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Pheromone Response Elements Are Necessary and Sufficient for Basal and Pheromone-Induced Transcription of the *FUS1* Gene of *Saccharomyces cerevisiae*

DAVID C. HAGEN, GRETCHEN McCAFFREY,† AND GEORGE F. SPRAGUE, JR.*
Institute of Molecular Biology and Department of Biology, University of Oregon, Eugene, Oregon 97403

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The *FUS1* gene of *Saccharomyces cerevisiae* is transcribed in a and α cells, not in a/α diploids, and its transcription increases dramatically when haploid cells are exposed to the appropriate mating pheromone. In addition, *FUS1* transcription is absolutely dependent on *STE4*, *STE5*, *STE7*, *STE11*, and *STE12*, genes thought to encode components of the pheromone response pathway. We now have determined that the pheromone response element (PRE), which occurs in four copies within the *FUS1* upstream region, functions as the *FUS1* upstream activation sequence (UAS) and is responsible for all known aspects of *FUS1* regulation. In particular, deletion of 55 bp that includes the PREs abolished all transcription, and a 139-bp fragment that includes the PREs conferred *FUS1*-like expression to a *CYC1-lacZ* reporter gene. Moreover, three or four copies of a synthetic PRE closely mimicked the activity conferred by the 139-bp fragment, and even a single copy of PRE conferred a trace of activity that was haploid specific and pheromone inducible. In the *FUS1* promoter context, four copies of the synthetic PRE inserted at the site of the 55-bp deletion restored full *FUS1* transcription. Sequences upstream and downstream from the PRE cluster were important for maximal PRE-directed expression but, by themselves, did not have UAS activity. Other yeast genes with PREs, e.g., *STE2* and *BAR1*, are more modestly inducible and have additional UAS elements contributing to the overall activity. In the *FUS1* promoter, the PREs apparently act alone to confer activity that is highly stimulated by pheromone.

Mating in the yeast *Saccharomyces cerevisiae* involves the fusion of **a** and α haploid cell types to form an **a**/α diploid cell type. The secretion and response to peptide pheromones is crucial to the mating response. Each haploid cell type produces a unique peptide pheromone (**a**-factor from **a** cells or α-factor from α cells) that can bind to a receptor present on the surface of the other cell type. Binding of pheromone to receptor activates an intracellular signal transduction pathway that elicits physiological changes in the responding cell. These changes are similar, if not identical, in **a** and α cells and include altered transcription of a group of genes whose products are involved in mating, arrest of the cell division cycle late in the G_1 phase, and alteration of the cell surface (for reviews, see references 20, 21, and 48). These latter physiological events themselves appear to be the result of transcription changes, so, to a first approximation, the pheromone pathway is a pathway whereby the pattern of transcription is regulated (9, 56). In this report we identify the promoter sequences responsible for increased transcription of a particular pheromone-responsive gene, *FUS1* (34, 53).

Genetic and physiological studies have identified several genes that encode likely components of the intracellular pheromone response pathway. An early step in response involves a heterotrimeric, GTP-binding protein (G protein) encoded by *GPA1* (*SCG1*), *STE4*, and *STE18* (8, 23, 38, 55). This G protein is thought to interact with the receptors and to become activated when the receptors are bound by ligand (5). The nature of the effector protein that is stimulated by the activated G protein is not known, but the *STE12* gene product is thought to be an ultimate target of the pathway. STE12 is required for response (18, 26) and is part of protein-DNA complexes that form at the promoter region (upstream activation sequence [UAS]) of several genes whose transcription is stimulated as a result of activation of the pheromone response pathway (10, 12). Two other genes required for pheromone response, *STE7* and *STE11*, apparently encode protein kinases (40, 52), so one possibility is that STE12 is a transcription activator whose activity is modulated by phosphorylation.

Genes whose expression increases upon pheromone reception have been shown to share a common sequence element, PRE (pheromone response element; 30, 54): 5'-ATGAAACA (sometimes reported as 5'-TGAAACA). Two lines of experimentation point to a role for PRE in pheromone-stimulated transcription. First, deletion analysis of the upstream control region from *BAR1* has revealed that the PREs are important for pheromone-mediated transcription induction (30). Second, in cases where it has been examined, the site of binding of STE12 to DNA corresponds closely to the PRE sequences (10, 12). However, these experiments do not address whether the PREs are sufficient to confer regulation by pheromone nor whether the PREs can function alone as UAS elements. In fact, at several genes the PREs occur in conjunction with known UAS elements. For example, the *BAR1* upstream region contains a binding site for the transcription activator, MCM1. Moreover, the binding of STE12 to DNA in vitro is highly cooperative with other DNA-binding proteins, in particular MCM1 (12).

* Corresponding author.
† Present address: Department of Pharmacology, University of California, San Francisco, CA 94143.

In this report, we have examined the sequences required for regulated transcription of *FUS1*, a gene required for efficient fusion of cells during mating (34, 53). *FUS1* transcription is affected in two ways by the pheromone response pathway. First, transcription increases sevenfold or more upon stimulation by pheromone. Second, basal transcription in unstimulated cells requires that the pheromone response pathway be intact; that is, no *FUS1* transcript is detected in *ste4*, *ste7*, *ste11*, or *ste12* mutants (34). This latter observation suggests that transcription of *FUS1* is tightly controlled by the signal transduction pathway and that the level of expression in unstimulated cells is due to endogenous, pheromone-independent signaling. The *FUS1* upstream region contains a cluster of four PRE sequences. We show that these sequences are necessary and sufficient for transcription and its regulation. Other sequences within the *FUS1* upstream region contribute to maximal expression, but these sequences alone do not have UAS activity.

MATERIALS AND METHODS

Yeast and bacterial strains. Most of the yeast strains used in this study are from three congenic collections. Strains related to HR125-5d (*MAT***a** *leu2-3 leu2-112 ura3-52 his3 his4 trp1*) were SY816 (*MAT*α) and SY817 (*MAT***a**/*MAT*α), both from R. Jensen, YY751 (34; *MAT*α *fus1*Δ::*URA3*), SY1409 (*MAT*α *fus1*Δ*137*::*URA3*), SY1416 (*MAT*α *fus1*Δ*137*), SY1439 (*MAT*α *fus1*Δ*137*::4xPRE), SY1593 (*MAT*α *fus1*Δ*106*), SY1551 (*MAT*α *fus1*Δ*55*), SY1595 and SY1596 (*MAT*α *fus*Δ*55*::4xPRE), and SY1549 (*MAT*α *fus1*Δ*6*). The last eight strains were constructed by standard gene replacement techniques for yeasts (41), using *FUS1* DNA fragments derived from appropriate plasmids.

Strains congenic with EG123 (*MAT***a** *leu2 ura3 his4-519 trp1 can1-101*) were 246.1.1 (*MAT*α) and 1788 (*MAT***a**/*MAT*α), provided by K. Tatchell, and DC41 (*MAT*α), DC130 (*MAT*α *ste7*Δ*1*), and DC39 (*MAT*α *ste11*Δ*1*), provided by D. Chaleff.

