Identification of Genes Required for α2 Repression in *Saccharomyces cerevisiae*

Madhu Wahi and Alexander D. Johnson

Department of Microbiology, University of California, San Francisco, California 94143-0502

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ABSTRACT

Transcriptional repression of the **a**-specific genes in *Saccharomyces cerevisiae* α cells involves the concerted action of several proteins. The homeodomain protein α2, together with MCM1, recruits two general transcriptional repressors, SSN6 and TUP1, to the promoters of **a**-specific genes. SSN6 and TUP1 then mediate repression of the **a**-specific genes. SIN4, another general negative regulator, is required for this repression, but unlike *tup1* or *ssn6* deletions, *sin4* deletions cause only partial loss of repression. We have screened for other genes required for **a**-specific gene repression in α cells. In addition to recovering multiple alleles of previously identified genes required for this process (referred to as α2 repression), we have identified four other genes, designated *ARE1, ARE2, ARE3,* and *ARE4* (for *a*lpha2 *re*pression). Recessive mutations in the *ARE* genes cause partial loss of **a**-specific gene repression and cause pleiotropic phenotypes similar to those resulting from mutations in *SSN6, TUP1,* or *SIN4,* suggesting that the *ARE* genes are general negative regulators. Based on our initial analysis, we propose that two distinct classes of general negative regulators cooperate to bring about full levels of α2 repression. The sequence of *ARE1* revealed that it encodes a CDC28-related protein kinase, identical to *UME5,* and thus suggests that protein phosphorylation plays a role in α2 repression.

CELL differentiation is a complex process that typically requires cell-type control of gene expression. This control often involves gene repression since a differentiated cell must prevent the expression of genes specific to other cell types. The yeast *Saccharomyces cerevisiae* provides a simple system for studying this aspect of cell-type control. Its two haploid cell types, **a** and α, have several phenotypic differences. For example, **a** cells secrete **a**-factor and mate with α cells, while α cells secrete α-factor and mate with **a** cells. Despite these differences, their genomes are identical except at one locus, the mating type or *MAT* locus. In α cells the *MAT* locus encodes two transcriptional regulatory proteins, α1 and α2. α1 turns on the transcription of α-specific genes and α2, a homeodomain protein, turns off the transcription of the **a**-specific genes (for review see HERSKOWITZ *et al.* 1992).

α2 repression of the **a**-specific genes is known to require several other proteins. α2 binds cooperatively with MCM1 to a conserved DNA sequence, called the α2-MCM1 operator, located upstream of each **a**-specific gene (KELEHER *et al.* 1988). Operator-bound α2-MCM1 is believed to recruit at least two proteins to the promoter: SSN6 and TUP1 (KELEHER *et al.* 1992; KOMACHI *et al.* 1994). These two proteins are associated together in a high molecular weight complex (WILLIAMS *et al.* 1991) and function together as general transcriptional repressors (KELEHER *et al.* 1992; reviewed in JOHNSTON and CARLSON 1992; TRUMBLY 1992).

The SSN6-TUP1 repressor complex is essential for α2 repression. Null mutations in *SSN6* or *TUP1,* like those in *MATα2,* cause α-cell-type-specific defects, such as α-specific sterility (WICKNER 1974; LEMONTT *et al.* 1980; ROTHSTEIN and SHERMAN 1980; CARLSON *et al.* 1984). However, because SSN6 and TUP1 are involved in additional repression pathways, mutations in these two genes, unlike those in *MATα2,* cause pleiotropic phenotypes including slow growth, sporulation deficiency, and clumpiness (SCHAMHART *et al.* 1975; ROTHSTEIN and SHERMAN 1980; TRUMBLY 1986; SCHULTZ *et al.* 1990).

Another general negative regulator involved in α2 repression is SIN4. Because mutations in *SIN4* cause a spectrum of phenotypes similar to those caused by certain *spt* mutations or by depletion of histones, it has been suggested that *sin4* mutations alter chromatin structure and thereby cause global transcriptional defects (JIANG and STILLMAN 1992). Whereas α2 repression is eliminated in *matα2, ssn6,* or *tup1* null mutants, this repression is only partially defective in *sin4* null mutants (CHEN *et al.* 1993a). How SIN4 contributes to α2 repression is unclear. One possibility is that SIN4 mediates a repressive change in the chromatin structure at the promoter of **a**-specific genes in α cells. Work by SIMPSON *et al.* has demonstrated that a nucleosome is positioned adjacent to the α2-MCM1 site in α cells but not in **a** cells (SHIMIZU *et al.* 1991). The positioning of this nucleosome requires SSN6 and TUP1 in addition to α2, indicating that α2 repression may involve changes in chromatin structure (COOPER *et al.* 1994).

In this paper, we describe the identification and analysis of mutations in genes required for full levels of α2 repression. As expected, we isolated multiple alleles of previously identified genes involved in α2 repression. In addition, we identified recessive mutations in four

other genes (*ARE* genes). These mutations, like those in *SSN6, TUP1,* and *SIN4,* cause pleiotropic phenotypes. Mutations in at least three of the *ARE* genes can affect transcription from promoters other than those of the **a**-specific genes. These results suggest that the *ARE* genes are general negative regulators. *ARE1* encodes a CDC28-related protein kinase, indicating that protein phosphorylation is important for $\alpha 2$ repression. We have recently learned that *ARE1* is identical to a component of the RNA polymerase II holoenzyme (see DISCUSSION), suggesting that $\alpha 2$ repression is mediated, at least in part, through the general transcription machinery.

MATERIALS AND METHODS

Media, growth conditions, and genetic methods: Liquid and solid media have been described (SHERMAN *et al.* 1979). Sporulation plate medium consists of 0.1% yeast extract, 1% potassium acetate, 0.05% dextrose, and 2% agar. Unless indicated otherwise, cells were grown at 30° and in either rich YEPD medium or in synthetic SD drop-out medium. Clumpy cells were dispersed by adding EDTA to 25 mM before measuring optical density. Standard genetic methods for mating, sporulation, tetrad analysis, and curing plasmids were employed (MORTIMER and HAWTHORNE 1969; SIKORSKI and BOEKE 1991). Yeast cells were transformed by the lithium acetate method (ITO *et al.* 1983). The Luria-Bertani medium for growth of *Escherichia coli* has been described (MILLER 1972).

Strains: *S. cerevisiae* strains used in this study are listed in Table 1. All strains are derived from 246-1-1 and EG123, which are isogenic except at the *MAT* locus (SILICIANO and TATCHELL 1984). Strains SM1196 and SM1179 have been described (HALL and JOHNSON 1987). Strains of the genotype *areMFA2* were recovered from crosses between *areMFA2::lacZ* strains and 246-1-1 or EG123.

MWY2 and MWY4, used in linkage analyses, were derived from strains AJY165 and AJY158 (KELEHER *et al.* 1992), respectively, by cotransforming with the nonselectable *MFA2::lacZ*-bearing plasmid pSM38 cut with *Hin*dIII and the selectable *URA3*-marked plasmid, YEp24 (which was later cured). Transformants containing an integrated *MFA2::lacZ* fusion were isolated by screening transformants for β-galactosidase activity. MWY1 was recovered from a cross between MWY2 and SM1196.

