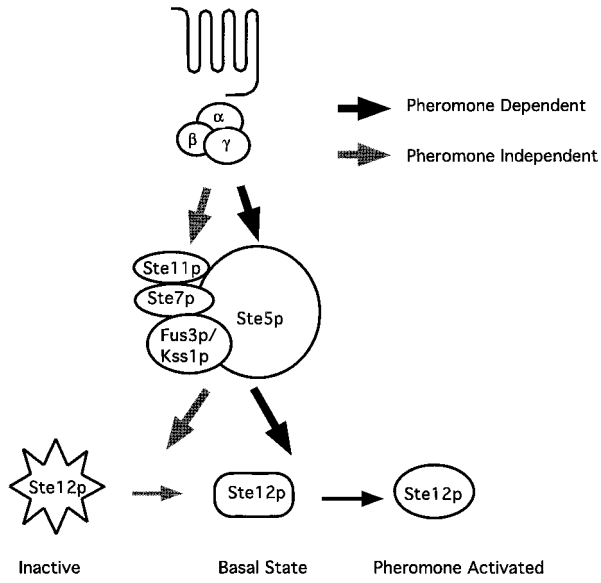


**Figure 6.** Model for the genetic interactions that regulate Ste12p activity. The inability of the Ste12p hyperactive mutants to activate transcription in a *ste4* or *ste5* background suggests the existence of a step that converts Ste12p into a competent factor (able to act as regulator of transcription); the 'licensing' of Ste12p requires an early interaction with components of the mating pathway. The pheromone independent activity of the $\beta\gamma$ dimer flows through Ste5p (probably the protein kinases that interact with it) and changes Ste12p from an inactive moiety to a competent state which is able to regulate transcription, both at the basal level and in response to pheromone

pathway were deleted. Deletion of *STE2* produced an increase in the activity of both the wild-type and the mutant proteins (Figure 5). Loss of either *FUS3* or *KSS1* also led to an increase in activity, with the effect more pronounced for *KSS1* than for *FUS3*. However, deletion of *STE5* resulted in an almost complete elimination of the activity of the wild-type and mutant Ste12p proteins (Figure 5); a similar result was observed with deletion of *STE4* (data not shown). Deletion of both *FUS3* and *KSS1* eliminated the activity of the wild-type protein and the 1–15 mutant, but had only a modest effect on the Δ19N mutant. These results indicate that the functions of at least some of the upstream components of the signalling cascade are essential for the constitutive phenotype, and that the Ste12p mutants cannot bypass the normal response pathway requirement for Ste12p and, in the case of the 1–15 mutant, for Fus3p or Kss1p.

## Discussion

Previous work has indicated that the Ste12p transcription factor undergoes both positive and negative regulation by the upstream components of the pheromone signal pathway (e.g. Song *et al.*, 1991; Elion *et al.*, 1993; Bardwell *et al.*, 1998). As shown in Figure 6, there appear to be three levels of Ste12p activity: (a) a repressed inactive form of Ste12p in cells that lack spontaneous signalling activity through the pheromone pathway; (b) a partially active form that results from the spontaneous activity of the pheromone pathway in the absence of pheromone; and (c) a fully induced form of Ste12p in cells treated with pheromone. The regulation of Ste12p was examined in this study by identifying *ste12* mutations that result in a high basal level of transcription of cell type-specific genes. All of the mutations affected the amino-terminal 19 residues of Ste12p, and probably act to disrupt the function of the amino-terminal domain. Consistent with this idea, deletion of the 19 amino-terminal residues also resulted in an elevated basal level of signalling that was, in fact, higher than that observed with the point mutants. While amino-terminal sequences can affect the stability of proteins in yeast (e.g. Bachmair *et al.*, 1986), the

mechanism by which the mutant Ste12 proteins enhanced transcription was not due to their overproduction, as they were produced at levels similar to wild-type Ste12p. The increased basal activity of the mutants was also not likely to be due to changes in their subcellular localization, because Ste12p is constitutively localized to the nucleus (Hung *et al*., 1997). Thus, the mutations appear to alter the function of the Ste12p and not its abundance in the nucleus.

To better define the role of the amino-terminus, we carried out double mutant analysis to examine the activity of the mutant Ste12 proteins in cells carrying mutations in other elements of the pheromone signal pathway. Deletion of *STE2*, encoding the α-factor receptor, caused an increase in the basal level of signalling by both the mutant and wild-type Ste12p, indicating that the mutant Ste12 proteins were still responsive to basal levels of signalling. Deletion of *STE2* elevates the basal level of signalling because the unoccupied α-factor receptors are thought to prevent spontaneous activation of the signal pathway by stabilizing the pheromone-responsive G proteins into inactive RG pre-activation complexes (Dosil *et al*., 2000). The elevated basal activity of the mutant Ste12 proteins did not appear to be due to increased sensitivity to the pheromone signal, because dose–response analyses indicated that the *ste12* mutants displayed normal pheromone sensitivity. These results are consistent with the observations that the domain that mediates pheromone-responsive transcriptional induction maps to the central region of Ste12p and not the amino-terminus (Kirkman-Correia *et al*., 1993; Pi *et al*., 1997). In contrast, mutations that block the basal signalling through the pheromone pathway, such as in *STE5* (encoding a scaffold protein) or in both *FUS3* and *KSS1* (encoding MAP kinases), caused reduced levels of basal signalling. These results suggest that the mutant Ste12 proteins, like the wild-type Ste12p, require ligand-independent activity of the of the pheromone pathway for full basal transcriptional activity.

