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Negative Regulation of *STE6* Gene Expression by the α2 Product of *Saccharomyces cerevisiae*

KATHERINE L. WILSON* AND IRA HERSKOWITZ

Department of Biochemistry and Biophysics, University of California, San Francisco, California 94143

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The α2 product of the α mating type locus of *Saccharomyces cerevisiae* is proposed to be a negative regulator of a set of dispersed genes concerned with specialized properties of **a** cells. This set of genes includes those, termed **a**-specific *STE* genes (*STE2*, *STE6*, and *STE14*), which are required for mating by **a** cells but not by α cells. We cloned the *STE6* gene to determine whether its expression is limited to **a** cells and, if so, whether its expression is inhibited in α cells by the α2 product. Expression of *STE6* was assayed in two ways: by blot hybridization, RNA and by β-galactosidase activity in strains carrying a *STE6-lacZ* hybrid gene. We found that *STE6* expression was limited to **a** cells and was negatively regulated by the α2 product. *STE6* RNA was not detectable in strains containing the wild-type α2 gene product. Expression of *STE6* was at least 150-fold lower in α cells than in **a** cells, based on β-galactosidase activities in **a** and α cells carrying the *STE6-lacZ* gene. These results confirmed that the α2 product is a negative regulator of gene expression and showed that it acts at the level of RNA production. We also examined the phenotype of a mutant carrying an insertion mutation of the *STE6* gene, the *ste6::lacZ* allele. In addition, an **a**-specific defect in mating, this mutant was greatly reduced (but not completely deficient) in **a**-factor production. Other phenotypes characteristic of **a** cells—Barrier activity, agglutination, and response to α-factor—were normal. *STE6* thus appears to be necessary for biosynthesis of **a**-factor.

The three cell types of yeast (**a**, α, and **a**/α) exhibit many differences in phenotype, although they differ genetically at only a single locus, *MAT*, the mating type locus. For example, **a** and α cells each secrete an oligopeptide pheromone called **a**-factor and α-factor, respectively, and respond to the pheromone of the opposite type (2, 10; reviewed in reference 34). In addition, **a** cells degrade α-factor in a process requiring the *BAR1* gene (17, 35). In contrast, **a**/α diploids neither produce nor respond to sex pheromones but are instead capable of undergoing meiosis and sporulation under the appropriate conditions. α cells carry the *MAT*α allele, **a** cells carry the *MAT***a** allele, and **a**/α cells (formed by mating between **a** and α cells) contain both *MAT* alleles.

MacKay and Manney proposed that *MAT* is a regulatory locus controlling the expression of genes unlinked to it, based on the finding that mutations affecting mating ability map to loci distinct from *MAT* (24, 25). Some genes, such as *STE5*, are required for mating of both **a** and α cells. Others have a phenotype in only one cell type. Mutations at three loci—*STE2* (24, 25), *STE6* (35), and *STE14* (L. C. Blair, Ph.D. thesis, University of Oregon, Eugene, 1979)—affect mating ability only in *MAT***a** strains; these genes are termed "**a**-specific" *STE* genes. Similarly, mutations at four loci—*STE3* (24, 25), *STE13* (37), *KEX2* (23), and *TUP1* (42)—affect mating only by *MAT*α cells and define a set of α-specific *STE* genes. It is plausible to propose that expression of **a**- or α-specific genes may be limited to their respective cell type. The **a**- and α-specific *STE* genes represent some but not all of the genes whose expression might be limited to one cell type: the *BAR1* gene might be cell-type regulated, as the Barrier phenotype is exhibited only by **a** cells and not by the other cell types.

Strathern et al. (38) proposed a specific model for control of cell type in which the mating type locus alleles code for three regulatory activities: α1, α2, and **a**1-α2 (Fig. 1). *MAT*α codes for two proteins, α1 and α2: α1 is a positive regulator of at least some genes that are required for the α cell type; α2 is a negative regulator of genes that are required for the **a** cell type. In *MAT***a** cells the **a**-specific genes are expressed constitutively because there is no α2 protein; α-specific genes are silent due to the absence of α1 protein. **a**/α cells contain the third regulatory activity, **a**1-α2, which requires α2 from *MAT*α and the **a**1 gene product of *MAT***a**. Mating is turned off in **a**/α cells because α2 inhibits expression of **a**-specific genes and because **a**1-α2 turns off production of α1. α2 thus has a dual role, by itself as the negative regulator of the **a**-specific family and together with the **a**1 protein as the negative regulator of genes turned off in **a**/α diploids.

At what level do the regulatory activities encoded by *MAT* act? α1 and **a**1-α2 have been shown to regulate RNA synthesis. Production of RNA from the α-specific *STE3* gene is dependent upon α1 protein (36); similar results have been obtained for *MFα1*, the major structural gene for α-factor (R. Jensen, K. Wilson, and S. Fields, unpublished data). RNA production from *HO* (18), *MATα1* (20, 29), and *STE5* (V. MacKay, J. Thorner, and K. Nasmyth, personal communication) has been shown to be negatively regulated by **a**1-α2. We note that expression of *STE* genes is not necessarily limited to one or another cell type: the α-specific *STE13* gene is expressed in all yeast cell types (G. Sprague, Jr. and R. Jensen, unpublished data; 19). *TUP1* and *KEX2*, despite being α-specific genes with respect to mating phenotype, must not be limited in expression to one cell type because mutations at these loci exhibit phenotypes (unrelated to mating) in all three cell types (23, 42).