To create MWY5, MWY6, and MWY7, the *sin4Δ::LEU2* allele was introduced into 246-1-1, SM1196, and SM1179, respectively, by transforming with M1381 (a kind gift of D. STILLMAN) cut with *Bam*HI. Leu$^+$ transformants were screened for clumpiness. This disruption removes almost the entire *SIN4* open reading frame. Disruption was confirmed by PCR analysis of the *SIN4* locus (see PCR assay below).

α and **a** *are1Δ* strains MWY13 and MWY14 were created in two steps. First, the *are1Δ::LEU2* allele was introduced into diploid MWY33, generating MWY37, by transforming with pMW14 cut with *Sna*BI and *Hin*dIII and selecting for Leu$^+$ transformants. This disruption removes *ARE1* sequence from +58 to +1588. Correct integration was confirmed by PCR analysis. MWY37 was then sporulated and *MATα* and *MAT***a**Leu$^+$ derivatives (MW13 and MW14) were recovered from dissected tetrads.

The *matΔ::URA3* allele contains a deletion of *MATα1* and a 1.1-kb *URA3* insertion at the *Bgl*II site in *MATα2*. This *matΔ::URA3* allele was introduced into α cells by transforming with pKK146 (a kind gift of K. KOMACHI) cut with *Hin*dIII. Ura+ transformants were then screened for the secretion of **a** factor by halo assay (SPRAGUE 1991). The *ssn6Δ9* (SCHULTZ *et al.* 1990) and *tup1Δ::LEU2* (KELEHER *et al.* 1992) alleles have been described.

The *Escherichia coli* strain DH5α was used for propagating plasmids.

Plasmids: Nonstandard plasmids used in this study are listed in Table 2. Plasmid pKK146, which was used to delete the *MAT* locus, was constructed by K. KOMACHI as follows. A *MATα Hin*dIII fragment (with a *Nde*I fragment deleted for *MATα1*) was subcloned into pGEM3 (with the *Eco*RI site in the polylinker destroyed). This vector was then cut with *Bgl*II within the *MATα2* sequence. The ends were filled in using Klenow fragment and ligated to a 1.1-kb *Sma*I-*Hin*dIII fragment (blunted with Klenow fragment) containing *URA3*.

Plasmid pMW15 contains the original *ARE1* clone isolated from a YEp24-based yeast genomic DNA library (CARLSON and BOTSTEIN 1982). Plasmid pMW16 is pMW15 with a $\gamma\delta$ transposon insertion located at about nucleotide +490 in the *ARE1* open reading frame. Plasmid pMW17, which was used to test whether *are1* is linked to the gene encoding the protein kinase, was created by subcloning a 3.0-kb *Bgl*II-*Bam*HI fragment from pMW16 (using a *Bgl*II site within the yeast genomic DNA and a *Bam*HI site within the transposon insertion) into the *Bam*HI site of YIp5. Plasmid pMW11, which was used to test whether the gene encoding the protein kinase complements the *are1* defect when carried on a low copy plasmid, was created by subcloning a 2.8-kb *Eco*RV-*Sna*BI fragment, containing the entire kinase open reading frame plus 583 nucleotides upstream and 530 nucleotides downstream, into the *Sma*I site of pRS316. pMW14, which carries the *are1Δ::LEU2* null allele, was constructed in two steps. First, a 5.8-kb *Bgl*II-*Nhe*I fragment from pMW15 was subcloned into the *Bam*HI-*Xba*I site of pUC18, creating pMW13. pMW13 was then cut with *Stu*I and *Sac*I, deleting 1.5-kb of *ARE1* sequence, and then ligated to a 2.2-kb *LEU2* PCR product with PCR-introduced *Stu*I and *Sac*I ends.

The following plasmids were constructed for sequencing *ARE1*. Plasmid pMW2 contains a 4.3-kb *Bgl*II-*Nhe*I fragment (the *Bgl*II site is within the transposon insertion) from pMW16 subcloned into pVZ-1. Plasmid pMW3 contains the 3.0-kb *Bgl*II-*Bam*HI fragment (the *Bam*HI site within the transposon insertion) of pMW16 subcloned into pVZ-1. Plasmid pMW10 contains the 5.8-kb *Bgl*II-*Nhe*I fragment of pMW15, the original *ARE1* clone, subcloned into pVZ-1.

Isolation of *are* mutants: Strain SM1196 (*MATα MFA2::lacZ*) was mutagenized with EMS as described (LAWRENCE 1991). Two populations of cells, one mutagenized to 80% survival and the other to 34% survival, were screened for derepression of *MFA2::lacZ* using an X-Gal filter assay (see β-galactosidase assays below). To reduce the frequency of recovering mutations in known genes, SM1196 carried extra copies of *SSN6* (pLN113-3) and *MATα* (pAJ195) on episomal plasmids at the time of mutagenesis.

The mutagenized cells were plated for single colonies (~200 per plate) on SD −Ura −Leu plates and incubated at room temperature for several days before replica plating to nitrocellulose filters overlayed on SD −Ura −Leu plates. The transferred colonies were grown 1–2 days at room temperature, and then X-Gal filter assays were performed to screen for blue (derepressed) colonies.

Even though the starting strain had extra copies of *SSN6* and *MATα*, multiple alleles of *ssn6* and *matα2* were recovered. We later realized that this result should have been expected. Complementation of a mutation by the wild-type gene carried on a plasmid can be detected only early (usually within the first 30 min) in the X-Gal filter assay. Eventually, perhaps because some cells in the mutant colony have lost the complementing plasmid and are thus derepressed, the colony turns blue. Because the X-Gal filter assay was allowed to proceed

TABLE 1

***S. cerevisiae* strains**

Strain	Genotype
Haploids	
SM1196	*Matα MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
SM1179	*Mata MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
246-1-1	*Matα can1 gal2 his4 leu2 suc2Δ trp1 ura3*
EG123	*Mata can1 gal2 his4 leu2 suc2Δ trp1 ura3*
AJY165	*Mata tup1Δ::LEU2 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
AJY166	*Matα tup1Δ::LEU2 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
AJY158	*Mata ssn6Δ9 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY1	*Matα tup1Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY2	*Mata tup1Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY4	*Mata ssn6Δ9 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY5	*Matα sin4Δ::LEU2 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY6	*Matα sin4Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY7	*Mata sin4Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY8	*Matα are1-5 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY9	*Mata are1-5 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY10	*Matα are1-41 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY11	*Matα are1-41 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY12	*Mata are1-41 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY13	*Matα are1Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY14	*Mata are1Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY15	*Matα are2-13 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY16	*Matα are2-13 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY17	*Mata are2-13 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY18	*Matα are2-30 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY19	*Mata are2-30 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY20	*Matα are2-40 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY21	*Mata are2-40 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY22	*Matα are2-59 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY23	*Mata are2-59 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY24	*Matα are3-57 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY25	*Matα are3-57 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY26	*Mata are3-57 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY27	*Matα are4-87 can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY28	*Matα are4-87 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY29	*Mata are4-87 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
Diploids	
MWY30	MWY8 × MWY9
MWY31	MWY13 × MWY14
MWY32	MWY16 × MWY17
MWY33	SM1179 × SM1196
MWY35	MWY25 × MWY26
MWY36	MWY28 × MWY29
MWY37	*Mata are1Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3* *Matα ARE1 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*

for over half an hour, any complementation would not have been detected. Candidate mutants were cured of the pLN113-3 and pAJ195 plasmids carrying *SSN6* and *MATα*, respectively, before further analysis.