Strains congenic with SY1187 (4; *MAT*α *leu2 ura3 his6 trp1am ade1 ade2-1oc met14 cry1* or *CRY1*) were YY1152 (*ste3*Δ::*URA3*), SY1600 (*ste4*Δ::*URA3*), SY1431 (*ste5*Δ::*URA3*), and SY1602 (*ste12*Δ::*URA3*), all from our laboratory collection.

Two additional yeast strains were used: DC5 (*MAT***a** *leu2-3 leu2-112 his3 can1 gal2*), provided by J. Strathern, and YY754 (34; *MAT***a** *fus1*Δ::*URA3 ura3 ade2 leu1 can1-1 cyh2-1 rme1-1*).

Escherichia coli strains used have been described previously: SB69 (17), SB115 (22), JM101 (35), DH5F′ (Bethesda Research Laboratories), and CJ326 (31).

Standard media and methods were used for propagating yeast and bacterial strains (42, 46). YEPD, SD-Leu, and SD-Ura media have been described elsewhere (25, 46).

Plasmids. The plasmids used in this research were constructed by standard methods (42). The plasmids can be categorized generally as *FUS1-CYC1-lacZ* plasmids, *FUS1-lacZ* plasmids, and plasmids used for *FUS1* gene replacements. *FUS1-CYC1-lacZ* fusion plasmids were derivatives of pSL709 or pΔSS.

Plasmid pSL709 (24; see Fig. 1B and Results) is a 2μm-based, *LEU2* plasmid related to pJDB207 (2); it has a cluster of restriction sites (polylinker) upstream from a 147-bp fragment of the yeast *CYC1* gene, which provides TATA sequences and an ATG initiation codon that is connected in frame to the *lacZ* gene of *E. coli*. Plasmid pSL857, which has the 139-bp *FUS1* UAS fragment in the pSL709 context, was constructed from pSL758 (see below) by the elimination of a 117-bp *Sph*I-*Sph*I fragment, 12 bp of polylinker, plus 105 bp of nonessential *CYC1* DNA. Derivatives of pSL709 in which various copy numbers and orientations of the synthetic PRE duplex oligonucleotide (D193/D194; see below) were ligated into the unique *Bam*HI site of the pSL709 polylinker are presented in Table 1 (see Results). The copy numbers and orientations were determined by dideoxy DNA sequencing (43) after first subcloning appropriate fragments of these vectors into M13mp18 or M13mp19 (57). Control plasmid pSL853 was constructed (by J.-J. Hwang-Shum) from pSL709 by the addition of a 429-bp *Xho*I-*Xho*I fragment, derived from pSL330 (25) and including the *CYC1* UAS, at the unique *Xho*I site.

Plasmids pSL1332 and pSL1334 were also derived from pSL709 but have a synthetic oligomer related to the binding site of the mammalian transcription factor ATF/CREB cloned into the polylinker region. To make these plasmids, the oligomer (D800/D801; see below) was first coloned into the *Pst*I site of the Bluescript M13$^+$ KS plasmid (Stratagene), sequenced to confirm the copy number and orientation, and then subcloned as a *Sal*I-*Bam*HI fragment into the *Xho*I and *Bam*HI sites of pSL709. Plasmid pSL1332 has the oligomer oriented as it is found naturally in the *FUS1* upstream region (33); pSL1334 has the oligomer inverted.

Plasmid pSL745 (25) is closely related to pSL709; it has an additional 12 bp of polylinker DNA and 105 bp of *CYC1* DNA. Plasmid pSL758 was constructed from pSL745 by ligating a 139-bp *Pst*I-*Dra*I fragment, first made blunt by the action of T4 DNA polymerase, into the unique *Sma*I site. The *Pst*I-*Dra*I fragment contains the *FUS1* UAS and was excised from pSL307 (34).

Plasmid pΔSS (27; see Fig. 1C and Results) is a 2μm-based, *URA3* plasmid related to YEp24 (7). Plasmid pΔSS has a 252-bp fragment of the *CYC1* gene that provides TATA sequences and an ATG initiation codon that is connected in frame to a *lacI-lacZ* hybrid gene. Plasmids pSL974 and pSL975 were constructed from pΔSS by ligating a *Xho*I-*Sal*I fragment from pSL758, which contains the *FUS1* UAS, into the unique *Xho*I site of pΔSS; pSL975 has the fragment in the inverted orientation. Versions of pΔSS bearing various combinations of the PRE oligonucleotide (see Table 1 and Results) were constructed by ligating *Xho*I-*Sal*I fragments from the corresponding pSL709 derivatives into the *Xho*I site of pΔSS. Plasmid pLGΔ-312S (15), a precursor of pΔSS that includes the *CYC1* UAS region, served as a control.

The *FUS1-lacZ* plasmids are all related to plasmid pSL555 (34; see Fig. 1A and Results). Plasmid pSL555 is a 2μm-based, *LEU2* plasmid related to pJDB207 (2); it has approximately 2.1 kbp of *FUS1* DNA, including 409 bp of the coding region, fused in frame to *lacZ* sequences. Control plasmid pSL1146 is identical to pSL555 except that it lacks the *FUS1* fragment. Plasmid pSL553 was constructed from pSL307 (34), the precursor of pSL555, and plasmid pSL24 (25). pSL553 is similar to pSL555 except that 1.4 kbp of *FUS1* upstream sequences have been eliminated and a *Hin*dIII-*Pst*I fragment of the yeast *URA3* gene, a remnant of pSL307, replaces a *Hin*dIII-*Bam*HI fragment of pBR322 (6) origin. Plasmid pSL642 is identical to pSL553 except that a 4.4-kbp *Pst*I-*Pst*I fragment was inverted in order to change the DNA context upstream from the *FUS1* fragment.

Several plasmids were constructed that were identical to pSL555 except for insertions, deletions, or substitutions within the *FUS1* fragment. Plasmid pSL626 was constructed

in several steps from pSL307, pSL24, and M13 sequencing vectors; the net result was a plasmid identical to pSL555 except that a *Pst*I-*Sal*I-*Xba*I-*Bam*HI-(*Sma*I) polylinker, derived from M13mp19 (57), was inserted at the *Pst*I site of the *FUS1* segment (construction details not presented). Plasmid pSL606 was similarly constructed except that the polylinker was inserted between the *Pst*I and *Dra*I sites of the *FUS1* fragment—a deletion of 137 bp (*FUS1* coordinates −259 to −123; see Fig. 2A). Plasmids pSL1268 and pSL1269 are separate isolates that harbor a 55-bp deletion (coordinates −208 to −154; see Fig. 2A). They were constructed by replacing a *Pst*I-*Eco*RI fragment of pSL606 with a *Pst*I-*Eco*RI fragment from plasmid pSL1245 (see below). Plasmid pSL1286 has a deletion of 106 bp (coordinates −259 to −154; see Fig. 2A); it was constructed by replacing a *Hin*dIII-*Bgl*II fragment of pSL1269 with a *Hin*dIII-*Bam*HI fragment of pSL606. Plasmid pSL1264 has a deletion of 6 bp (coordinates −249 to −244; see Fig. 2A); it was constructed by replacing a *Pst*I-*Eco*RI fragment of pSL606 with a *Pst*I-*Eco*RI fragment of plasmid pSL1241 (see below).