The focus of this paper is the regulation of an **a**-specific gene by the α2 product of *MAT*α. The proposal that α2 is a negative regulator of **a**-specific genes comes from analysis of yeast mutants defective in the *MATα2* gene. In particular, *matα2*$^-$ mutants exhibit several properties characteristic of **a** cells: they mate (inefficiently) with α cells, they exhibit the

* Corresponding author.

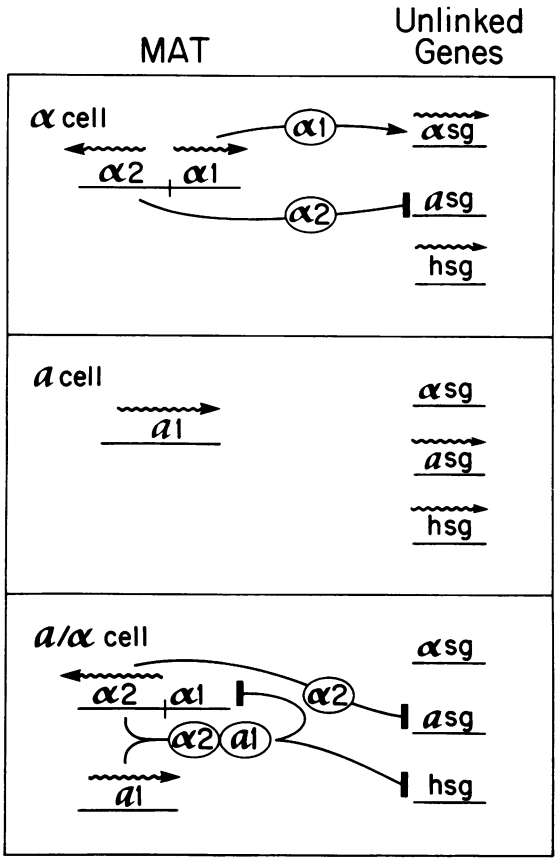

FIG. 1. The α1-α2 hypothesis for control of cell type by the mating type locus. The structure and expression of the mating type locus (*MAT*) alleles in **a**, α, and **a**/α cells are shown on the left. Unlinked genes whose expression is controlled by *MAT* are shown on the right. Wavy lines indicate gene expression. A line with an arrowhead represents stimulation of gene expression; lines ending in a bar represent inhibition of gene expression. Abbreviations: α*sg*, α-specific genes; **a***sg*, **a**-specific genes; *hsg*, haploid-specific genes. Circled symbols indicate the regulatory gene products that carry out stimulation or inhibition as described in the text.

a-cell-specific Barrier function, and they produce **a**-factor (17, 38; reviewed in reference 16). Since the *matα2* mutations tested are recessive to *MATα2*, it was proposed that α2 is a negative regulator of functions specific to **a** cells. Subsequent genetic and physiological analyses (21, 35, 38) support this view.

We ask here whether expression of the **a**-specific gene *STE6* is limited to **a** cells and whether its expression is turned off by the α2 product. We describe the cloning of *STE6* and its use in addressing these questions. We find that production of stable *STE6* mRNA is limited to **a** cells and is under negative regulation by α2. In addition, we have constructed an insertion mutation of *STE6* and studied its phenotype to assess the physiological role of *STE6* in mating.

MATERIALS AND METHODS

Strains, plasmids, and media. Yeast strains used are listed in Table 1. *Escherichia coli* strains were DB6507 (*thr leuB pro recA pyrF*::Tn*5 hsdR hsdM*; obtained from D. Botstein) and MC1066 (*leuB600 trpC9830 pyrF*::Tn*5 lacX74 rpsL hsdR galU galK*; obtained from M. Casadaban via M. Hall). Yeast strains HR125-5d and 1369, constructed by mating type interconversion from HR125-5d, were kindly provided by Rob Jensen.

Plasmids used were pBR322 (3), YEp13 (pBR322 containing the yeast *LEU2* gene and portions of 2μ DNA [4]), YIp5 (pBR322 containing the yeast *URA3* gene [40]), YCp50 (YIp5 carrying yeast centromere *CEN4* and an *ARS* sequence; obtained from C. Mann), and pMC1871 (pBR322 containing a *lacZ* gene fragment flanked by polylinkers; obtained from M. Casadaban [5]).

The *ste6*::*lacZ* fusion was constructed as follows (Fig. 2). The 5.5-kilobase-pair (kbp) yeast DNA fragment of plasmid YEpSTE6 was transferred into pBR322 via the vector *Hin*dIII and *Sph*I sites. The resultant plasmid pBR322-STE6 was partially digested with *Pst*I, ligated with *Pst*I-cut plasmid pMC1871 (containing the *lacZ* gene), and transformed into *Escherichia coli* strain MC1066. Transformants were selected by ampicillin resistance and screened for β-galactosidase activity by including the chromogenic substrate XG (5-bromo-4-chloro-3-indolyl-β-D-galactoside; Boehringer Mannheim Biochemicals) in the plates. Plasmid DNA was isolated from blue Ap^r colonies and screened by restriction analysis. Of six candidates, all contained the 3-kbp *lacZ Pst*I fragment inserted as shown in Fig. 2. One of these candidates, designated pBR322-ste6::lacZ, was used for the gene replacement analysis.