Genetic analysis: To test whether any of the mutations were in genes known to be required for α2 repression, each isolate was transformed with plasmids bearing either *MATα* (CYp60, pAJ166, or pAJ195), *SSN6* (pLN113-3), *TUP1* (pFW28), *SIN4* (M1305), or *MCM1* (pAJ169 or pAJ170). Transformants were scored for complementation of the *MFA2::lacZ* derepression and/or clumpiness by comparing the phenotype of a mutant carrying a plasmid containing one of these known genes to the phenotype of the same mutant carrying a corresponding control plasmid (YEp24, YEp13, pRS315, or pRS316). Observed complementation (13 candidates) was distinguished from suppression (four candidates) by performing allelism tests using corresponding test strains: *MATa* (SM1179), *tup1Δ* (MWY2), *ssn6Δ* (MWY4), and *sin4Δ* (MWY7) for testing linkage to the *MAT, TUP1, SSN6,* and *SIN4* locus, respectively.

Some isolates undergoing the allelism test were found to be deficient in mating to the test strain. For other isolates, mating was possible, but the diploid derived from the cross was deficient in sporulation. Therefore, the isolates carried the complementing plasmid during mating to the test strain, and the derived diploids still carried the complementing plasmid during the subsequent sporulation and tetrad dissection. After tetrad dissection, each segregant was cured of its plasmid before analysis of the mutant phenotype.

TABLE 2

Plasmids

Name	Description	Source
pVZ-1	Bluescript vector pBS+ from Strategene with expanded polylinker	S. HENIKOFF
pSM38	*MFA2::lacZ* fusion in pUC18	S. MICHAELIS
pKK146	pGEM derivative containing a *MATα* *Hin*dIII fragment with a *URA3* insertion in *MATα2* and a *Nde*I fragment containing *MATα1* deleted	K. KOMACHI
CYp60	*Hin*dIII fragment containing *MATα, URA3, CEN4, ARS1*	I. HERSKOWITZ lab
pAJ166	*Hin*dIII fragment containing *MATα* in YEp13	K. KOMACHI
pAJ195	*Hin*dIII fragment containing *MATα* in YCp50	C. KELEHER
pFW28	3.5-kb *Xho*I-*Sph*I fragment containing *TUP1* in YEp24	WILLIAMS and TRUMBLEY (1990)
M1305	4.8-kb *Bam*HI fragment containing *SIN4, URA3,* 2μ origin	JIANG and STILLMAN (1992)
M1381	*SIN4* disruptor construct containing the *SIN4Δ::LEU2* allele	D. STILLMAN lab
pLN113-3	Contains original clone of *SSN6* isolated from a YEp24 genomic library	SCHULTZ and CARLSON (1987)
2μ ML (pAJ169)[a]	3.4-kb *Bam*HI-*Xho*I fragment containing *MCM1* in YEp13	EBLE and TYE (1991)
2μ MU (pAJ170)	3.4-kb *Bam*HI-*Xho*I fragment containing *MCM1* in YEp24	EBLE and TYE (1991)
pLGΔ-312S (pAJ1)	*CYC1-lacZ* reporter, *URA3,* 2μ origin	GUARENTE and HOAR (1984)
pAJ3	pAJ1 with an α2-MCM1 site inserted into the unique *Sal*I site in the *CYC1* promoter	KELEHER *et al.* (1988)
pΔSS	Derived by deleting the *CYC1* UAS elements from pAJ1	JOHNSON and HERSKOWITZ (1985)
M740	UAS-less *HO-lacZ* reporter, *URA3,* 2μ origin	JIANG and STILLMAN (1992)
pTXL6	3.5-kb *Hin*dIII-*Pst*I fragment containing *TUP1* in a pUC-derived plasmid	WILLIAMS and TRUMBLY (1990)
pAJ122	4.8-kb *Bam*HI fragment containing *LYS2* in pBR derivative	Original source unknown
pAB539	4.3-kb fragment containing *STE2* in YEp13	BURKEHOLDER and HARTWELL (1985)
pBAR2	9.0-kb yeast genomic fragment containing *BAR1* in YEp13	MACKAY *et al.* (1988)
pMW15	Original *ARE1* clone isolated from a YEp24-based yeast genomic library (CARLSON and BOTSTEIN 1982)	This work
pMW16	pMW15 containing a transposon insertion located at about +490 in the *ARE1* sequence	This work
pMW17	3.0-kb *Bgl*II-*Bam*HI fragment from pMW16 in YIp5	This work
pMW11	2.8-kb *Eco*RV-*Sna*BI *ARE1* fragment in pRS316	This work
pMW13	5.8-kb *Bgl*II-*Nhe*I fragment from pMW15 in pUC18	This work
pMW14	pMW13 with a substitution of a 2.2-kb *LEU2* fragment for 1.5-kb of *ARE1* sequence deleted with *Stu*I and *Sac*II	This work
pMW2	4.3-kb *Bgl*II-*Nhe*I fragment from pMW16 in pVZ-1	This work
pMW3	3.0-kb *Bgl*II-*Bam*HI fragment of pMW16 in pVZ-1	This work
pMW10	5.8-kb *Bgl*II-*Nhe*I fragment of pMW15 in pVZ-1	This work

[a] Parentheses indicate that plasmid is referred to by its Johnson lab number in this study.

β-galactosidase assays: The X-Gal filter assay has been described previously (SCHENA *et al.* 1989). In brief, colonies or patches of cells are replicated onto nitrocellulose or Whatman filter paper overlaying an appropriate medium plate. After colony growth, the filter is dipped into liquid nitrogen for 10 sec to permeabilize the cells and then placed onto Whatman filter paper saturated with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$ and 0.027% β-mercaptoethanol) plus 0.03% X-Gal (5-bromo-4-chloro-indolyl-β-D-galactopyranoside). Filters are incubated at room temperature until the reaction is stopped by removing the filter and drying.

Quantitiative liquid β-galactosidase assays were performed as described (MILLER 1972) with the modifications described in KELEHER *et al.* (1988), except that glucose was added to 2% to all samples grown in SD medium lacking uracil 1 hr before assaying. For each sample, two to three independent transformants (for strains carrying reporter plasmids) or colonies (for strains containing the integrated *MFA2::lacZ* fusion)

were assayed in triplicate on different days. Numbers represent averages and SD was generally <15–25% of the mean (see appropriate table and figure legends).

RNA isolation and Northern analysis: RNA was isolated as described (NASMYTH 1983), except that the RNA buffer consisted of 50 mM Tris HCl pH 7.4, 100 mM NaCl, and 10 mM EDTA. RNA was isolated from 246-1-1 (α *wild-type*), EG123 (**a** *wild-type*), AJY166 (α *tup1*), MWY10 (α *are1-41*), MWY15 (α *are2-13*), MWY24 (α *are3-57*), and MWY27 (α *are4-87*). RNA (1 μg per lane) was run on a 1% agarose gel containing formaldehyde as described (MANIATIS *et al.* 1982). Hybridization and wash solutions have been described (CHURCH and GILBERT 1984). RNA was cross-linked to GeneScreen nylon membranes by 40-sec exposure to UV irradiation in a Stratagene UV Stratalinker. Probes were radioactively labeled with ^{32}P by random priming using an Amersham Megaprime DNA labeling kit.

