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Genetic links between alpha2 repression and the general transcription machinery in saccharomyces cerevisiae

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Genetic Links between alpha2 Repression and the General Transcription Machinery in Saccharomyces cerevisiae

by

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by

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Genetic Links between alpha2 Repression and the General Transcription Machinery in *Saccharomyces cerevisiae*

Madhu Wahi

Abstract: Cell differentiation typically requires cell-type control of gene expression. In eukaryotes, this control primarily occurs at the transcriptional level and is directed by specific DNA-binding regulatory proteins, which can activate or repress transcription. Currently, much more is known about how these proteins activate transcription than about how they prevent it.

The yeast *Saccharomyces cerevisiae* is an ideal system for studying transcriptional repression in the context of cell-type control of gene expression. In *S. cerevisiae* haploid α cells, transcription of genes specific to the other haploid cell type, the **a** cell, is turned off. Transcriptional repression of the **a**-specific genes is known to require several proteins. The homeodomain protein α2 binds cooperatively with MCM1 to an operator site located upstream of each **a**-specific gene. Operator-bound α2-MCM1 recruits a general repressor complex, comprised of SSN6 and TUP1, to the DNA. SSN6-TUP1 then mediates transcriptional repression of the **a**-specific genes. SIN4, another general negative regulator, is also required for this repression.

However, unlike null *ssn6* or *tup1* mutations, null *sin4* mutations only cause partial loss of repression. The mechanism by which the **a**-specific genes are repressed is unknown.

I performed a genetic screen for other genes required for this process (referred to as α2 repression), and in doing so identified four genes, which I designated the *ARE* genes for alpha2 repression. Recessive *are* mutations lead to partial loss of repression and pleiotropic phenotypes similar to those resulting from mutations in *SSN6*, *TUP1*, and *SIN4*. Based on initial analysis, I propose that two classes of general negative regulators cooperate to bring about full levels of α2 repression. Once class includes SIN4, ARE3, and ARE4 and the other includes SSN6, TUP1, ARE1, and ARE2.

I focused on ARE1 and ARE2 since they belong to the same class as SSN6 and TUP1. Cloning and sequencing of *ARE1* revealed that it encodes SRB10, a CDC28-related protein kinase that is a component of the general transcription machinery. Cloning of *ARE2* revealed that it encodes SRB8, another component of the transcription machinery. My results lead to the proposal that SSN6-TUP1 exerts α2 repression by interacting with SRB proteins.

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Introduction

Cell differentiation is a complex process that typically requires cell-type control of gene expression. This requirement can present an enormous challenge to a multicellular organism. For example, the human platelet cell must express eight platelet-specific genes while preventing the expression of over 31,000 genes specific to other cell types (Adams et al., 1995). In eukaryotes, cell-type control of gene expression most often occurs at the transcriptional level, where it is typically directed by regulatory proteins that bind to specific sites upstream of their target promoters. These regulatory proteins can either activate or repress transcription, depending on the precise assembly of proteins on the DNA which in turn is determined by the DNA sequence of the gene regulatory region and the cell type. Currently, much more is known about how these regulators activate transcription than about how they bring about transcriptional repression. An ideal system for studying the mechanism of transcriptional repression is the yeast *Saccharomyces cerevisiae*. In *S. cerevisiae* haploid α cells, transcription of genes specific to **a** cells, the other haploid cell type, is turned off by an unknown mechanism.

This thesis began with a genetic screen to identify new components required for repression of the **a**-specific genes in α cells (often referred to as α2 repression) in the hope that they would point towards a mechanism of transcriptional repression. Mutations in four genes which encode new components were identified and analyzed (Wahi and Johnson, 1995). These genes were designated the *ARE* genes for alpha2 repression. *ARE1* and *ARE2*

were cloned and characterized further. The results of this study lead to a specific model for the mechanism of α2 repression.