TABLE 1. Strains and crosses

Strain	Genotype	Source
70	*MATα thr3-10*	F. Sherman
227	*MATa lys1-1*	J. Hicks
1369	*MATα*; isogenic with HR125-5d	R. Jensen
HR125-5d	*MATa leu2-3 leu2-112 ura3-52 trp1-am his3 his4*	R. Jensen
K39-3b	*MATa ste6-21* (RSA21 allele of J. Rine) *leu2-3 leu2-112 his4 ade6 lys2 can1*	This work
K49-4b	*MATα ste6-21 his4-am trp1-am ade2 ade6 lys2-oc tyr1-oc thr SUP4-3-ts*	This work
K69-1d	*MATa ste6-21 ura3-52 his4 lys2*	This work
K72-33a	*MATα ste6-21 ura3-52 leu2-3 leu2-112 aro2 lys5 ade5 his4 thr trp5*	This work
K77	*MATa ste6*::*lacZ*; isogenic with HR125-5d	This work
K88-1, K88-2	*MATa STE6*::YIp5-STE6 (two independent Ura$^+$ integrants isogenic with HR125-5d)	This work
RC757	*MATα sst2-1 met1 his6 can1 cyh2*	R. Chan
VC2	*matα1-2 ade6*; other markers same as XP8-4a	V. MacKay
VC73	*matα2-1 ade6*; other markers same as XP8-4a	V. MacKay
XMB4-12b	*MATa sst1-1 ilv3 arg9 ura1* killer$^+$	L. Blair
XP8-4a	*MATa leu1 trp5 his6 met1*	P. Kushner
XP8-18b	*MATα*; isogenic with XP8-4a	P. Kushner
XP11	*MATa*/*MATα*; isogenic with XP8-4a	P. Kushner
XT1177-S245c	*MATα ade6 his6 leu1 met1 trp5-1 gal2 can1 rme*	T. Manney
Diploids	Parents	
K59	K77 × K49-4b	
K61	K77 × 1369	
K73-1, K73-2	K88-1 × K72-33a, K88-2 × K72-33a	

Yeast media YEPD and SD were prepared as described previously (17). Media containing XG were prepared as previously described (12, 30), except that yeast XG plates contained ca. 0.7 mg of XG per ml.

Genetic manipulations. Genetic manipulations were performed as previously described (35). Matings involving mating-deficient strains were performed by selection for prototrophy or were facilitated by including the YEpSTE6 plasmid (containing the *STE6* gene) in the *ste6* strain. In the latter case, further analysis was performed on diploids that had lost the plasmid and which were obtained by screening for cells that became Leu$^-$ after growth on rich medium.

Isolation of yeast DNA fragment carrying the *STE6* gene. A yeast clone bank constructed in vector YEp13 (4) by *Sau*3A partial digestion of DNA from yeast strain AB320 (28) was kindly provided in *E. coli* by K. Nasmyth. Plasmid DNA was isolated and used to transform *ste6* strain K39-3b by the method of Beggs (1). Yeast transformants were selected as leucine prototrophs on synthetic medium lacking leucine and were then collected, replated, and tested for their ability to mate as **a** by replica plating with α tester strain 70. Strain K39-3b is somewhat temperature sensitive for mating and gives rise to numerous prototrophic colonies on a patch test for mating at 30°C. To facilitate screening transformant colonies, mating was performed at 32.5°C, which reduces the background of mating. Plasmid DNA was isolated as previously described (28) from the one colony that exhibited mating and was used to transform *E. coli* strains by standard methods.

Preparation and hybridization of yeast RNA. Yeast RNA was isolated as described by Jensen et al. (18). Polyadenylated RNA was isolated with polyuridylic acid–Sepharose-4B and fractionated on 1.5% agarose–6% formaldehyde (9), except that HEPES buffer (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid; pH 7.8) was used instead of borate buffer. RNA was transferred to nitrocellulose (Sartorius) and hybridized with probe (41), except that dextran sulfate was omitted from the hybridization solutions. Probe was prepared by nick translation of plasmid YIp5-STE6 DNA with a New England Nuclear nick translation kit. Washed filters were autoradiographed for 48 to 72 h with Kodak XAR-2 film and a DuPont 1 Lightning Plus intensifying screen. Sizes of the RNA species were estimated by comparison of the autoradiograph with the rRNA bands in an ethidium bromide-stained lane of the original gel. Autoradiographs were scanned with a Zeineh Soft Laser scanning densitometer; peak areas were determined by cutting out and weighing.