Northern probes were a 1.0-kb *Bam*HI-*Bgl*II *TUP1* fragment isolated from pTXL6, a 219-bp PCR fragment of the α2 homeodomain sequence (a kind gift of M. STARK), a 2.8-kb *Bgl*II-*Bam*HI fragment of *LYS2* isolated from pAJ122, a 1.1-kb *Pvu*II fragment of *STE2* isolated from pAB539, and a 1.5-kb *Eco*RI fragment of *BAR1* isolated from pBAR2.

RNA transcripts were quantified using an Applied Biosystems PhosphorImager using ImageQuant software. In each sample, the amount of transcript from the gene of interest was normalized to the amount of *LYS2* control transcript; this normalized value was then compared to that of the sample derived from the wild-type strain 246-1-1.

DNA sequencing: *ARE1*-bearing plasmids pMW2, pMW3, and pMW10 were sequenced by the Biological Resource Center DNA Sequencing Facility at University of California, San Francisco, using Taq cycle sequencing with dye terminators. Both strands of the *ARE1* locus were completely sequenced.

Transposon mutagenesis: Tn1000 (γδ) transposon mutagenesis has been described (GUYER 1978; SANCAR and RUPP 1979). Donor and recipient strains were kind gifts of F. BANUETT. pMW15 was transformed into the donor bacterial strain E8037 (Amp^s Str^s/F'128) and transformants were selected on LB plates containing ampicillin. The transformed donor strain was grown at 37° to an optical density at 600 nm (OD_{600}) of 0.3. The recipient strain WA8067 (Amp^s Str^r/F^-) was grown at 37° to an OD_{600} of 0.5. Two milliliters of WA8067 were added to 0.5 ml of the E8037 transformant and the mixture was incubated at 37° for 2 hr while shaking. Serial dilutions of cells were then plated onto LB plates containing streptomycin (25 μg/ml) and ampicillin (50 μg/ml). The Amp^r Str^r colonies carry pMW15 containing γδ transposon insertions.

PCR assays: The PCR amplification protocol and three primers used to determine which *MAT* allele is present have been described (HUXLEY *et al.* 1990). In this assay, PCR amplification of the *MAT***a** locus produces a 544-bp fragment, while amplification of the *MATα* locus produces a 404-bp fragment. The fragments are visualized on an 0.8% agarose gel. Two modifications that improved the PCR reaction efficiency were used: the PCR reaction volumes were 50 to 100 μl instead of 5 μl, and instead of combining all three primers in one reaction, two separate reactions were set up for each strain, one to amplify *MAT***a** and one to amplify *MATα*.

To confirm integration of *sin4Δ::LEU2, are1Δ::LEU2,* and pMW17, a PCR amplification protocol developed by P. SORGER was used. In brief, a yeast colony is picked and resuspended in 99.5 μl of PCR reaction mix that consists of 63.5 μl H_2O, 10 μl 10× PCR buffer (100 mM Tris HCl pH 8.3, 500 mM KCl, 15 mM $MgCl_2$), 16 μl 1.25 mM each dNTP, 5 μl 2 μM primer 1, and 5 μl 2 μM primer 2. Samples are boiled 5 min and then spun down briefly. One-half a microliter of Taq polymerase (2.5 U) is added and reactions are overlayed with oil. Amplification is for 30 cycles of 94° 1 min, 42° 2 min, 65° 4 min. Following PCR amplification, reaction products are run on an 0.8% agarose gel to visualize the diagnostic fragments.

Cloning of *ARE1*: Strain MWY11 (α *are1-41 MFA2::lacZ*) was transformed with a YEp24-based yeast genomic library (CARLSON and BOTSTEIN 1982). Transformants were plated at ~200 colonies per SD −Ura plate. The X-Gal filter assay was used to screen for white (repressed for *MFA2::lacZ*) transformants. As a secondary screen, transformants that appeared white were tested for clumpiness in liquid SD −Ura medium. Out of ~9,300 transformants screened by X-Gal filter assay, one transformant, 41-6, was both white on X-Gal and nonclumpy in liquid SD −Ura medium. Both mutant phenotypes were restored upon curing 41-6 of its plasmid (pMW15).

Sporulation efficiency: Freshly grown patches of cells were replicated to sporulation medium plates and then incubated at 30° for 5 days. Sporulation efficiency was determined by the ratio of the number of asci (containing either one to four spores) present to the total number of cells. Cells were counted in a hemacytometer at ×400 magnification. Two independent diploid colonies for each sample were tested and the sporulation efficiency of each was averaged.

RESULTS

Isolation and initial analysis of mutants defective in α2 repression: To isolate mutants defective in α2 repression, we used an α strain that contains a chromosomal *MFA2::lacZ* reporter gene. Because *MFA2* is an **a**-specific gene, this *MFA2::lacZ* fusion is under cell-type control: it is expressed in **a** cells but not in α cells. Wild-type α yeast colonies appear white in X-Gal filter assays, whereas α yeast colonies defective in α2 repression express this reporter and thus appear blue. We mutagenized the starting α strain with EMS and screened for mutants that were derepressed using an X-Gal filter assay. Out of ~8800 colonies screened, 130 blue colonies were isolated, 21 of which were chosen for further study. Nine of these isolates were chosen because they appeared the most derepressed, and 12 were chosen because, although less derepressed than the first group, they shared the clumpy phenotype of *ssn6, tup1,* and *sin4* mutants.

To determine whether any of these isolates bear mutations in genes already known to be involved in α2 repression, each mutant strain was transformed with plasmids carrying *MATα, SSN6, TUP1, SIN4,* or *MCM1* and scored for complementation of the derepression phenotype. Any complementation observed with a given plasmid was subsequently confirmed by an allelism test using a corresponding test strain containing the *MFA2::lacZ* reporter. For this linkage analysis, a wild-type *MAT***a** strain (for testing linkage to *MATα*) or a *tup1, ssn6,* or *sin4* deletion mutant was used (see MATERIALS AND METHODS). After tetrad dissection (10 to 40 asci per cross, from one to four independent diploids), the mutant phenotype of segregants (either derepression of *MFA2::lacZ* in α segregants or clumpiness in both **a** and α segregants) was analyzed. As indicated in Table 3,

TABLE 3

Isolation of genes required for α2 repression

Mutation	No. of alleles
α2	6
SSN6	2
TUP1	3
SIN4	2
ARE1	2
ARE2	4
ARE3	1
ARE4	1

Twenty-one isolates defective for α2 repression were identified and analyzed as described in the text. Of these 21 isolates, 13 were found to carry mutations in genes known to be required for α2 repression. The eight remaining isolates were found to carry mutations comprising four separate genetic loci, designated *ARE1, ARE2, ARE3,* and *ARE4* (for *a*lpha2 *re*pression).

multiple alleles of all the expected, previously identified genes were isolated. No mutations in *MCM1* were recovered, an unsurprising result since *MCM1* is an essential gene.