Several pSL555-like *FUS1-lacZ* plasmids were constructed with four copies of the PRE oligomer duplex, in the rightward orientation (see below), replacing deleted *FUS1* DNA. Plasmid pSL1148 has the PREs at the site of the 137-bp deletion; it was made by replacing a *Hin*dIII-*Eco*RI fragment of pSL606 with a *Hin*dIII-*Eco*RI fragment of pSL1088 (see below). Plasmid pSL1282 has the PREs at the site of the 106-bp deletion; it was made by replacing a *Hin*dIII-*Bgl*II fragment of pSL1268 with a *Hin*dIII-*Bst*YI fragment of plasmid pSL1088 (see below). Plasmid pSL1319 has the PREs at the site of the 55-bp deletion; it was made by replacing a *Bgl*II-*Bam*HI fragment of pSL1269 with a *Pst*I-*Bam*HI fragment of pSL1282.

A collection of plasmids served as sources of DNA fragments useful for replacing the wild-type *FUS1* promoter at the chromosomal locus with *FUS1* promoter mutations made in vitro. Plasmid pSL996 was constructed by cloning a *Hin*dIII-*Eco*RI fragment from pSL606, which carries the 137-bp *FUS1* deletion, into the *Hin*dIII and *Eco*RI sites of plasmid pSP65 (Promega Biotec). Plasmid pSL1088 with the *FUS1* 137-bp deletion replaced by four rightward PREs was constructed by cloning a *Sma*I-*Bst*YI fragment of pSL940 into the *Sal*I and *Bam*HI sites of pSL996 after first making the *Sal*I site blunt by the action of *E. coli* DNA polymerase Klenow fragment. Other plasmids used for replacement of the chromosomal *FUS1* promoter were constructed by oligomer-directed mutagenesis. First, the *Eco*RI-*Eco*RI *FUS1* fragment from pSL555 was cloned into the Bluescript M13$^+$ KS plasmid (Stratagene), creating pSL1206. Plasmid pSL1245 with the 55-bp deletion and pSL1241 with the 6-bp *FUS1* deletion were made from pSL1206 by using oligonucleotides D813 and D799 (see below), following the method of Kunkel et al. (22, 31). DNA preparations were made from separate transformants and screened for the acquisition of diagnostic restriction sites. Mutant candidates were confirmed by DNA sequencing (43).

Two plasmids were used for making ^{32}P-labeled RNA probes (see below). Plasmid pSL589, for probing *FUS1* mRNA, was constructed by cloning a 2.1-kbp *Pst*I-*Pvu*II fragment of *FUS1* DNA from plasmid pSL324 (34) into the *Pst*I *Sma*I sites of plasmid pSP65 (Promega Biotec). Plasmid pSL613, for probing *URA3* mRNA, was constructed (by A. Bender) by cloning a 1.0-kb *Pst*I-*Hin*dIII fragment of *URA3* DNA from plasmid YEp24 (7) into the *Pst*I and *Hin*dIII sites of plasmid pSP64 (Promega Biotec).

Oligonucleotides. Oligonucleotides were synthesized by the University of Oregon Biotechnology Laboratory on a model 380B Applied Biosystems synthesizer (phosphoramidite method). The PRE oligonucleotide duplex, with *Bam*HI-complementary ends, was a combination of oligonucleotides D193 (5′-GATCATGAAACA) and D194 (5′-GATC TGTTTCAT). We define a rightward PRE insert as having the orientation as D193 is entered above and with TATA sequences and the direction of transcription to the right. The ATF/CREB site oligonucleotide duplex, with *Pst*I-complementary ends, was a combination of oligonucleotides D800 (5′-TTGACGTATGCA) and D801 (5′-TACGTCAATGCA). Oligonucleotides D813 (5′-CACTTTTCGCGCAGATCTAA TAACGTAATTCTCG) and D799 (5′-TGCCCTTTTCTCGA GATTGAATGG) were designed to create mutations within the *FUS1* segment of plasmid pSL1206. D813 was used to delete 55 bp (coordinates −208 to −154) of *FUS1*, including the four naturally occurring PRE sequences, and insert 6 bp, a *Bgl*II restriction site. D799 was used to delete 6 bp (coordinates −249 to −244) of *FUS1*, the ATF/CREB-like site of *FUS1* (33), and insert 6 bp, a *Xho*I restriction site.

β-Galactosidase assays. For β-galactosidase assays, plasmid-bearing yeast strains were grown at 30°C overnight to saturation in SD-Leu or SD-Ura selective medium. The cells were diluted into YEPD medium to a density of about 10^7 cells per ml and grown at 30°C for one doubling (3 to 4 h). The cells were then mixed 1:1 with prewarmed, fresh YEPD medium, with **a**-factor, or with α-factor and incubated at 30°C with agitation for an additional 2.5 h. The **a**-factor preparation was the culture filtrate of strain DC5 grown to saturation in YEPD medium at 30°C (17). The α-factor preparation was synthetic α-factor purchased from Sigma and made up to 1.25 μg/ml in YEPD medium. The cells were prepared and assayed, and the units of β-galactosidase activity were calculated as previously described (25). For experiments not involving pheromone treatment, the overnight cultures were simply diluted into SD-Leu medium to about 10^7 cells per ml and grown for 5 h before harvesting for the assays.

Quantitative mating assay. A quantitative mating assay was devised previously to distinguish Fus1$^+$ and Fus1$^-$ phenotypes (34); here the method was modified slightly. Strain YY754 (*MAT***a** *fus1*Δ::*URA3*) served as the standard test strain to which a series of *MAT*α strains were mated. Each strain was grown in YEPD medium at 30°C to a density of about 3 × 10^7 cells per ml; the cells were centrifuged and resuspended in YEPD medium diluted 10-fold in water. Mixtures of 10^7 cells of YY754 plus 10^6, 10^5, or 10^4 α cells were made in 250-μl volumes and spread on plates of SD medium, selecting for diploid colonies. The separate suspensions of **a** and α cells were also titered on plates of YEPD medium. In this mating protocol, 1 to 4% of wild-type α cells (SY816) formed diploid colonies; the mating efficiency of mutant α cells is expressed relative to the mating of SY816 performed in parallel experiments.