This introductory chapter presents relevant background on transcriptional regulation and α2 repression. Two leading models for the mechanism of α2 repression are analyzed. Chapter 2 describes the genetic screen and analysis of isolated mutants. In addition, the cloning and sequencing of *ARE1* are detailed. In the supplement to Chapter 2, further analysis of the *ARE* genes is presented as well as data which were described but not shown in Chapter 2. Chapter 3 describes the cloning and characterization of *ARE2*. Chapter 4 summarizes the results and puts forward a model for the mechanism of α2 repression based on these results. Finally, biochemical experiments to test and further refine the model are proposed.

Transcription Initiation

In eukaryotes, cell-type control of gene expression primarily occurs at the transcriptional level. Many studies on transcriptional regulation have focused on genes transcribed by RNA polymerase II (pol II). Central to these studies has been the general transcription machinery, a complex comprised of at least five general transcription factors (TFIIB, TFIID, TFIIE, TFIIF, and TFIIH), pol II, and, at least in the yeast *Saccharomyces cerevisiae*, pol II-associated proteins, which include the SRB proteins (McKnight and Yamamoto, 1992; Thompson et al., 1993; Koleske and Young, 1994; Kim et al., 1994; Liao et al., 1995; Hengartner et al., 1995).

A general understanding of transcription initiation is important for understanding how transcription is regulated. Transcription initiation can be divided into several steps, each of which could be subject to regulation. The first step involves the ordered assembly of the general transcription machinery at pol II-transcribed gene promoters, that is, at the DNA region containing a TATA box (in most cases) and the transcriptional start site(s) (reviewed in McKnight and Yamamoto, 1992). There are two models, both based on *in vitro* studies, for how this process occurs. In one model, TFIID first binds to the TATA box. Binding of TFIID is followed by binding of TFIIB. Next, pol II, TFIIE, TFIIF, and TFIIH, assemble onto the TATA-TFIID-TFIIB complex in multiple steps. The other model for the assembly of the transcription complex is derived from recent findings that some of these factors can be copurified from *S. cerevisiae* in a complex termed the pol II holoenzyme. The holoenzyme is comprised of TFIIB, TFIIF, TFIIH, pol II, the SRB proteins, and at least one other protein, GAL11 (Thompson et al., 1993; Koleske and Young, 1994; Kim et al., 1994; Liao et al., 1995; Hengartner et al. 1995). In this model, a preassembled pol II holoenzyme binds to the TATA-TFIID complex. Following binding of the holoenzyme, TFIIE binds to the TATA-TFIID-holoenzyme complex, completing the assembly of the general transcription machinery. As pointed out by M. Carey (1994), this second model is not dramatically different from the first; it is, however, a more simple way of viewing the process.

Once the transcription machinery has assembled, mRNA synthesis can begin. The transcription machinery undergoes a transition from a closed complex to an open complex in which a short stretch of promoter DNA is melted (Wang et al., 1992; Choy and Green, 1993). This transition resembles that which occurs in prokaryotes. Namely, after the bacterial RNA polymerase holoenzyme (which consists of a core polymerase and a sigma subunit) binds to the promoter, the holoenzyme-promoter DNA complex isomerizes from a closed complex in which the DNA is unmelted to an open complex in which a local stretch of 10 to 15 base pairs of DNA double helix is melted (reviewed in von Hippel et al., 1984 and in McClure, 1985). In eukaryotes, unlike in prokaryotes, this isomerization requires ATP hydrolysis (Wang et al., 1992; Choy and Green, 1993). Following open complex formation, mRNA synthesis begins and the polymerase leaves the promoter (often termed promoter clearance) to begin the processive elongation phase of transcription (reviewed in Mcknight and Yamamoto, 1992).