After elimination of mutants defective in known genes required for α2 repression, the remaining eight mutants were studied further. Genetic analysis was facilitated by the following: all of the mutants could mate despite being defective for α2 repression, and α mutant segregants could be followed in outcrosses by using the X-Gal filter assay. In this assay, the α segregants defective in α2 repression turn blue but more slowly than the wild-type **a** cells, a result consistent with the observation that they are not fully derepressed (see below). PCR analysis of the *MAT* locus of randomly selected segregants was performed to check mating type (MATERIALS AND METHODS), and in 97% (57/59) of the cases, the assignment of mutant α or wild-type **a** as determined by X-Gal filter assay was correct, indicating that the X-Gal filter assay is an accurate method for detecting the mutant α segregants.

The eight newly isolated mutants have pleiotropic phenotypes; most notably, like *ssn6, tup1,* and *sin4* mutants, they are clumpy. The *MFA2::lacZ* derepression cosegregates with the clumpiness upon outcrossing to a wild-type strain carrying the *MFA2::lacZ* reporter (22 to 32 α segregants tested per cross). To test whether the cosegregating phenotypes were due to a single gene defect, each mutant was crossed to a wild-type strain of opposite mating type. Tetrad analysis (9–15 asci tested per cross) after sporulation of these heterozygous diploids demonstrated that the clumpy phenotype segregated 2:2 and that approximately half of all α segregants were derepressed, indicating that in each mutant, the phenotype is due to a single genetic lesion. Heterozygous diploids derived from crossing the eight isolates to the wild-type strain of opposite mating-type were not clumpy, indicating that each of the eight mutations is recessive.

Complementation and linkage analysis: To determine the number of complementation groups represented by these eight mutations, the *MAT* locus in each mutant was deleted and then each *MAT*-deleted mutant was crossed pairwise (*matΔ* strains mate as *MAT***a** strains) to all of the original *MATα* mutants. Diploids were selected, and then complementation of the clumpy phenotype was scored. The eight mutations comprise four complementation groups designated *are* for *a*lpha2 *re*pression (Table 4).

Tetrad analysis after sporulation of representative diploids derived from crossing members of different *ARE* groups showed that each complementation group is unlinked to the others, demonstrating that the *ARE* genes define four separate genetic loci. Standard tetrad analysis indicated tight linkage between the two members of *ARE1* and between the four members of *ARE2*, suggesting that two alleles of *are1* and four alleles of *are2* were recovered (Table 5).

Together, the complementation and linkage analysis indicate that the eight *are* mutations comprise four independent genetic loci, and that there are two alleles of *are1,* four alleles of *are2,* and one allele each of *are3* and *are4* (Table 3). Alleles of *are1* and *are2* display unlinked noncomplementation, indicated by the only partial complementation of the clumpy phenotype in diploids that are doubly heterozygous for recessive *are1* and *are2* mutations (Table 4). Such a genetic interaction suggests a functional relationship between these two gene products (VINH *et al.* 1993).

Expression of *MFA2::lacZ* in wild-type and mutant strains: To determine the level of derepression in each of the *are* mutants and to compare it to the level of derepression in some of the previously characterized mutants, we performed quantitative β-galactosidase assays. Each mutant was backcrossed twice to a wild-type strain carrying the *MFA2::lacZ* reporter (SM1196 and/or SM1179). **a** and α mutant segregants were identified by the derepression of *MFA2::lacZ* and/or clumpy phenotype. Mating-type was confirmed by PCR analysis of the *MAT* locus. We quantified repression as the ratio of β-galactosidase activity in an **a** strain to that in the isogenic α strain. In an α *tup1* deletion mutant repression is reduced 200-fold relative to wild-type, *i.e.,* there is no detectable repression (Table 6). In an α *sin4* deletion mutant, repression is reduced 38-fold but not eliminated. In comparison, repression is reduced 7- to 24-fold in the *are* mutants, with the *are2-13* mutation having the strongest effect.

***are* mutations act independently of the pheromone response pathway:** It was possible that the aberrant expression of *MFA2::lacZ* in α *are* mutants was due to an increase in **a**-specific gene activation rather than to a defect in α2 repression. While the **a**-specific genes are subject to α2 repression, they are also under the control

TABLE 4

Complementation analysis of *are* mutants

	Matα							
MatΔ	*are1-5*	*are1-41*	*are2-13*	*are2-30*	*are2-40*	*are2-59*	*are3-57*	*are4-87*
are1-5	−	−	±	+	+	+	+	+
are1-41	−	−	±	+	+	+	+	+
are2-13	±	±	−	−	−	−	+	+
are2-30	+	±	−	−	−	−	+	+
are2-40	+	+	−	−	−	−	+	+
are2-59	+	+	−	−	−	−	+	+
are3-57	+	+	+	+	+	+	−	+
are-87	+	+	+	+	+	+	+	−

Complementation analysis of the eight recessive *are* mutants. The *MAT* locus in each *are* mutant was disrupted with a *URA3* insertion (see MATERIALS AND METHODS). Each *matΔ are* mutant was then crossed pairwise to each of the α *are* mutants, which were carrying a *LEU2*-marked plasmid. Diploids were selected on SD plates lacking uracil and leucine. The clumpy phenotype was then determined by growing the selected diploids in liquid SD media lacking uracil and leucine at 30°. −, clumpy; +, not clumpy; ±, indicates partially clumpy, suggesting possible unlinked noncomplementation.

of an activation pathway, known as the pheromone response pathway (reviewed in HOEKSTRA *et al.* 1991). In addition to the single α2-MCM1 operator, the upstream control region of **a**-specific genes contains multiple activation sites, called pheromone response elements (PREs), through which the pheromone response pathway acts. To test whether the *are* mutations have an effect on α2 repression in the absence of PREs, we used a reporter gene in which the α2-MCM1 operator has been removed from its endogenous chromosomal context. This reporter gene consists of the yeast *CYC1* gene fused in frame to the *lacZ* gene. The promoter of this test gene, containing the two *CYC1* upstream activation sites (UASs) plus the *CYC1* TATA region, drives the expression of *lacZ*. An α2-MCM1 operator inserted between the UAS elements and the TATA region brings the *CYC1-lacZ* reporter under negative control by α2-MCM1 (JOHNSON and HERSKOWITZ 1985).

The chromosomal *MFA2::lacZ* gene was outcrossed from a representative of each *ARE* complementation group by backcrossing to an *ARE MFA2* strain of opposite mating type (246-1-1 or EG123). *MATα are MFA2* segregants were identified by PCR analysis of the *MAT* locus, by the presence of clumpiness, and by appearing

TABLE 5

Linkage analysis

Diploid	PD[a]	NPD	TT	Ratio of derepressed α to wild-type α segregants
are1-5 / *are1-41*	ND[b]	ND	ND	46/46
are2-13 / *are2-30*	11	0	0	21/22
are2-13 / *are2-40*	24	0	0	ND
are2-13 / *are2-59*	36	0	0	ND

Multiple alleles of *are1* and *are2* were recovered. Each *are1* and *are2* mutant was backcrossed twice to a wild-type *MFA2::lacZ*-bearing strain of opposite mating type (SM1179 or SM1196). **a** and α *are* mutant segregants were identified based on the derepression of *MFA2::lacZ* and/or clumpiness; mating type was confirmed by PCR analysis of the *MAT* locus. These backcrossed segregants were then crossed to generate the diploids described here. After sporulation and tetrad dissection, segregation of the clumpy phenotype was scored and/or the fraction of α segregants that were derepressed for *MFA2::lacZ* was determined.