RNA analysis. For analysis of *FUS1* mRNA levels, plasmid-free cells were grown exponentially in YEPD medium at 30°C to about 3 × 10^7 cells per ml, then mixed 1:1 with prewarmed YEPD medium or with **a**-factor, and grown for an additional 30 min before harvesting for RNA preparation. RNA was isolated and probed as described previously (49) except that total RNA rather than poly(A)$^+$ RNA was used. Samples containing 20 μg of RNA were fractionated by gel electrophoresis. The gel was blotted to nitrocellulose, the blot was hybridized with ^{32}P-labeled RNA probes for *FUS1* and *URA3*, and the RNA-RNA hybrids were visualized by

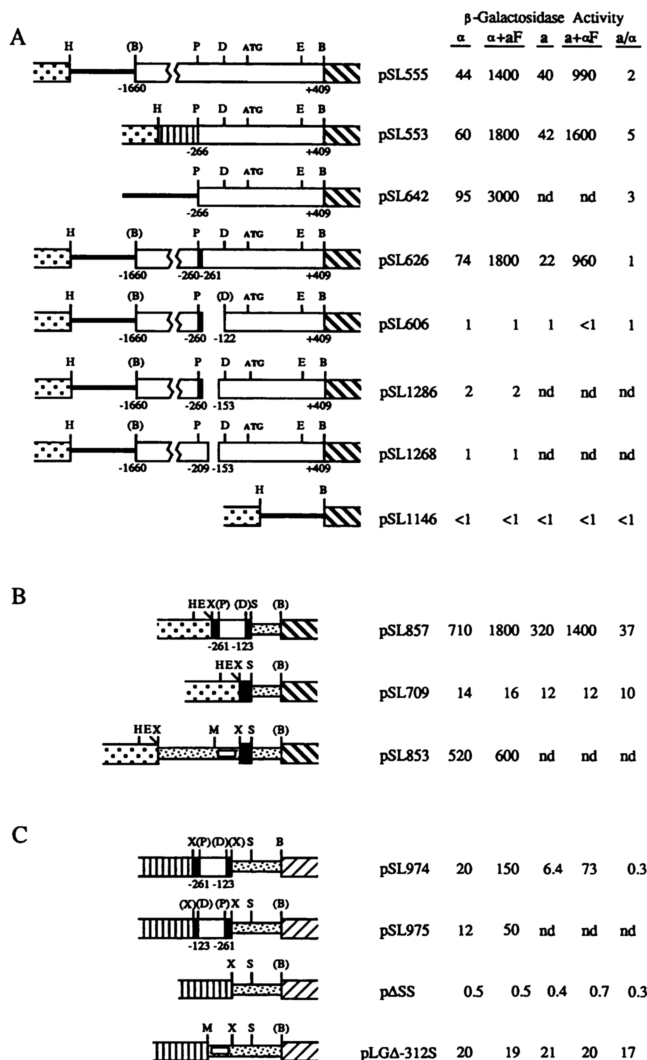


FIG. 1. *FUS1-lacZ* fusions and *FUS1-CYC1-lacZ* fusions. In panel A, *FUS1* DNA segments (▭), including the *FUS1* ATG initiation codon, have been connected in frame to *lacZ* (▧) from the ninth codon onward. The origins of segments upstream of *FUS1* are indicated as follows: pBR322 (—), 2μm plasmid (⁘), and *URA3* (▥). Polylinker segments of plasmids pSL626, pSL606, and pSL1286 are indicated by vertical black bars. In panels B and C, a DNA segment of *FUS1* containing the UAS is connected upstream to a segment of the *CYC1* gene (▩) that includes TATA elements and an ATG codon connected in frame to *lacZ*. Other symbols are as in panel A. The *lacZ* segments (▨) of plasmids in panel C differ from the other *lacZ* segments. Control plasmids pSL853 and pLGΔ-312S have *CYC1* fragments that include the *CYC1* UAS (indicated by a small open box) and no *FUS1* DNA. In all panels, coordinates are relative to the *FUS1* ATG and indicate the nucleotides included in the fragments. Reference restriction sites are indicated as follows: *Hin*dIII, H; *Bam*HI, B; *Pst*I, P; *Dra*I, D; *Eco*RI, E; *Xho*I, X; *Sph*I, S; and *Sma*I, M. Restriction sites marked with parentheses were used in constructing the plasmids but are no longer present. Restriction sites within the polylinker segments are not indicated, and the *Xho*I site indicated for pLGΔ-312S is actually a *Xho*I-*Sal*I-*Xho*I restriction site cluster. Each plasmid was introduced into strains 246.1.1, EG123, and 1788 (A) or strains SY816, HR125-5d, and SY817 (B and C), and the transformants were assayed for β-galactosidase activity. Haploid cells were assayed after growth with or without pheromone (a-factor [aF] or α-factor [αF]). Assays were performed as described in Materials and Methods, and activities are expressed in modified Miller units (25, 37). In all instances the values reported are the means of at least two determinations; in most cases three independent transformants were assayed. One standard deviation, for values of 20 U or greater, was typically 30% of the mean value. nd, assays not done.

autoradiography. The signals were evaluated quantitatively by scanning the autoradiograph with a Biomed Instruments scanning densitometer; the values for *FUS1* mRNA were standardized relative to *URA3* mRNA of the same lane. The RNA probes were made by using an SP6 transcription system purchased from Promega Biotec and plasmids pSL589 (for *FUS1*) linearized with *Hin*dIII and pSL613 (for *URA3*) linearized with *Bam*HI.

RESULTS

DNA segment necessary for *FUS1* expression. *FUS1* is a gene that is expressed only in **a** and α yeast cell types, not in **a**/α diploid cells. Furthermore, in **a** and α cells, *FUS1* mRNA levels increase sevenfold or more when the cells are exposed to the pheromone produced by the opposite cell type (34, 53). We sought to identify the DNA sequences upstream of the *FUS1* coding region responsible for conferring this pattern of regulation. Previously we reported the construction of a *FUS1-lacZ* gene fusion which placed the *E. coli lacZ* gene under the control of the *FUS1* promoter (34; Fig. 1A, plasmid pSL555). This original fusion included 1,660 bp of DNA upstream from the putative initiation codon of *FUS1*. Here, by deletion analysis, we further define the region necessary and sufficient for control of *FUS1*.

To define a region necessary for *FUS1* transcription, we created additional deletions in the context of the *FUS1-lacZ* gene fusion. A deletion to −266 with respect to the translation initiation codon retained the characteristic pattern of *FUS1* expression (Fig. 1A). Two plasmids with this deletion endpoint, but differing in the sequence adjacent to *FUS1-lacZ*, exhibited similar levels of expression, implying that neighboring sequences contribute little to the activity of the *FUS1-lacZ* construction (plasmids pSL553 and pSL642). The expression conferred by this deletion implies that sequences downstream of −266 are required for *FUS1* transcription. To test this possibility, we created three internal deletions in the context of the original large *FUS1* segment. These deletions removed 137 bp (coordinates −259 to −123), 106 bp (coordinates −259 to −154), or 55 bp (coordinates −208 to −154) of *FUS1* DNA (Fig. 2A), and all resulted in the lack of expression of *FUS1-lacZ* (Fig. 1A; compare plasmids pSL606, pSL1286, and pSL1268 with pSL626 or pSL555). Thus, the region defined by these deletions contains sequences essential for *FUS1* expression.