Phosphorylation of the CTD and Its Possible Role in Transcription Initiation

The largest subunit of pol II contains a C-terminal domain (CTD), which is comprised of a tandem series of highly conserved heptapeptide repeats, the number of which generally increases with the complexity of the organism (reviewed in Dahmus, 1995). Two forms of this subunit of pol II exist *in vivo*. One contains an unphosphorylated CTD (subunit IIa) and the other contains an extensively phosphorylated CTD (subunit IIo).

The phosphorylation state of the CTD changes during transcription. Biochemical evidence suggests that the form of polymerase recruited to promoters (i.e., incorporated into the general transcription machinery) contains an unphosphorylated CTD. This evidence includes the selective inhibition of transcription initiation over transcription elongation using antibodies that have ten-fold higher reactivity to IIa than IIo (Laybourn and Dahmus, 1989), and the finding that pol II which contains the IIa subunit is incorporated into the general transcription machinery preferentially over polymerase containing IIo (Lu et al., 1991; Chesnut et al., 1992). On the other hand, results from *in vitro* cross-linking studies and *in vivo* immunofluorescence microscopy studies using antibodies directed against IIo and IIa suggest that pol II engaged in elongation contains a phosphorylated CTD (Bartholomew et al., 1986; Cadena and Dahmus, 1987; Kang and Dahmus, 1993).

Since the CTD is in the unphosphorylated state at the beginning of transcription initiation and is in the phosphorylated state during elongation, it has been proposed that phosphorylation of the CTD is required for the general transcription machinery to leave the promoter to begin elongation (Laybourn and Dahmus, 1990; reviewed in Dahmus, 1995). As pointed out by M. E. Dahmus (1995), it is not difficult to imagine that the extensive phosphorylation of the CTD would alter the conformation of the CTD; and that these changes could disrupt many of the contacts pol II makes with the

promoter or with other holoenzyme components, thereby facilitating promoter clearance.

However, there is no conclusive evidence to support the view that CTD phosphorylation is required for transcription initiation. In fact, there is evidence to the contrary. Some genes can be transcribed in minimal *in vitro* transcription systems in which the CTD is not phosphorylated (Parvin and Sharp, 1993; Serizawa et al., 1993; reviewed in Dahmus, 1995). Also, results from an *in vitro* study of the *Drosophila melanogaster* hsp70 gene suggests that, in this particular case, the phosphorylation of the CTD might occur shortly after elongation (O'Brien et al., 1994).

Certainly, at some promoters the CTD might be phosphorylated during transcription initiation. Even so, it would be difficult to determine whether this phosphorylation is essential for pol II to leave the promoter or whether it simply coincides with transcription initiation. Therefore, at this point in time, the requirement for phosphorylation of the CTD during transcription initiation *in vivo* remains to be proven.

How a Gene is Turned On

As mentioned above, any step in transcription initiation, from assembly of the general transcription machinery to promoter clearance, may be subject to regulation. Transcription of pol II-transcribed genes is typically directed by regulatory proteins that bind to specific sequences located upstream of the promoter region (reviewed in McKnight and Yamamoto, 1992). Transcriptional activation, at least at some promoters, is thought to involve

two steps (reviewed in Wolffe, 1994). In the first step, DNA-bound activator proteins (activators) displace repressive chromatin structures from promoters upon binding to their specific site, and thereby give transcription machinery components access to the promoter. For example, the promoter region of the *S. cerevisiae PHO5* gene is incorporated into four nucleosomes. These nucleosomes occlude the TATA box, as well as a DNA-binding site for the transcriptional activator PHO4 (Almer et al., 1986; Rudolph and Hinnen, 1987; Vogel et al., 1989). PHO4 binds to the accessible PHO4 site located between two of the nucleosomes, and in doing so, initiates a chain of events leading to the disruption of all four nucleosomes (Svaren et al., 1994; Venter et al., 1994). The promoter region is then accessible to other DNA-binding regulatory proteins as well as to general transcription machinery components.