[a] PD, parental ditype; NPD, nonparental ditype; TT tetratype. Clumpiness is scored.

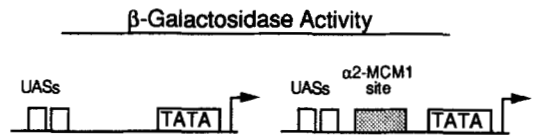
[b] ND, not determined.

TABLE 6

Expression of *MFA2::lacZ* in wild-type and mutant strains

Genotype	Units of β-galactosidase activity		Repression
	Mata	*Matα*	
wild-type	140	0.7	200
tup1Δ::LEU2	110	110	1.0 (200×)
sin4Δ::LEU2	90	17	5.3 (38×)
are1-5	200	13	15 (13×)
are1-41	220	8.1	27 (7.4×)
are2-13	100	12	8.3 (24×)
are2-30	80	3.8	21 (9.5×)
are2-40	120	7.1	17 (12×)
are2-59	110	8.5	13 (15×)
are3-57	150	11	14 (14×)
are4-87	180	9.0	20 (10×)

Numbers represent units of β-galactosidase activity in *MFA2::lacZ*-bearing strains of the indicated genotype. Results are averages of triplicate assays performed on at least three independent colonies on different days. The SD was typically <25% of the mean. Numbers in parentheses indicate fold decrease in repression relative to wild-type. All strains were grown in YEPD media.



Genotype	pAJ1	pAJ3	Repression (pAJ1/pAJ3)
α wild type	180	1.3	140
α *tup1Δ::LEU2*	180	120	1.5 (93×)
α *sin4Δ::LEU2*	600	37	16 (8.7×)
α *are1-41*	93	3.2	29 (4.8×)
α *are2-13*	200	29	6.9 (20×)
α *are3-57*	190	20	9.5 (15×)
α *are4-87*	470	15	31 (4.5×)

FIGURE 1.—Effect of *are* mutations on α2 repression of the *CYC1-lacZ* reporter gene. Numbers represent units of β-galactosidase activity in *MATα* strains of the indicated genotype. Results are averages of triplicate assays performed on at least three independent transformants on different days. The SD was typically <20% of the mean. Transformants were grown in glucose medium under uracil selection.

white in the X-Gal filter assay. Each was then transformed with a plasmid bearing the *CYC1-lacZ* reporter with no α2-MCM1 site (pAJ1) or bearing the *CYC1-lacZ* reporter with the site (pAJ3), and quantitative β-galactosidase assays were performed. Repression was quantified as the ratio of β-galactosidase activity in a strain carrying the reporter with no α2-MCM1 site to that in the same strain carrying the reporter with the site. In an α *tup1* deletion mutant, α2 repression of this test gene is essentially eliminated (Figure 1), as is also the case for repression of *MFA2::lacZ*. The effect of each *are* mutation on α2 repression of this test gene (5- to 20-fold reduction in repression) is similar to the effect each mutation has on the *MFA2::lacZ* reporter (7- to 24-fold reduction), indicating that the *are* mutations can act independently of the pheromone response pathway.

In addition, it should be noted that the *are4* mutation increases activated transcription of the *CYC1-lacZ* reporter lacking the α2-MCM1 site by ~2.5-fold (compare *CYC1-lacZ* expression in *are4* and wild-type strains in Figure 1, column pAJ1). Notably, the *sin4* deletion has a similar effect (threefold), whereas the *are1, are2* and *are3* mutations do not.

Derepression of a-specific genes in *are* mutants: To determine whether the defect in repression lies at the transcriptional level and is not specific to *lacZ* gene expression, we tested whether some of the endogenous **a**-specific genes in the *are* mutants were expressed inappropriately. Northern analysis revealed that both *STE2* and *BAR1* genes were expressed in the α *are1* and α *are2* mutants but not in the α *ARE* strain (data not shown). The level of derepression was significantly lower than that seen in an α *tup1* deletion mutant, consistent with the smaller effect on reporter gene repression caused by the *are* mutations.

Suppression of *are* mutations by known genes: In the course of the genetic analysis, we noted that some of the *are* mutations are suppressed by overexpression of previously identified genes. In each case, the derepression of *MFA2::lacZ,* but not the clumpiness, is suppressed. This suppression was determined qualitatively by X-Gal filter assay. *TUP1* overexpression suppresses one allele of *are2.* (*TUP1* overexpression also suppresses the two alleles of *ssn6* recovered in our screen.) *SIN4* overexpression suppresses *are4-87.* This genetic interaction between *SIN4* and *ARE4* is consistent with the fact, discussed above, that both the *sin4* and *are4* mutations increase activated transcription of *CYC1-lacZ. MATα,* even when on a low copy plasmid, strongly suppresses both alleles of *are1.* Overexpression of *TUP1, SIN4,* or *MATα* did not suppress any of the other *are* mutations. Furthermore, neither overexpression of *SSN6* nor *MCM1* suppressed any of the *are* mutations.

The phenotype of the *are* mutants could be due to lower expression levels of some of the previously identified genes required for α2 repression. The fact that *MATα, TUP1,* and *SIN4* suppress some of the *are* mutations could indicate that the *ARE* genes are transcriptional activators of these genes. To test this possibility, we examined the RNA levels of *MATα2* and *TUP1* in the α *are* mutant strains by Northern analysis. When compared to expression levels in an α wild-type strain (246-1-1), *TUP1* and *MATα2* expression levels were not significantly altered in any of the *are* mutants analyzed, with one exception: the level of *MATα2* expression was

reduced to 65% of wild-type levels in the α *are1-41* mutant (data not shown). It is not known whether such a reduction in the level of *MATα2* expression would affect α2 repression in α cells. It is possible that ARE1 acts as a positive regulator of *MATα2*, in addition to having other regulatory roles in the cell.

Some *are* mutations derepress UAS-less *CYC1-lacZ* and *HO-lacZ* fusion genes: The genetic interactions observed suggest an initial way to group the *ARE* genes: one group includes *SSN6, TUP1, ARE1* and *ARE2* (given the unlinked noncomplementation between alleles of *are1* and *are2*, indicated in Table 4) and another group includes *SIN4* and *ARE4*. No genetic interactions between *ARE3* and the other *ARE* genes or previously identified genes were observed.

Another way to group the *ARE* genes is based on whether mutations in these genes derepress basal transcription, *i.e.*, transcription in the absence of a known UAS element. It has been shown previously that *sin4* mutations derepress at least four different UAS-less test genes (JIANG and STILLMAN 1992; CHEN *et al.* 1993b). Because TUP1 is thought to be recruited to the promoters on which it acts by specific DNA-binding proteins (KELEHER *et al.* 1992), *tup1* mutations are not expected to strongly affect basal transcription of these test genes, which lack α2-MCM1 sites.