Definition of sequences sufficient for the *FUS1* pattern of expression. To define more precisely the element(s) responsible for control of *FUS1* transcription, we took two approaches. First, we asked whether a small fragment from the *FUS1* upstream region, corresponding closely to the 137-bp segment deleted above, could confer the *FUS1* pattern of expression to *CYC1-lacZ* reporter genes. The *CYC1* component of these genes contributes a TATA sequence and signals for transcription and translation initiation but lacks a UAS. A 139-bp DNA segment from *FUS1* (coordinates −261 to −123) was ligated immediately upstream of the *CYC1-lacZ* gene fusions. In two plasmid backgrounds, pSL709 and pΔSS, this fragment conferred haploid-specific, pheromone-

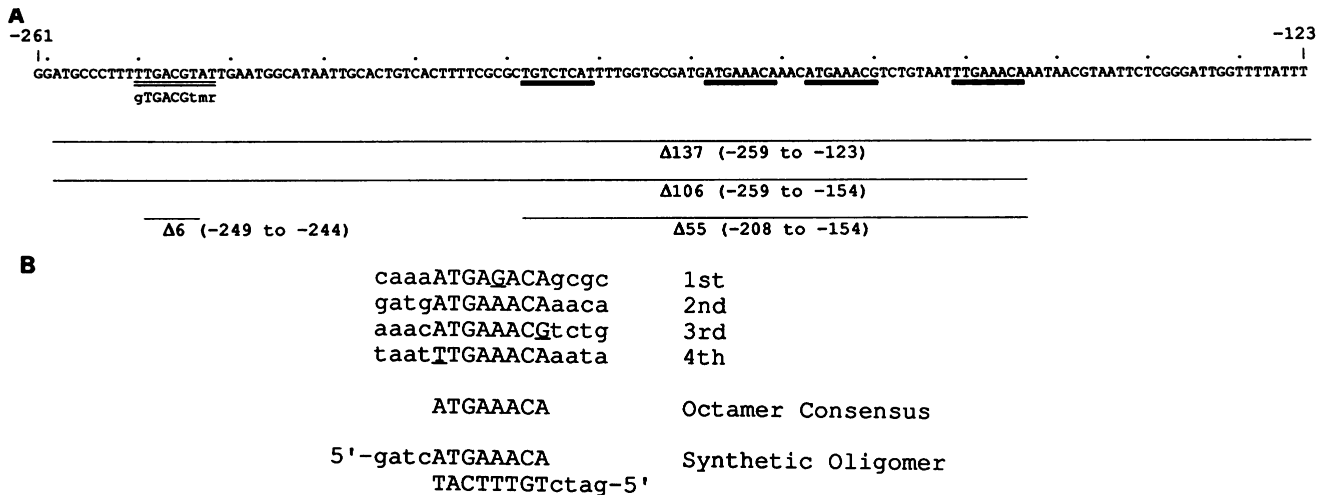


FIG. 2. *FUS1* UAS and *FUS1* PREs. (A) Sequence of a 139-bp fragment of DNA from the *FUS1* upstream region that is sufficient to confer *FUS1*-like expression to a heterologous gene (e.g., the *CYC1-lacZ* reporter gene). Coordinates are relative to the initiation codon of *FUS1*. The four PRE sites are underlined with bars. An ATF/CREB-like site (33) is doubly underlined with the consensus sequence (32) printed below; bases in lowercase are less highly conserved; M = A or C, R = A or G. The extents of four deletions, referred to in the text, are also indicated. The sequence is identical to our previously reported *FUS1* sequence except that a sequencing error was discovered and corrected: −262 GGATCGCCC (34) is now −261 GGATGCCC; the new sequence is in complete agreement with the *FUS1* sequence of Trueheart et al. (53). (B) Alignment of the four PREs of *FUS1* The sequence of the first PRE has been inverted. The PRE octamer consensus sequence (30, 54) and the PRE oligonucleotide duplex used in this study are also shown.

inducible production of β-galactosidase (plasmids pSL857 and pSL974 in Fig. 1B and C, respectively). The same fragment when inverted also conferred pheromone-inducible activity but was two- to threefold less active (plasmid pSL975; Fig. 1C). Gene fusions that lacked a UAS or contained the natural *CYC1* UAS did not show the characteristic *FUS1* pattern of expression. The 139-bp fragment thus contains the *FUS1* UAS.

The two different plasmid backgrounds used in Fig. 1B and 1C yielded data that were qualitatively similar but quantitatively quite dissimilar. The pSL709- and pΔSS-based plasmids differ from each other in several ways: in the *lacZ* fragments that they contain, in the spacing of the UASs from the TATA region, in the sequences immediately upstream of the UASs, and in the number of copies per yeast cell. Each of these differences contributes substantially to the observed discrepancies in the activities conferred (data not presented). Nevertheless, each system has its relative advantages and disadvantages. Plasmid pSL709 with a UAS inserted confers a high level of β-galactosidase activity to cells, but because a ceiling of activity is often attained, regulatory inputs are not fully apparent. Thus, with the *FUS1* UAS fragment (pSL857; Fig. 1B), expression was stimulated by pheromone only 2.5-fold in α cells and 4.4-fold in **a** cells. In contrast, *FUS1* mRNA production from the chromosome increases sevenfold or more in response to pheromone reception. On the other hand, plasmids derived from pΔSS give rise to lower β-galactosidase activities, but the activities often parallel more closely the regulation characteristic of the chromosomal locus. Thus, with the *FUS1* UAS fragment (pSL974; Fig. 1C), expression was stimulated by pheromone 7.5-fold in α cells and 11-fold in **a** cells. Both types of plasmids were again used in the following experiments.

The second approach to define the sequences sufficient for the *FUS1* pattern of expression tested the UAS activity of a repeated sequence element, PRE (5'-ATGAAACA), found within the 139-bp *FUS1* segment and within the upstream region of other pheromone-inducible genes (30, 54). The *FUS1* UAS contains four copies of this PRE sequence, one copy matching the consensus perfectly and three copies differing from consensus at single (but different) positions (Fig. 2). The internal deletions discussed above all remove the four PREs. To determine whether PREs can function as UAS elements, we cloned single and multiple copies of a synthetic version of the octanucleotide (Fig. 2B) into the *CYC1-lacZ* vectors. Table 1 presents β-galactosidase assays performed on cells harboring derivatives of plasmid pSL709 (group A) or derivatives of pΔSS (group B) with one to four copies of PRE. The assay values clearly demonstrate that PRE by itself conferred UAS activity that is haploid specific and pheromone inducible. A single copy conferred a trace of activity (detected only in the pSL709 background); additional copies conferred much more activity in a nonadditive, cooperative fashion (compare UASs labeled R, RR, RRR, and RRRR). Both orientations were active, although one orientation was more than twice as active as the other (compare UASs labeled R to L, compare RR with LL, and compare RRR with LLL in group A). Plasmids bearing multiple copies of PRE mimicked very closely the activation levels conferred by the *FUS1* UAS fragment (compare the *FUS1* UAS with UASs labeled RRR and RRRR, for example).