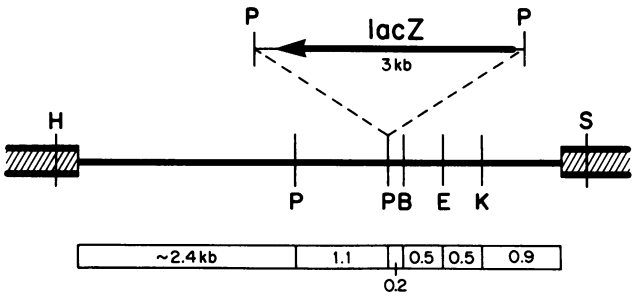

FIG. 2. Restriction map of yeast DNA containing *STE6*, showing position of *lacZ* insertion. Shown is the restriction map of the original 5.5-kbp yeast DNA insert of YEpSTE6 drawn to physical scale. Enzyme cleavage sites are as follows: P, *Pst*I; B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; K, *Kpn*I; and S, *Sal*I. Enzymes that do not cut within the yeast DNA include *Ava*I, *Bgl*II, *Cla*I, *Hin*dIII, *Hpa*I, *Nae*I, *Pvu*I, *Pvu*II, *Sac*I, *Sal*I, *Sma*I, *Sph*I, and *Xho*I.

Gene replacement with *ste6::lacZ*. The *STE6* gene of HR125-5d was replaced with the *ste6::lacZ* allele by the method of Rothstein (31), with the following modifications. To generate a linear fragment containing yeast sequences at both ends, we digested plasmid pBR322-ste6::lacZ at the unique *Hin*dIII site in the vector (Fig. 2), removed ca. 1 kbp from the end by BAL 31 exonuclease digestion to leave yeast sequences at that end, made a second cut at the unique *Kpn*I site in the yeast DNA, and used the entire mixture to transform strain HR125-5d. The linear fragments were cotransformed with uncut plasmid YEp13 containing the selectable *LEU2* marker, and Leu$^+$ transformants were screened as described below.

Assays of mating phenotype. Mating type was determined (35) by replica plating patches of the strains to be tested (which are auxotrophic) onto SD minimal agar plates previously spread with lawns (in rich broth) of either **a** (strain 227) or α (strain 70) testers containing complementary auxotrophic mutations. Diploids are prototrophic and grow on minimal medium. Mating type **a** was indicated by the formation of prototrophs after mating with the α lawn, and mating type α was indicated by formation of prototrophs with the **a** lawn.

a-factor production was assayed by replica plating strains to YEPD plates previously spread with a thin lawn (1×10^5 to 5×10^5 cells per plate; L. C. Blair, personal communication) of strain RC757 (7, 8) followed by incubation at room temperature (22 to 25°C). **a**-factor production was judged by halo size relative to a standard **a** strain (either HR125-5d or 227) on the same plate. α-factor production was tested by bioassay on plates at room temperature as previously described (35) with strain XMB4-12b. α-Factor response of single cells (17) and the plate assay for Barrier activity (35) were performed as described, with strain 70 as the source of α-factor.

Agglutination assays were performed in rich medium as described by Sprague et al. (37) by mixing exponentially growing cultures and visually monitoring the production of large aggregates over the ensuing 2 h. The strains tested were the HR125-5d isogenic series: **a**, α (strain 1369), and **a** *ste6::lacZ* (strain K77) in every pairwise combination. Quantitative efficiency of mating was determined as described previously (35). Known numbers of cells of the strains being tested were spread on minimal medium agar plates with 3×10^7 cells of mating tester strain XT1177-S245C and incubated at 30°C. Cells (4×10^7) of each strain were also spread alone on minimal plates to test for reversion of auxotrophic markers: no prototrophs were observed. Efficiency of mating was calculated as the ratio of the titer of cells able to mate to the titer of total cells as assayed on YEPD plates.

β-Galactosidase assays. β-Galactosidase assays were performed as described by Miller (27) with chloroform-sodium dodecyl sulfate to permeabilize the cells. For each segregant shown in Table 2, one culture was grown and divided into five portions; β-galactosidase activity was assayed over a 3-h period with each portion used for one time point.

RESULTS

Isolation of a cloned DNA segment that complements the mating defect of a *ste6* mutant. We isolated a plasmid that carried *STE6* by screening for plasmids that allow *MAT***a** *ste6* strains to mate. A *MAT***a** *ste6 leu2* strain (K39-3b) was transformed with a plasmid pool that consisted of random *Sau*3A genomic yeast DNA fragments inserted into the

TABLE 2. β-Galactosidase activity in segregants from diploid K61

Strain	*MAT*	*STE6*	β-Galactosidase Activity (U)[a]
K61-2a	α	*STE6*	<0.03
2b	**a**	*STE6*	<0.03
2c	α	*ste6::lacZ*	<0.03
2d	**a**	*ste6::lacZ*	4.0
K61-3a	α	*STE6*	<0.03
3b	**a**	*STE6*	<0.03
3c	**a**	*ste6::lacZ*	4.7
3d	α	*ste6::lacZ*	<0.03
K61	**a**/α	*STE6/ste6::lacZ*	<0.03

[a] β-Galactosidase assays performed as described in the text.

LEU2$^+$ vector YEp13. Independent Leu$^+$ transformants (1.8×10^4) were collected, replated on medium lacking leucine, and screened for their ability to mate as **a** as described above. One such colony was obtained. It contained a plasmid (YEpSTE6) which allowed the recipient *ste6* cells to mate with α tester strain 70. The restriction map of the 5.5-kbp yeast DNA insert of YEpSTE6 is shown in Fig. 2.