Nucleosome disruption is generally insufficient to produce full levels of activation (reviewed in Wolffe, 1994). It is thought that in the second step of transcriptional activation, activators stimulate transcription by promoting the assembly of the general transcription machinery. Activators typically contain an activation domain that is separate from the DNA binding domain. It was once thought that activation domains were one of three types: acidic, glutamine-rich, or proline-rich. It is now evident, however, that activation domains can also be rich in serine, threonine, isoleucine, and basic amino acids (reviewed in Triezenberg, 1995). Furthermore, it may be the pattern of distribution of bulky hydrophobic amino acids within the activation domain rather than the abundance of one particular amino acid that is most

important in conferring the ability to activate transcription (Triezenberg, 1995). These activation domains are thought to interact with transcription machinery components. Acidic activation domains have been shown to interact directly with TFIIB, TFIID, and TFIIH (Lin et al., 1991; Roberts et al., 1993; Goodrich et al., 1993; Xiao et al., 1994). Furthermore, acidic, proline-rich, or glutamine-rich activation domains can stimulate the recruitment of TFIIB to the TFIID-TATA complex (Choy and Green, 1993).

Transcriptional activators may also stimulate transcription initiation after the general transcription machinery has assembled. Although not rate-limiting *in vitro* (Choy and Green, 1993), the ATP hydrolysis required for isomerization of the closed complex to open complex could be stimulated by an activator. If, in some cases, phosphorylation of the CTD is required for pol II to leave the promoter, then this phosphorylation event could be stimulated by an activator.

How Are Genes Turned Off?

In contrast to transcriptional activation, relatively little is known about how specific DNA-binding proteins turn off transcription. Some DNA-binding repressor proteins (repressors) work simply by interference with an activator (reviewed in Goodburn, 1990; and in Clark and Docherty, 1993). A repressor might bind to a specific site that overlaps an activator binding site and thereby prevent the activator from binding. Some homeodomain regulatory proteins in *Drosophila* may work in this way, as some bind to sites that overlap with, or are identical to, activator sites (Hoey and Levine, 1988;

Desplan et al., 1988). Repressors can also interfere with activation by forming an inactive heterodimer with an activator. The mammalian proto-oncogene product MYC must heterodimerize with another protein, MAX, to bind efficiently to DNA and activate transcription (Littlewood et al., 1992; Amati et al., 1993). However, when the partner of MAX is the MAD or MXI1 protein, the heterodimer is inactive despite retaining DNA-binding ability (Ayer et al., 1993; Zervos et al., 1993). Direct interference with activators by specific DNA-binding repressors is unlikely to be a predominant mechanism of repression. Every specific DNA-binding activator would have to be matched with a specific DNA-binding repressor whose site was identical to or overlapped with the activator site. This requirement would greatly increase the number of required regulatory proteins.

Another way specific DNA-binding repressors could exert repression is by inhibiting a step during transcription initiation. Some might inhibit the assembly of the general transcription machinery. One method could be by steric occlusion, whereby a repressor binding site overlaps essential elements in the promoter region. There are no clear examples of this in eukaryotes; however, many examples can be found in prokaryotes. For instance, one of the lambda phage cI repressor sites overlaps the P_R promoter; the binding of a cI dimer to this site prevents the bacterial RNA polymerase from binding to the promoter (Hawley et al., 1985). Possible but unproven examples of steric occlusion in eukaryotes include negative regulation of the simian virus 40 (SV40) early promoter by SV40 T antigen and negative regulation by the

thyroid hormone receptor. The binding site of the SV40 T antigen (Rio et al., 1980) and, in some cases, the binding site of the thyroid hormone receptor (Krishna et al., 1989) overlap their target promoters. A second method of inhibiting the assembly of the general transcription machinery could be by directly interacting with transcription machinery components. Biochemical evidence suggests that the *Drosophila* homeodomain repressor protein EVE acts in this way although the target of EVE is unknown (Johnson and Krasnow, 1992). Furthermore, it has recently been shown that an interaction between the *Drosophila* repressor protein Krüppel and TFIIE leads to repression *in vitro* (Sauer et al., 1995). A third way that a repressor could inhibit the assembly of the transcription machinery is by inducing a repressive chromatin structure which would occlude the promoter from general transcription machinery components. There is evidence, to be discussed below, that this type of repression contributes to α2 repression (Shimizu et al., 1991; Roth et al. 1992; Cooper et al., 1994).