The *FUS1* UAS, as can be seen in Fig. 2, contains four PREs, and they are spaced 12, 3, and 8 bp apart. Additionally, three of the PREs are imperfect matches to the consensus sequence, and one has the leftward orientation. The PREs in the artificial clusters that we have made are all perfect matches to the consensus, and the spacing is always 4 bp. We have not yet studied the consequences of single base substitutions within the sythetic PREs or variations in the spacing between adjacent PREs, and although we have demonstrated that the natural cluster of PREs is essential for

TABLE 1. Activities of UAS fragments and synthetic PRE oligonucleotides in *CYC1-lacZ* plasmids[a]

Plasmid	UAS[b]	β-Galactosidase activity				
		α	α + aF	a	a + αF	a/α
Group A						
pSL709[c]	None	14	16	12	12	10
pSL857[c]	*FUS1*	710	1,800	320	1,400	37
pSL844	R	29	82	25	75	14
pSL845	L	16	35	ND[d]	ND	ND
pSL938	RR	150	870	ND	ND	ND
pSL875	LL	41	320	ND	ND	ND
pSL943	RRR	590	1,800	240	1,300	33
pSL879	LLL	180	1,000	ND	ND	ND
pSL940	RRRR	940	1,900	ND	ND	ND
pSL853[c]	*CYC1*	520	600	ND	ND	ND
pSL1332	ATF	5	6	ND	ND	ND
pSL1334	ATF^{-1}	6	5	ND	ND	ND
Group B						
pΔSS[c]	None	0.5	0.5	0.4	0.7	0.3
pSL974[c]	*FUS1*	20	150	6.4	73	0.3
pSL975[c]	$FUS1^{-1}$	12	50	ND	ND	ND
pSL976	R	0.6	0.5	ND	ND	ND
pSL980	RR	0.6	2.2	ND	ND	ND
pSL982	RRR	1.7	44	0.9	17	0.2
pSL984	RRRR	5.1	150	1.8	50	0.3
pLGΔ-312S[c]	*CYC1*	20	19	21	20	17

[a] Plasmids were introduced into congenic *MAT*α, *MAT***a**, and *MAT*α/*MAT***a** strains derived from HR125-5d. Groups A and B represent constructions harbored in different plasmid backgrounds, pSL709 or pΔSS. The α cells were grown with or without **a**-factor (**a**F), and the **a** cells were grown with or without α-factor (αF). Cells were assayed for β-galactosidase activity as described in Materials and Methods. Activities are reported in modified Miller units (25, 37). In each case, three independent transformants were assayed; the mean activities are listed.

[b] The UASs used are the *FUS1* UAS (a 139-bp fragment, described in the text and Fig. 2A), the *CYC1* UAS (described for plasmids pSL853 and pLGΔ-312S in Materials and Methods), and various combinations of the PRE oligonucleotide duplex. R and L indicate rightward and leftward PREs; the orientations are defined in Materials and Methods. In addition to the PRE combinations listed, LR, LRR, LRL, LLR, RRRL, RRLL, LLRR, LLLR, and clusters of more than four PREs were also isolated in the pSL709 background. These PRE combinations generally gave β-galactosidase activities comparable to those of homogeneous clusters of the same size; for clarity of presentation, the heterogeneous clusters are not included. $FUS1^{-1}$ indicates a *FUS1* UAS inverted relative to the natural orientation. ATF indicates an oligonucleotide duplex matching the *FUS1* ATF/CREB-like sequence (33; see text). ATF^{-1} indicates the ATF/CREB-like sequence inverted relative to the natural orientation.

[c] Assay values are presented also in Fig. 1.

[d] ND, assays not done.

FUS1 expression, we have not studied the relative contributions of the individual PREs of the *FUS1* UAS.

In summary, the synthetic PRE sequence (in three or four copies) closely duplicated the gene control conferred by the bona fide 139-bp *FUS1* UAS sequence. Expression was observed only in α and **a** cells, not in **a**/α cells, and expression in the haploid cells was induced to higher levels when the cells were exposed to the pheromone of the opposite cell type.

PRE-directed expression depends on *STE4*, −*5*, −*7*, −*11*, and −*12* genes. Another characteristic of *FUS1* expression is its absolute dependence on products of the *STE4*, *STE5*, *STE7*, *STE11*, and *STE12* genes required for pheromone response (34). The UAS activity of the synthetic PREs also showed a strong requirement for the *STE* gene products. Virtually no β-galactosidase activity was observed when *lacZ* reporter plasmids containing either the *FUS1* UAS or the synthetic UAS (three copies) were harbored in strains bearing null alleles of *STE4*, *STE5*, *STE7*, *STE11*, or *STE12* (Table 2), and, as expected, there was no pheromone response (data not shown). Because *FUS1* expression in unstimulated cells is fully dependent on the products of these genes, it appears that *FUS1* is tightly controlled by the signal transduction pathway, with control being exerted through the PRE sequences.

TABLE 2. Effects of sterile mutations on *FUS1*- and PRE-directed expression[a]

Strain (genotype)	β-Galactosidase activity			
	pSL709, none[b]	pSL857, *FUS1*	pSL943, RRR	pSL853, *CYC1*
Group A				
DC41 (wild type)	21	590	500	590
DC130 (*ste7*)	24	9	30	850
DC39 (*ste11*)	20	8	21	820
Group B				
SY1187 (wild type)	9	320	230	800
SY1600 (*ste4*)	7	6	18	630
SY1431 (*ste5*)	6	3	14	430
SY1602 (*ste12*)	8	1	14	610
YY1152 (*ste3*)	5	270	180	460

[a] Plasmids were introduced into the indicated strains; group A and group B strains represent two different congenic collections. The cells were assayed for β-galactosidase activity as described in Materials and Methods. Activities are reported in modified Miller units (25, 37). In each case, the mean activity of three independent transformants is reported.

[b] Plasmid and UAS. The UASs (where used) were the 139-bp *FUS1* UAS fragment, three rightward PRE oligonucleotides (RRR), and a 429-bp *CYC1* UAS fragment. See Materials and Methods for plasmid descriptions.