YEpSTE6 carries the *STE6* gene. To determine whether the 5.5-kbp insert in YEpSTE6 carries the *STE6* gene and not a different gene with overlapping function, we allowed a plasmid containing the putative *STE6* DNA to recombine with homologous sequences in the chromosome and mapped the site of integration. If the cloned DNA segment contains *STE6*, then the plasmid should integrate at the *STE6* locus. For this analysis, the entire 5.5-kbp insert of YEpSTE6 was subcloned into the *URA3*$^+$ plasmid YIp5, and the resultant plasmid (YIp5-STE6) was introduced into *MAT***a** *STE6 ura3-52* cells (strain HR125-5d) by transformation. Stable Ura$^+$ transformants result from integration of the plasmid into the genome; integration at the *ura3* locus is greatly reduced by using the *ura3-52* mutation in the recipient strain (32). Transformants with a stable Ura$^+$ phenotype were crossed to *MAT*α *ste6 ura3* strain K72-33a. The resultant diploids were sporulated, and the haploid meiotic products were analyzed for mating phenotype and Ura phenotype. In crosses with two independently isolated Ura$^+$ integrants (K73-1 and K73-2), the Ura$^+$ phenotype contributed by YIp5-STE6 was tightly linked to the *STE6* locus: no recombinants were observed in 65 tetrads—all *MAT***a** spores were either mating proficient and Ura$^+$ or mating deficient and Ura$^-$, like the parents. These results showed that *URA3* is now located at the *STE6* locus and therefore that the 5.5-kbp segment contains nucleotide sequences present at this locus. Even though YIp5 is incapable of autonomous replication (39), plasmid YIp5-STE6 yielded unstable transformants with high efficiency, indicating that the yeast insert may contain a sequence (an *ARS* sequence [39]) that allows autonomous replication of YIp5.

One-step gene replacement creates an insertion mutation in *STE6*. We used the one-step gene replacement scheme of Rothstein (31) to replace the wild-type *STE6* gene with a *ste6::lacZ* allele. The *ste6::lacZ* mutation was constructed by inserting a 3-kbp *Pst*I-*Pst*I fragment containing *lacZ* into a *Pst*I site of the cloned DNA (Fig. 2). The *lacZ* fragment contains the entire *lacZ* coding sequence beginning at the eighth amino acid (5). Thus, expression of this *lacZ* segment in yeast cells is dependent upon an external promoter and an in-frame translation initiation codon. The *ste6::lacZ* fusion was constructed for the following reasons. First, we anticipated that the *lacZ* insertion would disrupt the normal *STE6* gene to create a null mutation at *STE6*. Second, determination of the chromosomal locus in which the *ste6::lacZ* allele is introduced would confirm that the cloned DNA indeed contains *STE6* sequences. Finally, we could infer the orientation of the *STE6* gene on the cloned DNA if an active fusion protein were made.

The *ste6::lacZ* DNA fragment, linearized as described above, was mixed with uncut YEp13 in a molar ratio of ca. 100:1 (fragment YEp13) and transformed into a mating-proficient *MAT***a** *STE6 leu2* yeast recipient (HR125-5d). The *LEU2*$^+$ plasmid YEp13 was included as a selectable marker for cells that successfully took up DNA. Leu$^+$ transformants were replated for single colonies and screened for mating ability, since we anticipated that cells that had undergone one-step gene replacement with the *ste6::lacZ* DNA might show a Ste6$^-$ phenotype. This was indeed the case: 0.5% of the transformants had become mating deficient, and all of these transformants contained the *lacZ* insert. The presence of the *lacZ* insert was scored by its enzymatic activity (blue colonies on XG indicator plates.)

We analyzed one such mating-deficient transformant (strain K77) by the method of Southern (33), using as a hybridization probe the plasmid pBR322-ste6::lacZ (data not shown). The data were consistent with a simple substitution of *STE6* by *ste6::lacZ*.

Confirmation that the *lacZ* insertion allele is located at the *STE6* locus was obtained in the following way: we analyzed the meiotic progeny of a cross (K59) between the sterile transformant K77 (*MAT***a** *ste6::lacZ*) and an α strain (K49-4b) carrying an *ste6* mutation. All *MAT***a** segregants obtained in 37 tetrads were unable to mate, indicating tight linkage between the *ste6* locus and the *ste* mutation contributed by the transformant. In addition, half of the *MAT***a** segregants (which must carry the *ste6::lacZ* allele) formed blue colonies on XG indicator plates.

The *ste* mutation caused by the *lacZ* insertion was a mutation of the *STE6* gene, because the *ste6::lacZ* allele, carried on the low-copy-number plasmid YCp50, failed to complement the mating defect of strain K69-1d (*MAT***a** *ste6-21*). Similarly, the *ste6::lacZ* allele at its chromosomal position failed to complement the *ste6-21* mutation in *MAT***a**/*MAT***a** diploid cells. (Control experiments showed that the *ste6::lacZ* mutation is recessive to *STE6*.) We noted that insertion of *lacZ* into the *Bam*HI site located ca. 200 base pairs from the *Pst*I insertion site also rendered the original YEpSTE6 plasmid unable to complement a *ste6* mutation (unpublished data).