The activity of Ste12p also undergoes negative regulation, which is mediated by the ability of the inactive form of the Kss1p MAP kinase to enhance the repression of Ste12p by the Dig proteins (Bardwell *et al*., 1998) to create a fully repressed form of Ste12p. Interestingly, deletion of *KSS1* increased the basal level of signalling mediated by both wild-type and the mutant Ste12 proteins. This result suggests that the mutant Ste12 proteins, like the wild-type, are sensitive to the repressive effects of Kss1p, and is consistent with the observation that the sequences critical for Kss1p-mediated repression map to the central region of Ste12p, and not to the amino-terminus (Bardwell *et al*., 1998). Deletion of the Fus3p MAP kinase also caused an increase in the basal activity of the Ste12 proteins, suggesting the existence of a redundant negative regulatory function carried out by both Fus3p and Kss1p. As the repressive effects of Fus3p have not been studied in detail, this result adds another layer of complexity to the regulation of gene expression by Ste12p.

Comparison of the activity of the Ste12p mutants in *ste5*Δ vs *fus3*Δ *kss1*Δ strains suggests that there is complex interplay between the different forms of regulation. A double *fus3*Δ *kss1*Δ deletion lowered the basal activity of the mutant Ste12 proteins, as expected for a mutant cell that lacks spontaneous flux through the pheromone signal pathway. However, the double *fus3*Δ *kss1*Δ did not lower the basal activity to the same extent as the *ste5*Δ mutation. The observation that Fus3p and Kss1p play both positive and negative roles in signalling may account for this difference in basal signalling. The *ste5*Δ strain lacks any signalling to promote the basal state of Ste12p, but it retains the inactive forms of the MAP kinases, which can promote repression. In contrast, the *fus3*Δ *kss1*Δ double mutant had a higher basal level of Ste12p activity in the strong constitutive mutant, suggesting that the mutant Ste12p represents a form that lacks both the positive effects of basal activity of the pathway and the negative regulation mediated by the inactive forms of the MAP kinases. Taken together, these results indicate that the Ste12p mutants described in this report are subject to the same regulatory mechanisms as the wild-type protein.

The effects of the amino-terminal mutations were examined further by constructing fusion proteins with the Gal4p transcriptional activator, such that the DNA-binding domain and transcriptional activation domains of Ste12p could be analysed separately. The results suggest that the increased activity of the amino-terminal Ste12p mutants appears to be due to an increase in DNA-binding by Ste12p. This conclusion derives principally from the behaviour of Ste12p DNA-binding domain–Gal4p activation domain hybrid proteins, in which the truncated Δ19N Ste12p DNA-binding domain

 

conferred increased expression to a Ste12p-dependent reporter. Conversely, the Δ19N Ste12p DNA-binding domain was not hyperactive at a Gal4p-dependent reporter in the context of hybrid proteins containing the Gal4p DNA-binding domain. In view of the apparent increase in activity of the DNA-binding domain, it is possible that the amino-terminal mutations directly increase the affinity of the mutant proteins for DNA. Alternatively, the amino-terminal mutations may indirectly change the DNA-binding properties of Ste12p by altering its interaction with other factors present at the promoters of pheromone-responsive genes, such as Mcm1p or the general transcription machinery. In this regard, it is interesting that the amino-terminal mutants also increased the basal transcription of an α-specific reporter gene. In the context of α-specific genes, Ste12p is thought to be recruited to the upstream activating sequence by its interaction with the Mcm1p and the Matα1p DNA-binding proteins, rather than by a direct interaction of Ste12p with DNA (Yuan *et al*., 1993). In either case, the effect of the amino-terminal mutations would be to enhance basal transcription of pheromone-responsive genes by increasing the presence of Ste12p in the promoter regions of the relevant genes.

Overall, the results of this study indicate that the amino-terminus of Ste12p protein acts by a mechanism that is distinct from previously identified regulatory mechanisms to lower the basal level of Ste12p activity. Although this effect could be a property specific to the mutant proteins, it is likely that the amino-terminal domain of the wild-type Ste12p functions in a dynamic way to regulate signalling during mating, just as other components of the pheromone pathway are under multiple forms of regulation. The high degree of regulation of the pheromone pathway components is important for enabling yeast cells to undergo mating with high efficiency, since disruption of normal regulation would lead to a decrease in mating. Our studies suggest that the regulation of Ste12p is complex, and that the amino-terminus of Ste12p contributes to the overall efficiency with which yeast cells undergo mating.

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