The observation that the basal level of transcription of *FUS1* requires an intact pheromone response pathway raises a question regarding the origin of the basal expression in unstimulated wild-type cells. Two possibilities seem reasonable. First, a low level of signal may be propagated along the intracellular pathway even in the absence of pheromone. For example, there may always be some G-protein molecules in the activated form or some receptor in a conformation that mimics the ligand-bound state. Alternatively, cultures of haploid cells may contain a low concentration of the opposite pheromone, for example produced by cells that have undergone mating-type switching events, which would lead to a weak stimulation of the pheromone pathway. To test whether the basal level of expression is due to this latter possibility, we transformed the *lacZ* reporter plasmids into an α strain bearing a null allele of *STE3*, which encodes the **a**-factor receptor (16). β-Galactosidase production in these transformants was not significantly lower than in the wild-type background (Table 2), an observation that suggests that the receptor plays no role in generating the basal level of *FUS1* transcription.

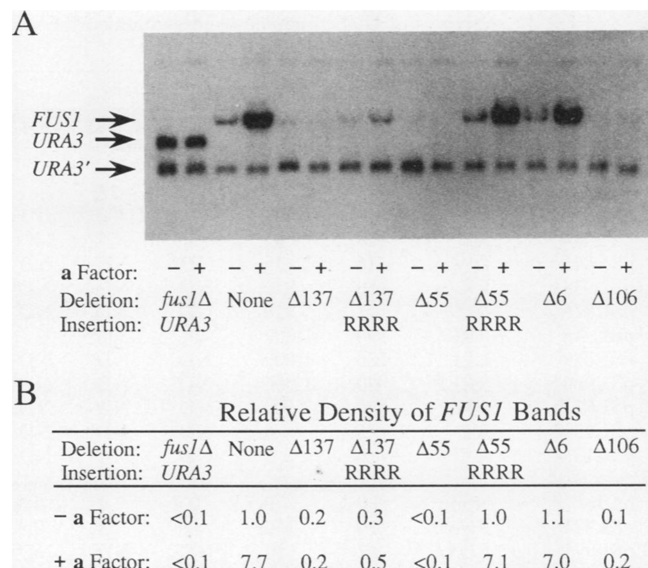
Sequences flanking PREs contribute to maximal *FUS1* expression. The octanucleotide PRE sequence, tandemly repeated in three or four copies, duplicated all properties characteristic of the *FUS1* UAS when tested above in *CYC1-lacZ* plasmid constructs. To test more rigorously the competence of the synthetic UASs, we assessed their ability to function in the setting of the chromosomal *FUS1* locus. Four copies of the PRE were inserted at the sites of the 137-, 106-, and 55-bp deletions within the *FUS1* upstream region. These new constructs were used to replace the chromosomal *FUS1* UAS, and their UAS activity was assessed by two

TABLE 3. Activities of *FUS1* UAS deletion and substitution mutations in *FUS1-lacZ* plasmids[a]

Plasmid	*FUS1* UAS	β-Galactosidase activity	
		No **a**-factor	**a**-factor
pSL555[b]	Wild type	44	1,400
pSL606[b]	Δ137	1	1
pSL1148	Δ137 + RRRR	3	110
pSL1286[b]	Δ106	2	2
pSL1282	Δ106 + RRRR	6	270
pSL1268[b]	Δ55	1	1
pSL1319	Δ55 + RRRR	51	1,200
pSL1264	Δ6	100	1,800

[a] Plasmids were introduced into *MAT*α strain 246.1.1. The cells were grown with or without **a**-factor and assayed for β-galactosidase activity as described in Materials and Methods. Activities are reported in modified Miller units (25, 37). In each case, the mean activity of three independent transformants is reported. The UAS deletions are depicted in Fig. 2A. RRRR indicates an insertion of four rightward PRE oligonucleotides. See Materials and Methods for plasmid descriptions.

[b] Assays values are presented also in Fig. 1A.



Deletion:	*fus1*Δ	None	Δ137	Δ137	Δ55	Δ55	Δ6	Δ106
Insertion:	*URA3*			RRRR		RRRR		
− **a** Factor:	<0.1	1.0	0.2	0.3	<0.1	1.0	1.1	0.1
+ **a** Factor:	<0.1	7.7	0.2	0.5	<0.1	7.1	7.0	0.2

FIG. 3. RNA analysis of strains bearing deletions and substitutions in the *FUS1* UAS region. Deletions Δ137, Δ55, Δ6, and Δ106, described in Materials and Methods and in Fig. 2A, are harbored in strains SY1416, SY1551, SY1549, and SY1593, respectively. Strains SY1439 and SY1595 contain four rightward copies of the synthetic PRE oligomer duplex (RRRR) inserted at the Δ137 or Δ55 deletion site. The deletion *fus1*Δ in strain YY751 serves as a negative control; it has the entire promoter region and most of the coding region of *FUS1* deleted. Strain SY816 is wild type and serves as a positive control. All of the strains are isogenic with SY816 except for the indicated deletions and insertions. Each strain was grown with or without **a**-factor, and total RNA was isolated and probed for *FUS1* and *URA3* mRNA as described in Materials and Methods. In panel A, the positions corresponding to *FUS1* and *URA3* mRNA on the autoradiograph are indicated. All of the strains carry the *ura3-52* allele of the *URA3* gene that gives rise to a truncated transcript, labeled *URA3'*. The strain bearing *fus1*Δ has, in addition, a wild-type *URA3* allele (inserted at the deleted *FUS1* locus) that gives rise to a full-length *URA3* transcript, labeled *URA3*. Each lane of the autoradiograph was scanned with a densitometer. (B) Density of each *FUS1* band, expressed relative to the density of the *URA3'* band of the same lane.

methods: *FUS1* transcript levels were measured, and mating assays that distinguish Fus1$^+$ from Fus1$^-$ cells were performed. In a parallel set of experiments, we constructed *FUS1-lacZ* fusions in which the PRE sequences were flanked on both sides by natural *FUS1* sequences. Cells carrying these plasmid-borne *FUS1-lacZ* fusions were assayed for β-galactosidase activity. As described below, we found that *FUS1* sequences immediately upstream and downstream of the natural PRE sequences are not essential but contribute significantly to maximal expression.

As noted earlier, strains bearing the 137-bp deletion in a *FUS1-lacZ* construct showed no β-galactosidase activity (Fig. 1A and Table 3). Likewise, α cells carrying this deletion at the chromosomal *FUS1* locus produced little or no *FUS1* mRNA even when exposed to **a**-factor (Fig. 3), and they showed a Fus1$^-$ phenotype in the mating assay (Table 4). Insertion of four PREs at this deletion site restored activity, but to no more than 10% of the wild-type levels by any of the three criteria: β-galactosidase levels (Table 3), *FUS1* mRNA production (Fig. 3), or mating ability (Table 4).