In addition, a repressor might act after the assembly of the general transcription machinery. No eukaryotic repressors are known with certainty to inhibit transcription initiation after this point. However, there are examples in prokaryotes. For example, the *E. coli lac* repressor LacR acts by enhancing a prexisting transcription pause site in the *lac* UV5 promoter such that RNA polymerase remains anchored at the promoter and cannot begin elongation (Lee and Goldfarb, 1991).

As a final note, repressors could work by opposing the action of an activator or could work irrespective of the activator. In the first case, for example, an activator could stimulate a kinase that then phosphorylates a transcription factor, while a repressor could stimulate a phosphatase that then dephosphorylates it. In theory, a repressor need not work by opposing the action of an activator since some repressors can turn off basal transcription, i. e., transcription in the absence of an activator (Herschbach et al. 1994).

A Model System for Studying Transcriptional Repression

The yeast *Saccharomyces cerevisae* is an ideal system for studying the mechanism(s) of transcriptional repression. First, many components of the general transcription machinery are conserved between this organism and higher eukaryotes (reviewed in Guarente and Bermingham-McDonogh, 1992). Thus, an understanding of transcriptional repression in yeast may ultimately lead to a better understanding of gene regulation in higher eukaryotes. Second, transcriptional repression can be readily analyzed on a genetic as well as biochemical level.

α2 repression of the a-specific genes is known to involve the concerted action of several proteins. α2 binds cooperatively with MCM1, a non-cell-type-specific protein, to an a-specific gene operator (asg) located upstream of each a-specific gene (Johnson and Herskowitz, 1985; Keleher et al., 1988). Operator-bound α2-MCM1 recruits a general repressor complex, comprised of SSN6 and TUP1 (Williams et al., 1991), to the promoter (Keleher et al., 1992;

Komachi et al., 1994; Tzamarias and Struhl, 1994). Once recruited to the promoter of the **a**-specific genes by operator-bound α2-MCM1, SSN6-TUP1 exerts transcriptional repression (Keleher et al., 1992; Tzamarias and Struhl, 1994). Unlike α2-MCM1, SSN6-TUP1 is required for repression of many different genes, including the DNA-damage inducible genes (reviewed in Elledge et al., 1993), the hypoxic genes (reviewed in Zitomer and Lowry, 1992), the haploid-specific genes in **a**/α cells (Mukai et al., 1991), and the glucose-repressible genes (Schultz and Carlson, 1987; reviewed in Trumbly, 1992). Because *ssn6* and *tup1* mutants are involved in the repression of many different genes, they have pleiotropic phenotypes including slow growth and clumpiness (Wickner, 1974; Schamhart et al., 1975; Lemontt et al., 1980; Rothstein and Sherman, 1980; and Carlson et al., 1984).

Another protein required for full levels of α2 repression is SIN4 (Chen et al., 1993a), a general transcriptional regulator that has either a positive or negative regulatory role, depending on the gene under its control (Jiang and Stillman, 1992; Jiang and Stillman, 1995). Like *ssn6* and *tup1* mutants, *sin4* mutants have pleiotropic phenotypes, including those mentioned above (Jiang and Stillman, 1992; Chen et al., 1993b; M. Wahi and A. D. Johnson, unpublished observations). It is not known whether the SIN4 protein interacts with any of the other known α2 repression components. Null *sin4* mutations only partially disrupt α2 repression (Chen et al., 1993a), whereas null *matα2*, *ssn6*, and *tup1* mutations eliminate it (Johnson and Herskowitz,

1985, Keleher et al., 1992). It is possible that the effect of *sin4* mutations on α2 repression is indirect; that is, *sin4* mutations may cause a decrease in *MATα2* expression and hence a decrease in levels of α2 protein (Jiang and Stillman, 1995; M. Wahi and A. D. Johnson, unpublished observations). For these reasons, most studies on α2 repression have focused on the role of SSN6-TUP1 rather than SIN4.