Phenotype of the *MAT*a** *ste6::lacZ* mutant.** Three previously isolated *ste6* mutants and eight new *ste6* mutants isolated in our laboratory (K. Wilson, unpublished data) were all leaky in phenotype: the ability to mate was greatly decreased but not completely abolished. The mating efficiency of strain K39-3b, for instance, was 3×10^{-3} at room temperature. It was therefore of interest to determine the phenotype of a null mutation, with the expectation that a 3-kbp insertion into *STE6* would severely inactivate this gene. The *ste6::lacZ* mutant manifested severe loss of mating ability: its mating efficiency was 5×10^{-7}. The wild-type parent mated with an efficiency of 0.86 (assayed as described above). Thus, the *ste6::lacZ* mutation affected mating much more than did previously existing mutations.

a strains carrying the *ste6::lacZ* mutation were defective in the production of the pheromone **a**-factor. The **a**-factor

deficiency cosegregated with the *ste6*::*lacZ* allele in all 39 tetrads from diploid K59. **a**-factor production from an isogenic set of strains—mutant *MAT***a** *ste6*::*lacZ*, its *MAT***a** *STE6* parent, and a *MAT*α control—is shown in Fig. 3. The **a**-factor halo produced by the *ste6* mutant was greatly reduced, but a small halo was visible. **a**-factor production is the only **a**-specific function that we have tested that is abnormal in the *ste6*::*lacZ* mutant. The mutant cells were normal in their ability to respond to α-factor by cell cycle arrest (14) and morphological change ("shmoo") as shown previously (J. D. Rine, Ph.D. thesis, University of Oregon, Eugene, 1979); furthermore, the mutant exhibited Barrier activity, and mutant and wild-type **a** cells agglutinated with α cells equally well by 1 h after mixing.

Control of *STE6* RNA synthesis by the mating type locus. To determine whether the mating type locus controls expression of the *STE6* gene, we used the cloned *STE6* gene to assay RNA production from this gene in the three cell types, **a**, α, and **a**/α, and in the two mutants, *matα1* and *matα2*. The strains, isogenic except at the mating type locus, were derivatives of strain XT1177–S245C.

Polyadenylated RNA, size fractionated as described above, was hybridized with a probe (YIp5-STE6) that contained *URA3* and *STE6*. We detected three species of RNA complementary to the probe in *MAT***a** cells (Fig. 4). The smallest (0.9 to 1.0 kilobases [kb]) corresponded to the *URA3* transcript (M. Rose, personal communication) and was present at similar levels in all strains. A second species (band X), ca. 3 kb in length, was also present in all strains. The largest transcript, ca. 4 kb in length, was present in *MAT***a** cells but was undetectable in *MAT*α, *MAT***a**/*MAT*α, or *matα1* cells. However, the 4-kb transcript is present in cells carrying the *matα2* mutation (including a *matα2*/*MAT***a** diploid [data not shown]). In other words, the 4-kb RNA was absent from strains that contained the wild-type α2 gene product (*MAT*α and *matα1* haploids and *MAT***a**/*MAT*α diploids).

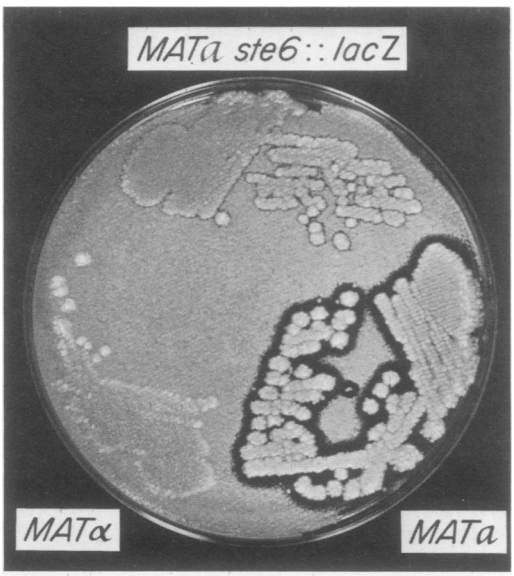

FIG. 3. Production of **a**-factor by an *ste6*::*lacZ* mutant. Strain K77 (carrying the *ste6*::*lacZ* mutation) and isogenic **a** and α strains (strains HR125-5d and 1369, respectively) are shown. Secretion of **a**-factor into the medium around a colony prevents growth of the supersensitive lawn (strain RC757), thus producing a halo or clear zone around the source of **a**-factor (see text).

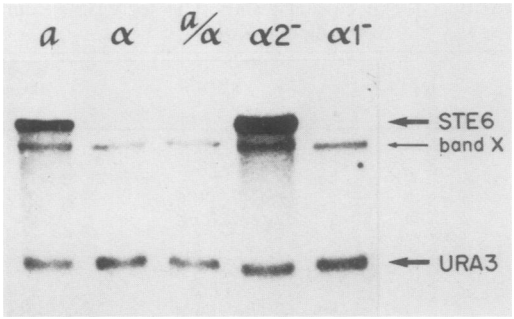

FIG. 4. Regulation of *STE6* RNA production by the mating type locus. Polyadenylated RNA was isolated from **a** (XP8-4a), α (XP8-18b), **a**/α (XP11), *matα2* (VC73), and *matα*1 (VC2) strains. RNA (1.5 μg) from each strain was fractionated by size by agarose gel electrophoresis, transferred to nitrocellulose paper, and hybridized with radioactively labelled YIp5-STE6 plasmid DNA (see the text). Positions of the *STE6* and *URA3* transcripts are indicated. The 3-kb transcript (band X) is not related to *STE6* (see text).