We studied the effect of the *are* mutations on two different UAS-less reporter genes: the UAS-less *CYC1-lacZ* and UAS-less *HO-lacZ* test genes. Figure 2 shows that *are3* and *are4* mutations strongly derepress basal transcription (17- to 28-fold and 16- to 23-fold, respectively) as does the *sin4* mutation (30- to 43-fold). On the other hand, *are1* and *are2* mutations have weaker effects (two- to eightfold and three- to sixfold, respectively), resembling the effect of the *tup1* mutation (one- to sixfold). The effects of the *are* mutations on basal transcription of these two UAS-less test genes suggest a grouping that is consistent with that indicated by the genetic interactions: one group includes *TUP1, ARE1,* and *ARE2,* and the other includes *SIN4, ARE3* and *ARE4.*

Additional phenotypes of the *are* mutants: The *are* mutants share additional phenotypes. Most grow more slowly than the wild-type strain at 30°, but none are temperature-sensitive for growth at 37°. Each has a similar abnormal cell morphology: cells are enlarged and sometimes fail to separate during cell division, resulting in chains of unseparated cells. Some single cells appear elongated or pear-shaped. This abnormal morphology is non-cell-type-specific and becomes more severe over time as the cells are incubated on YEPD plates. All of the *are* mutants except *are4-87* and *are2-59* have a "lacy" colony morphology (ROTHSTEIN and SHERMAN 1980). In addition, homozygous *are* diploids show a decrease in sporulation efficiency (2.5- to 10-fold).

Cloning and sequencing of *ARE1*: *ARE1* was cloned by transforming the original α *are1-41 MFA2::lacZ* strain MWY11 with a yeast genomic library and then screening

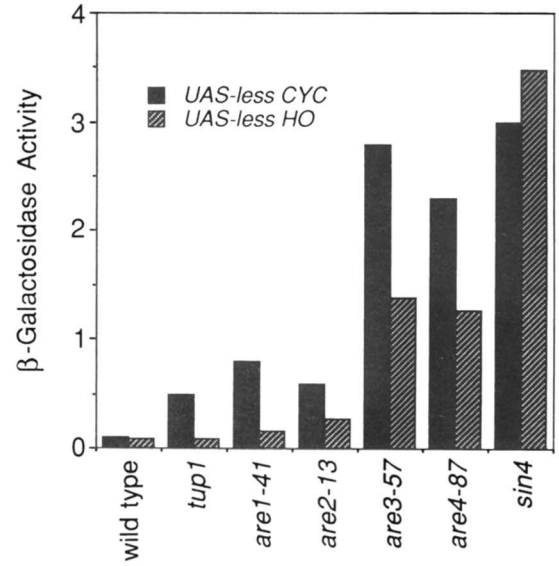


FIGURE 2.—Effect of *are* mutations on basal transcription. Bars show units of β-galactosidase activity in *MATα* strains of the indicated genotype that carry the UAS-less *CYC1-* or *HO-lacZ* reporter plasmids (pΔSS and M740, respectively). The UAS-less promoters driving the expression of *lacZ* contain the TATA elements derived from the indicated promoter. Neither UAS elements nor α2-MCM1 sites are present in these reporter genes. For each strain, results are averages of triplicate assays performed on two to three independent transformants on different days. The SD was typically <20% of the mean. Transformants were grown in glucose medium under uracil selection.

for complementation of the *MFA2::lacZ* derepression. One plasmid, pMW15, was isolated that complements both the *MFA2::lacZ* derepression and clumpiness of each *are1* allele. To determine whether pMW15 actually contains the *ARE1* gene, a *URA3*-integrating plasmid containing a subclone derived from the pMW15 genomic insert was linearized by cutting with *Sac*II and integrated into an **a** *ARE1 MFA2::lacZ ura3* strain (SM1179). After confirming integration by PCR analysis, the *URA3*-marked integrant was crossed to an α *are1-41 MFA2::lacZ ura3* strain; diploids were selected and sporulated. Tetrad analysis (27 asci tested from two independent heterozygotes) indicated that the insert integrated at the *ARE1* locus. (All wild-type α segregants were Ura+ and all mutant α segregants were Ura−).

To determine the location of the *ARE1* gene on pMW15, we subjected the plasmid to transposon mutagenesis (see MATERIALS AND METHODS). Plasmids with transposon insertions were tested for the ability to complement the α *are1-41* defect (*MFA2::lacZ* derepression and clumpiness). Most of the transposons that disrupted the complementation activity mapped to a region of ~3 kb. Sequence analysis in this region revealed an open reading frame encoding a putative CDC28-related protein kinase, recently identified as *UME5* (SUROSKY *et al.* 1994). To confirm that the protein kinase is *ARE1*, a 2.8-kb *Sna*BI-*Eco*RV fragment that con-

tains only the kinase sequence plus 583 nucleotides upstream and 530 nucleotides downstream was subcloned into a low copy yeast vector and shown to complement both phenotypes of each *are1* allele.

To confirm further that the protein kinase is identical to *ARE1*, we disrupted the chromosomal copy of the protein kinase gene and analyzed the phenotype. An *are1Δ::LEU2* allele was introduced into the wild-type **a**/α *MFA2::lacZ leu2* homozygous diploid strain MWY33. This disruption removes almost the entire *ARE1* coding sequence, leaving only 57 nucleotides upstream and 74 nucleotides downstream of the *LEU2* insertion. Disruption of the *ARE1* locus was confirmed by PCR (MATERIALS AND METHODS). Tetrad analysis (15 asci tested) after sporulation of the heterozygous diploid showed that the clumpiness cosegregated with the Leu^+ phenotype. Furthermore, in all Leu^+ α segregants, the *MFA2::lacZ* reporter was derepressed, while in all Leu^- α segregants, the *MFA2::lacZ* reporter was repressed, further indicating that *ARE1* encodes the protein kinase.

Phenotype of a known null *are1* allele: We used the *are1Δ::LEU2* allele to determine the phenotype of a null *are1* mutation. Although the *are1* disruption is not lethal, the phenotypes caused by the mutation indicate that *ARE1* is important for cell growth. First, the mutant spore colonies are clumpier and grow more slowly than either the *are1-5* or *are1-41* allele. Second, the mutant spore colonies rapidly acquire mutations that suppress the clumpy growth; *i.e.*, as the mutant spore colonies grow, sectors of faster growing cells appear at high frequency. Upon streaking out these spore colonies, two distinct types of colonies are generated, partially clumpy colonies (apparently arising from cells in the faster growing sectors) and smaller, very clumpy colonies.

To determine whether the *are1* disruption has a greater effect on α2 repression than the other two *are1* mutations, we identified both **a** and α *are1Δ:LEU2* segregants by PCR analysis of the *MAT* locus and then quantitated the level of *MFA2::lacZ* expression by performing β-galactosidase assays on the smaller, very clumpy colonies. As indicated in Table 7, the sevenfold decrease in repression caused by the *are1* disruption is not significantly different from that exhibited by the *are1-5* or *are1-41* mutants (seven- to ninefold).

TABLE 7

Expression of *MFA2::lacZ* in wild-type and *are1* strains

Genotype	Units of β-galactosidase activity		Repression
	*MAT***a**	*MATα*	
ARE$^+$	120	0.8	150
are1-5	170	9.8	17 (8.8×)
are1-41	180	8.2	22 (6.8×)
are1Δ::LEU2	110	4.9	22 (6.8×)

Numbers represent units of β-galactosidase activity in *MFA2::lacZ* strains of the indicated genotype. Results are averages of assays performed in triplicate. Two independent colonies for each sample were assayed. The SD is <15% of the mean. Numbers in parentheses indicate fold decrease in repression relative to the wild-type strains SM1179 (*MAT***a**) and SM1196 (*MATα*). All strains were grown in YEPD medium.