Current Understanding of the Mechanism of α2 Repression:

There are two leading models for the mechanism of α2 repression: the "nucleosome positioning" model and the "general transcription machinery" model. In the former, SSN6-TUP1 exerts α2 repression by positioning a nucleosome at the promoter of the a-specific genes. This nucleosome occludes the TATA box such that components of the general transcription machinery cannot assemble at the promoter. In the second model, SSN6-TUP1 exerts repression by directly interacting with component(s) of the general transcription machinery. This interaction interferes with the assembly of the general transcription machinery or with a later step during transcription initiation. In theory, these models are not mutually exclusive; both mechanisms could contribute to full levels of α2 repression.

These models have been widely discussed in the literature. Despite this discussion, however, there is no conclusive evidence to support either

model. First, the nucleosome positioning model will be considered. Evidence derived from micrococcal nuclease and DNAse I footprinting studies suggests that operator-bound α2-MCM1 stably positions a nucleosome over the TATA box of **a**-specific gene promoters in a TUP1- and SSN6-dependent manner (Shimizu et al., 1991, Roth et al., 1992; Cooper et al., 1994). The relevance of this positioned nucleosome to α2 repression is unclear. In one experiment, it was found that N-terminal deletions in histone H4 partially destabilize the positioning of this nucleosome and lead to partial loss of α2 repression (Roth et al., 1992). However, the validity of the repression data is unclear because repression was quantitated using nonisogenic strains. In another experiment, it was tested whether α2-MCM1 would be able to repress transcription of an **a**-specific gene if the TATA box of the **a**-specific gene promoter was not incorporated into a nucleosome (Patterton and Simpson, 1994). According to the nucleosome positioning model, α2-MCM1 would no longer be able to repress transcription of the gene. In the experiment, linkers of a 25 bp random DNA sequence were inserted between the α2-MCM1 operator site and the TATA box of an **a**-specific gene promoter fused to *lacZ*. This additional sequence did not interfere with the positioning of the nucleosome adjacent to the α2-MCM1 operator site, but had the desired effect of shifting the location of the TATA element to an internucleosomal region. In contradiction to the simple expectations predicted by the nucleosome positioning model, α2-MCM1 could still repress the test gene

even though the TATA box was not occluded by a nucleosome. Despite this contradiction, the result is inconclusive. On the one hand, the result could indicate that nucleosome positioning is not required for α2 repression, and that instead, α2 repression works by some other mechanism–perhaps by directly interacting with the general transcription machinery. On the other hand, it is possible that even though the TATA box is exposed, the two nucleosomes on either side of the TATA sterically hinder the assembly of the general transcription machinery.

Even if nucleosome positioning plays a role in α2 repression, its relative contribution to repression is unclear. In the absence of positioned nucleosomes, α2-MCM1 can nearly fully repress a test gene containing an α2-MCM1 site in the upstream control region (M. Redd, M. Stark, and A. D. Johnson, unpublished results). An unlikely possibility is that at some a-specific gene promoters α2 repression works by nucleosome positioning and that at other promoters by a different mechanism. Or, on the other hand, perhaps nucleosome positioning is irrelevant to α2 repression at any of the a-specific gene promoters.