The 4-kb RNA species appears to be the *STE6* transcript. We analyzed polyadenylated RNA from *ste6*::*lacZ* strain K77 and its isogenic *STE6* parent and found that the 4-kb RNA species is absent in the *ste6*::*lacZ* strain, replaced by one larger and two smaller RNA species (data not shown). The alteration in the 4-kb transcript as a result of the *lacZ* insertion mutation indicated that this species was the *STE6* transcript. Why the *lacZ* insertion into *STE6* resulted in three polyadenylated RNA species is not known. In contrast, the 3-kb band was unaltered in the *ste6*::*lacZ* strain and thus appeared to be unrelated to *STE6*.

These results indicated that the *STE6* transcript is cell type specific and that transcription of the *STE6* gene is under negative regulation by the *MATα2* gene product. We could not determine from this analysis whether the action of α2 blocked transcription of *STE6* or affected *STE6* RNA stability. The abundance of the *STE6* transcript in **a** cells, estimated from densitometric scans of the Northern autoradiograph, was ca. the same as that of the *URA3* transcript (ca. 3 copies per cell [22]). *STE6* RNA was undetectable in α cells. The level of *STE6* RNA in $\alpha2^-$ strains (normalized to *URA3* RNA) was at least 1.5 times greater than the level in **a** cells. The reason for the difference between $\alpha2^-$ and **a** cells is under study.

β-Galactosidase activity in *MAT*a** *ste6*::*lacZ* strains is under *MAT* control.** To quantitate the difference in *STE6* expression between different cell types and to determine whether β-galactosidase expression from the chromosomal *ste6*::*lacZ* gene is controlled by *MAT*, we analyzed segregants from diploid K61, which was formed by crossing the insertion mutant K77 (*MAT***a** *ste6*::*lacZ*) with strain 1369 (*MAT*α *STE6*.) Segregants from K61, which comprise an isogenic set of strains that differed only at *MAT* and *STE6*, were analyzed for mating phenotype and β-galactosidase activity. Because only half of the spores carried *ste6*::*lacZ*, the half that carried *STE6* constituted the negative controls for β-galactosidase activity. Results from two complete tetrads are shown in Table 2. In both tetrads, one of the **a** segregants carried *ste6*::*lacZ* and the other carried *STE6*. The presence or absence of the mutant allele was determined by outcrosses for all four α segregants (data not shown). β-galactosidase activity in the *MAT***a** *ste6*::*lacZ* segregants was ca. 4.3 U, whereas β-galactosidase activity in α and **a**/α strains carrying the *lacZ* allele was undetectable above the background of the

negative controls (less than 0.03 U). As expected, β-galactosidase activity was detectable in *matα2* strains carrying the *ste6*::*lacZ* allele (data not shown), confirming the result from RNA analysis that the α2 gene product is a negative regulator of *STE6* expression.

From the β-galactosidase assays, we estimated that expression of the fusion gene was at least 150-fold lower in α cells than in **a** cells. Interestingly, the *ste6*::*lacZ* gene was also fully regulated when carried on a high-copy-number plasmid: YEp13 carrying *ste6*::*lacZ* exhibited 150 U of β-galactosidase activity in an **a** cell and less than 0.03 U of activity in an α cell (K. Wilson, unpublished data).

The orientation of the *lacZ* insertion indicated that the *STE6* gene was oriented on the cloned DNA fragment as shown in Fig. 2. We showed that this orientation was correct by subcloning fragments into the single-stranded DNA phage M13; we were able to protect the predicted strand from S1 nuclease digestion when the DNA was hybridized with RNA from **a** cells (K. Wilson, data not shown). No fragment was protected with RNA from α cells. This analysis placed the 5′ end of the *STE6* transcript to the right of the *Kpn*I site (K. Wilson, data not shown). The *lacZ* insertion is thus located ca. 1.2 kbp from the 5′ terminus of the transcript.

DISCUSSION

The *STE6* gene is required for mating by **a** cells but not by α cells. We have cloned *STE6* and used it to study the function and regulation of *STE6*. A strain carrying the *ste6*::*lacZ* insertion mutation shows a large reduction in **a**-factor activity, which appears to be the sole reason for defective mating. *STE6* is thus necessary for **a**-factor biosynthesis. Expression of the *STE6* gene has been assayed in two ways: blot hybridization analysis of *STE6* RNA and β-galactosidase activity produced by the *STE6-lacZ* hybrid gene. We find that expression of *STE6* is limited to **a** cells and is negatively regulated by the α2 product of the α mating type locus.