DISCUSSION

In this study we have isolated mutations that confer defects in α2 repression. As expected, we recovered mutations in genes previously known to be required for α2 repression, namely, *MATα2, SSN6, TUP1* and *SIN4*. In addition, we recovered mutations in four other genes, designated *ARE* genes for *a*lpha2 *re*pression. Our results suggest that, like *SSN6, TUP1*, and *SIN4*, the *ARE* genes can regulate the transcription of genes other than those subject to α2 repression. First, the *are* mutations cause pleiotropic phenotypes including clumpiness, slow growth, decreased sporulation efficiency, and abnormal cell morphology. Second, two of the *are* mutations strongly derepress basal transcription of at least two different reporter genes. Finally, *ARE1* is identical to *UME5* (SUROSKY *et al.* 1994) and *SSN3* (S. KUCHIN and M. CARLSON, personal communication). *UME5* is required for the negative regulation of early meiosis-specific genes during mitotic growth (STRICH *et al.* 1989). *SSN3* is required for full levels of glucose repression (CARLSON *et al.* 1984; VALLIER and CARLSON 1994). Taken together, these results suggest that the *ARE* genes encode negative regulators that affect transcription of diverse genes.

The general negative regulators involved in α2 repression can be divided into two classes based on their ability to repress basal transcription, *i.e.*, transcription in the absence of known UAS elements (Figure 3). SIN4, ARE3, and ARE4 belong to the class that represses basal transcription. Mutations in *SIN4* strongly derepress at least four different UAS-less reporter genes (JIANG and STILLMAN 1992; CHEN *et al.* 1993b). Both mutations in *ARE3* and *ARE4*, like mutations in *SIN4*, strongly derepress basal transcription of the two UAS-less reporter genes tested in this study. Moreover, two observations are consistent with grouping SIN4, ARE3, and ARE4 together. First, both the *sin4* and *are4-87* mutations significantly increase activated transcription of the *CYC-lacZ* test gene, unlike mutations in *TUP1* or other *ARE* genes, and second, overexpression of *SIN4* suppresses the *are4-87* mutation.

SSN6, TUP1, ARE1, and ARE2 appear to belong to the other class. We found that neither *tup1, are1*, nor *are2* mutations significantly derepress at least two different UAS-less reporter genes. Furthermore, the genetic interactions we observed are consistent with this grouping. *TUP1* overexpression suppresses a mutation in *ARE2*, and alleles of *are1* and *are2* display unlinked noncomplementation. Because SSN6 and TUP1 function

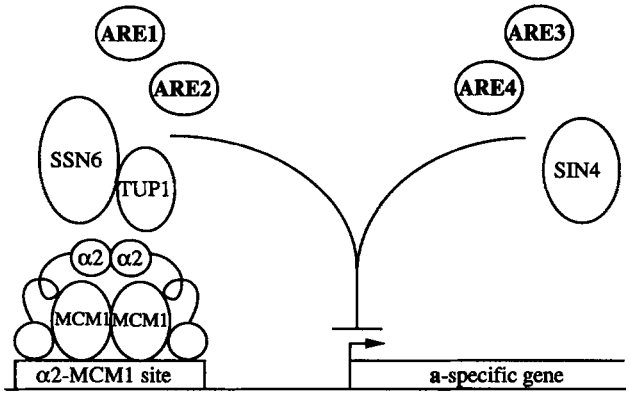


FIGURE 3.—Model for α2 repression of **a**-specific genes. Operator bound α2-MCM1 recruits the general repressors SSN6 and TUP1 to the promoters of **a**-specific genes. Once recruited, SSN6 and TUP1, which are known to be physically associated in a protein complex, mediate repression of the **a**-specific genes. Full repression requires at least five other general negative regulators: SIN4 and the *ARE* gene products. The general negative regulators required for α2 repression can be divided into two classes based on their ability to repress basal transcription, *i.e.*, transcription in the absence of known UAS elements. SSN6-TUP1-ARE1-ARE2, which do not repress basal transcription, are in one class and SIN4-ARE3-ARE4 are in the other. The genetic interactions we observed are consistent with this grouping. *TUP1* overexpression suppresses an *are2* mutation, alleles of *are1* and *are2* display unlinked noncomplementation, and *SIN4* overexpression suppresses the *are4-87* mutation. How these gene products interact to bring about full levels of α2 repression is unknown (see text for discussion).

together as transcriptional repressors (reviewed in JOHNSTON and CARLSON 1992; KELEHER *et al.* 1992; TRUMBLY 1992), SSN6 is not expected to repress basal transcription, although this was not tested directly.

Both classes, SSN6-TUP1-ARE1-ARE2 and SIN4-ARE3-ARE4, include components in which mutations cause only partial loss of α2 repression. It is clear that *SSN6* and *TUP1* are essential for α2 repression, as null mutations in either gene eliminate repression (WICKNER 1974; LEMONTT *et al.* 1980; ROTHSTEIN and SHERMAN 1980; CARLSON *et al.* 1984). However, null mutations in *SIN4* or *ARE1*, or mutations recovered in the other *ARE* genes, cause only partial loss of repression. The smaller effect on repression could be due to possible partial functional redundancies between these gene products. In addition, the *are2*, *are3*, or *are4* alleles may not be null. How these negative regulators interact to bring about full levels of α2 repression is unknown.

The molecular characterization of the *ARE* genes should help elucidate the mechanism of α2 repression. We have cloned the *ARE1* gene and have found that it is identical to *UME5/SSN3*, which encodes a CDC28-related protein kinase. Recently, we have learned that *ARE1* is identical to yet another gene, *SRB10* (S. LIAO, J. ZHANG and R. A. YOUNG, personal communication). The *SRB* genes were isolated as suppressors of a growth defect caused by C-terminal tail truncations of the large subunit of RNA polymerase II (NONET and YOUNG 1989; THOMPSON *et al.* 1993). Biochemical experiments have demonstrated that the *SRB* gene products are components of the RNA polymerase II holoenzyme (THOMPSON *et al.* 1993; KIM *et al.* 1994; KOLESKE and YOUNG 1994), which consists of RNA polymerase II subunits, SRB proteins, several general transcription factors, and other as yet unidentified proteins.

This finding suggests that SSN6 and TUP1 mediate repression, at least in part, through acting on the general transcription machinery and raises some interesting possibilities for the mechanism of α2 repression. For example, the SRB10 protein kinase could negatively regulate transcription initiation by phosphorylating a general transcription factor. SSN6 and TUP1 could stimulate this kinase activity. Another possibility, a refinement of the "locking" model proposed by KELEHER *et al.* (1988), is that once SSN6-TUP1 is recruited to the promoter of **a**-specific genes by operator-bound α2-MCM1, SSN6-TUP1 tightly binds to SRB10 and/or to other SRB proteins. This tight interaction would tether the RNA polymerase in place, thereby preventing transcription. Future studies will investigate the link between SSN6-TUP1 and the RNA polymerase II holoenzyme.

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