Evidence used to support the general transcription machinery model is also inconclusive. One result used to support this model is that α2-MCM1 can repress RNA polymerase I (pol I) and pol II transcription units (Hershbach and Johnson, 1993), but not those of RNA polymerase III (pol III) (Morse et al., 1992; Herschbach and Johnson, 1993). Herschbach and Johnson

(1993) argue that the fact that α2-MCM1 can repress both pol I- and pol II-transcribed genes indicates that α2-MCM1 does not work by interfering with an upstream activator. Activation of pol I- and pol II-transcribed genes is thought to occur by different mechanisms (Schreck et al., 1989). They go on to argue that the ability of α2-MCM1 to repress both pol I- and pol II-transcribed genes, but not those of pol III, suggests that the target of α2 repression is a factor common to both the pol I and pol II general transcription machineries but not common to that of pol III. This is an appealing interpretation of the results; however, it is just as likely that the differential repression by α2-MCM1 could be solely due to nucleosome positioning. As Morse et al. (1992) point out, the reason α2-MCM1 cannot repress pol III-transcribed genes could simply be that these genes, which have highly active promoters, are more resistant to nucleosome positioning than are pol I- or pol II-transcribed genes.

Other evidence used to support the general transcription machinery model is the observation that α2-MCM1 can repress a test gene five-fold, in an SSN6- and TUP1-dependent manner, in an *in vitro* transcription system (Herschbach et al. 1994; M. Arnaud and A. D. Johnson, unpublished results). In this system, repression by interference with an activator seems safely ruled out since the transcription reactions do not include exogenously added activators and the DNA template does not contain a binding site for any known activator. In addition, there is no apparent chromatin assembly step, suggesting that α2-MCM1 can repress transcription *in vitro* in the absence of

nucleosomes. Hershbach et al. (1994) thus propose that the repression complex (α2-MCM1-SSN6-TUP1) works in this transcription system by inhibiting the assembly or activities of the general transcription machinery.

Nevertheless, repression by nucleosome positioning in this *in vitro* transcription system cannot be ruled out. If SSN6-TUP1 actively promotes the assembly of a nucleosome, then a chromatin assembly step during the *in vitro* transcription reaction may be unnecessary. (Histones are likely present in this whole-cell extract transcription system). It is conceivable that if exogenous histones were added to those already present and if more time were provided for SSN6-TUP1 to organize a nucleosome, repression would be closer to the 100-fold repression seen *in vivo*. Thus, although this system will be useful for further testing these models, currently, the fact that α2 repression works in this *in vitro* transcription system does not support the general transcription machinery model over the nucleosome positioning model.

New Repression Components Suggest a Mechanism for α2 Repression

More experiments must be done in order to understand the mechanism(s) of α2 repression, and perhaps more definitive experiments could be designed if other components involved in α2 repression were known. I carried out a genetic screen to identify new components involved in α2 repression with the hope that they would point towards a mechanism. I identified mutations in genes required for full levels of α2 repression (Wahi and Johnson, 1995).

Multiple alleles of all the expected genes previously known to be involved in α2 repression were identified. In addition, I isolated mutants defective in four other genes, which I designated *ARE* for alpha2 repression. The *are* mutations cause pleiotropic phenotypes, such as slow growth, clumpiness, and sporulation deficiency, similar to those resulting from *ssn6*, *tup1*, and *sin4* mutations. I present evidence that the *ARE* gene products are general negative regulators that can be divided into two distinct classes.

I decided to pursue *ARE1* and *ARE2*, which fall into one of these two classes. *ARE1* encodes a serine/threonine protein kinase identical to SRB10 (Wahi and Johnson, 1995; Liao et al., 1995). *ARE2* is identical to *SRB8* and interacts genetically with *TUP1* (Wahi and Johnson, submitted). The SRB proteins are physically associated with pol II and several general transcription factors in a complex called the pol II holoenzyme (Thompson et al., 1993; Koleske and Young, 1994; Kim et al., 1994; Liao et al., 1995; Hengartner et al. 1995). My results provide genetic evidence that SSN6-TUP1 brings about α2 repression by interacting with the general transcription machinery, and suggest a specific model that can be tested biochemically.