The cloned segment contains the *STE6* gene. *STE6* was cloned by complementation of the mating defect of a *MAT***a** *ste6* mutant. Evidence that the plasmid with complementing ability (YEp13-STE6) contains the *STE6* gene is threefold. First, the insert directs plasmid integration at the *STE6* locus. Second, a DNA segment inserted into the cloned *STE6* DNA by in vitro methods destroys the complementing activity of the plasmid. Finally, replacement of homologous chromosomal sequences with this insertionally altered DNA (*ste6*::*lacZ*) utilizing the one-step gene replacement method results in a recessive mutation that is tightly linked to the *STE6* locus and unable to complement a *ste6* defect. The *ste6*::*lacZ* insertion mutation alters a single α2-regulated RNA species, which is thereby deduced to be the *STE6* transcript.

Function of the *STE6* gene. The original *ste6* mutant was isolated in a screen for mutants that are defective in mating (J. Rine, Ph. D. thesis, University of Oregon, Eugene, 1979). Because this mutant maintains its ability to respond to α-factor, it would not have been found in extensive mutant hunts in which nonmating mutants were selected for their resistance to α-factor (15, 26). The recessive *ste6*::*lacZ* insertion mutation produced in vitro causes a more severe mating defect than the original mutant allele does, but it also affects mating only by **a** cells. The only known phenotype of these mutants, aside from their defect in mating, is that they are defective in **a**-factor activity. The mutant responds normally to α-factor: it arrests cell division and gives the characteristic altered cell morphology. Furthermore, the mutant agglutinates with α cells and exhibits Barrier activity (inactivation of α-factor). Because the only known defect of the *ste6* mutant is in **a**-factor activity, it is likely that the *STE6* gene is involved in biosynthesis of **a**-factor. The bioassay used to detect **a**-factor activity does not allow us to distinguish whether, for instance, the cells secrete low levels of normal **a**-factor or normal levels of altered **a**-factor (6). *STE6* is unlikely to be a structural gene for **a**-factor because the *STE6* restriction map does not correspond to the maps of two cloned **a**-factor genes (A. Brake, personal communication). The *STE6* gene product might be necessary for expression of structural gene(s) for **a**-factor, for processing a putative precursor to **a**-factor, or for secretion of **a**-factor.

It is striking that the *ste6*::*lacZ* mutant, despite a severe defect in mating due to the insertion mutation, still produces measurable **a**-factor (Fig. 3). Perhaps the insertion mutation allows formation of an amino-terminal segment of the *STE6* protein that has partial activity. It is also possible that the *ste6*::*lacZ* mutation is a complete inactivation of the *STE6* gene but that another yeast gene product can partially substitute for *STE6*. More interesting possibilities are that unprocessed (or partially processed) **a**-factor precursor has some biological activity or that the *STE6* product is not absolutely required for expression of **a**-factor genes.

Control of expression of *STE6*. Expression of the *STE6* gene is limited to **a** cells: *STE6* RNA is found in **a** cells but is not detectable in α or in **a**/α cells. A variety of genetic and physiological observations indicate that the α2 gene product of *MAT*α is a negative regulator of genes involved in specialized **a**-cell functions (35, 38). In particular, *matα2* mutants are constitutive for several different **a** cell functions. We have shown that a *matα2* mutation allows α cells to produce full levels of *STE6* RNA. This result supports the previous inference that the α2 product is a negative regulator of certain **a**-specific genes and shows further that the α2 product acts to inhibit RNA synthesis from the *STE6* gene. Because we are assaying stable RNA species, we are unable to distinguish whether α2 acts to inhibit transcription or affects RNA stability. Evidence that the α2 product is located in the nucleus (13) is consistent with the idea that the *STE6* gene itself is the target of α2.

Studies with an *ste6*::*lacZ* fusion support the findings obtained with the hybridization assays. Our data show that the *ste6*::*lacZ* fusion, like the 4-kb RNA species, is regulated by α2; hence, assay of β-galactosidase is a legitimate measure of expression of the *STE6* locus. β-galactosidase activity in **a** cells is ca. 4.3 U but is not detectable in α or **a**/α cells. We estimate from β-galactosidase activities that *STE6* expression is at least 150-fold lower in cells containing the α2 product than in **a** cells. The *ste6*::*lacZ* fusion is also fully regulated when carried on a high-copy-number plasmid. These results indicate that negative regulation by α2 is potent. We note that assays of β-galactosidase activity might underestimate low-level expression of the *STE6* gene in α cells. Because β-galactosidase must tetramerize to be active (43), production of a low level of monomer as might occur in α cells would not be detected.

At least three genes are regulated by α2. In addition to *STE6*, expression of the *BAR1* (V. L. MacKay and T. R. Manney, personal communication) and *STE2* (A. Hartig and V. L. MacKay, personal communication) genes is limited to **a** cells and is inhibited by the α2 product. We anticipate that additional genes regulated by α2 will be identified.

The regulatory activities α1, α2, and **a**1-α2 have now all been demonstrated to regulate RNA synthesis (transcription or RNA stability) of their target genes: α1 is required for

expression of *STE3* (36) and *MFα1* (R. Jensen, K. Wilson, and S. Fields, unpublished data) and **a**1-α2 inhibits RNA synthesis from *MATα1* (20, 29), HO (18), *STE5* (V. L. MacKay, J. Thorner, and K. Nasmyth, personal communication), and the repeated element Ty1 (11). It is thus apparent that the mating type locus governs yeast cell type by controlling synthesis of RNA for genes dispersed throughout the genome via its agents, α1, α2, and **a**1-α2.

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