The two smaller deletions also destroyed UAS activity at the chromosomal locus. In particular, no *FUS1* transcript was detected (Fig.3) and the 55-bp deletion resulted in a Fus1$^-$ phenotype in mating assays (Table 4; the 106-bp deletion was not tested). Insertion of four PREs at each deletion site restored UAS activity. In the case of the 106-bp deletion, the activity was greater than seen for the 137-bp deletion, but the activity was still noticeably less than for the wild-type UAS (Table 3). However, in the context of the 55-bp deletion, the four PREs restored UAS activity to essentially wild-type levels (Tables 3 and 4 and Fig. 3).

Thus, sequences on both sides of the PREs contribute to maximal activity of the wild-type UAS. What might these sequences be? In the region downstream of the *FUS1* PRE cluster, there is an AT-rich region which is present in the 106- and 55-bp deletions but absent in the 137-bp deletion. This region may be part of the *FUS1* TATA region and therefore would be required for maximal expression. Upstream of the *FUS1* PRE cluster, a sequence resembling the mammalian ATF/CREB element has been noted by Lin and Green (33; underlined in Fig. 2A); furthermore, mammalian ATF/CREB elements have UAS activity in yeast cells in vivo, and yeast extracts contain a factor that binds to these elements (28, 33, 44). Nevertheless, for the following reasons, we feel that the ATF/CREB-like site in the *FUS1* UAS region does not play a role in *FUS1* expression: (i) the 55-bp deletion fully retains the ATF/CREB-like site yet has essentially no UAS activity (Tables 3 and 4 and Fig. 3); (ii) a 6-bp deletion (coordinates −249 through −244; Fig. 2A) of the ATF/CREB-like site is indistinguishable from wild type (Tables 3 and 4 and Fig. 3); and (iii) a synthetic copy of the ATF/CREB-like region of *FUS1* confers no UAS activity when cloned into a UAS-free *CYC1-lacZ* reporter vector, pSL709 (Table 1, group A). Thus, a sequence upstream of the PREs, other than the ATF/CREB-like sequence, contributes significantly to maximal *FUS1* expression.

DISCUSSION

In both haploid yeast cell types, an early response to pheromone is increased transcription from several genes. Previous work has suggested that a sequence element termed the PRE is an important component of the promoter

TABLE 4. Mating efficiencies of strains bearing deletion and substitution mutations in the *FUS1* UAS[a]

Strain	*FUS1* UAS	Relative mating frequency
SY1416	Δ137	3×10^{-4}, $<5 \times 10^{-5}$
SY1439	Δ137 + RRRR	2×10^{-2}, 2×10^{-2}
SY1551	Δ55	$<4 \times 10^{-5}$, 1×10^{-4}
SY1595, SY1596	Δ55 + RRRR	0.7, 1.0
SY1549	Δ6	0.7, 1.2
YY751	*fus1*Δ + *URA3*	$<4 \times 10^{-5}$, 6×10^{-5}

[a] The *MAT*α strains listed are all isogenic to strain SY816 except for the indicated deletions or substitutions in the *FUS1* UAS. Deletions Δ137, Δ55, and Δ6 are depicted in Fig. 2A; *fus1*Δ is a deletion of the entire promoter region and most of the coding region of *FUS1*. The insertion RRRR is four rightward copies of the synthetic PRE oligonucleotide. The α cells were mixed with an excess of **a** cells (strain YY754 [*MAT***a** *fus1*Δ::*URA3*]) and spread,in a limited amount of rich medium, on a plate selective for **a**/α diploid cells (see Materials and Methods). Mating frequencies are expressed relative to the mating frequency of wild-type strain SY816 (assigned a value of 1) determined in a parallel experiment. The wild-type strain mated with an absolute frequency of 0.1 to 0.4. Strains SY1595 and SY1596 are identical. Each was assayed for mating, and the values are listed on the same row. Each of the other strains was assayed twice; both values are listed.

regions of some pheromone-stimulated genes (30, 54). We have extended this analysis by determining the DNA sequences that are responsible for basal and pheromone-stimulated transcription of *FUS1*. This gene is transcribed in both haploid cell types, not in **a**/α diploids, and its transcription is highly induced by treating haploids with the appropriate pheromone (34, 53).

We find that a cluster of PRE elements in the *FUS1* upstream control region is both necessary and sufficient for *FUS1* transcription and all facets of its regulation. In particular, all expression is abolished when the four PREs in the *FUS1* upstream region are removed by a 55-bp deletion. This result is complemented by the finding that a 139-bp fragment of DNA including the PREs of *FUS1* can confer *FUS1*-like expression to a *CYC1-lacZ* reporter gene. Furthermore, a simple cluster of synthetic PREs can substitute for the *FUS1* UAS fragment either in the reporter gene constructions or in the natural *FUS1* context. Thus, the PREs alone can act as UAS elements to confer haploid-specific transcription that is highly stimulated by pheromone. Even so, at the natural *FUS1* locus, the UAS activity of the PREs is increased by neighboring sequences, which by themselves are not UAS elements. Whether these sequences are simply providing a more favorable context or are actually important for binding auxiliary proteins is not known.

Our finding that the PRE sequences serve as haploid-specific UAS elements is in contrast to a recent report from Sengupta and Cochran (45). They found that the PREs conferred **a**-specific expression and therefore had to invoke a second, non-PRE UAS to rationalize the haploid-specific expression of a gene like *FUS1*. We find no evidence for a second, α-specific UAS at *FUS1*. Rather, our results lead to a simple conclusion: *FUS1* contains a single PRE-based UAS, which confers haploid-specific expression.

PRE-directed and natural *FUS1* transcription are absolutely dependent on the *STE4*, *STE5*, *STE7*, *STE11*, and *STE12* gene products, known components of the pheromone response pathway. This property provides an explanation for the haploid-specific transcription that characterizes expression driven by the *FUS1* UAS. In particular, transcription of *STE4*, *STE5*, and to some extent *STE12* is repressed in **a**/α diploids through the action of **a**1-α2 (13, 14, 36, 55), a repressor activity present only in **a**/α cells. **a**1-α2 is presumed to act by binding to operator sites within the upstream control regions of at least some of these and other genes (11, 36, 47, 50). However, the *FUS1* upstream region does not contain **a**1-α2 operator sites. Thus, repression of *FUS1* in **a**/α diploids is not the consequence of the direct action of **a**1-α2 but rather is the indirect consequence of repression of *STE4*, *STE5*, and *STE12*, which are required for activity or PRE-based UASs.

Although PRE sequences alone can act as UAS elements, most genes inducible by pheromone have additional UAS elements contributing to the overall activity of their promoters. For example, the *BAR1* and *STE2* genes contain PREs in close proximity to a P box (12, 22, 29), a known UAS element to which the MCM1 protein binds (1, 3, 19, 24, 29, 39, 51). In **a** cells these genes are only moderately inducible by α-factor. If the P box is deleted and the PREs are left intact, expression is highly inducible although lower overall; if PRE function is abolished, expression is not inducible (22, 30). Thus, PREs can act alone to confer transcription highly stimulated by pheromone or can act in concert with other UAS systems to give more modest transcription stimulation.

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