References

Adams, M. et al., 1995 Initial assessment of human gene diversity and expression patterns based upon 83 million nucleotides of cDNA sequence in *Genome Directory,* Nature **377** Suppl., 3-174.

Almer, A., H. Rudolph, A. Hinnen and W. Hörz, 1986 Removal of positioned nucleosomes from the yeast *PHO5* promoter upon *PHO5* induction releases additional upstream activating DNA elements. EMBO J. **5:** 2689-2696.

Amati, B., M. W. Brooks, N. Levy, T. D. Littlewood, G. I. Evan and H. Land, 1993 Oncogenic activity of the c-MYC protein requires dimerization with MAX. Cell **72:** 233-245.

Ayer, D. E., L. Kretzner and R. N. Eisenman, 1993 MAD: A heterodimeric partner for MAX that antagonizes MYC transcriptional activity. Cell **72:** 211-222.

Bartholomew, B., M. E. Dahmus, and C. F. Meares, 1986 RNA contacts subunits IIo and IIc in HeLa RNA polymerase II transcription complexes. J. Biol. Chem. **261:** 14226-14231.

Cadena, D. L., and M. E. Dahmus, 1987 Messenger RNA synthesis in mammalian cells is catalyzed by the phosphorylated form of RNA polymerase II. J. Biol. Chem. **262:** 12468-12474.

Carey, M., 1994 Simplifying the complex. Nature **368:** 402-403.

Carlson, M., B. C. Osmond, L. Neigeborn and D. Botstein, 1984 A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107:** 19-32.

Chen, S., R. W. West, Jr., S. L. Johnson, H. Gans, B. Kruger *et al.*, 1993a TSF3, a global regulatory protein that silences transcription of yeast *GAL* genes, also mediates repression by α2 repressor and is identical to SIN4. Mol. Cell. Biol. **13:** 831-840.

Chen, S., R. W. West, Jr., J. Ma, S. L. Johnson, H. Gans *et al.*, 1993b *TSF1* to *TSF6*, required for silencing the *Saccharomyces cerevisiae GAL* genes, are global regulatory genes. Genetics **134:** 701-716.

Chesnut, J. D., J. H. Stephens, and M. E. Dahmus, 1992 The interaction of RNA polymerase II with the adenovirus-2 major late promoter is precluded by phosphorylation of the C-terminal domain of subunit IIa. J. Biol. Chem. **267:** 10500-10506.

Clark, A. R., and K. Docherty, 1993 Negative regulation of transcription in eukaryotes. Biochemical Journal **296:** 521-541.

Cooper, J. P., S. Y. Roth and R. T. Simpson, 1994 The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure. Genes Dev. **8:** 1400-1410.

Choy, B. and M. R. Green, 1993 Eukaryotic activators function during multiple steps of preinitiation complex assembly. Nature **366:** 531-536.

Desplan, C., Theis, J. and P. H. O'Farrell, 1988 The sequence specificity of homeodomain-DNA interaction. Cell **54:** 1081-1090.

Dahmus, M. E., 1995 Phosphorylation of the C-terminal domain of RNA polymerase II. Biochim. Biophys. Acta **1261:** 171-182.

Elledge, S. J., Z. Zhou, J. B. Allen and T. A. Navas, 1993 DNA damage and cell cycle regulation of ribonucleotide reductase. BioEssays **15:** 333-339.

Goodburn, S., 1990 Negative regulation of transcriptional initiation in eukaryotes. Biochem. Biophys. Acta **1032:** 53-77.

Goodrich, J. A., T. Hoey, C. J. Thut, A. Admon and R. Tjian, 1993 *Drosophila* $TAF_{II}40$ interacts with both a VP16 activation domain and the basal transcription factor TFIIB. Cell **75:** 519-530.

Guarente, L., and O. Bermingham-McDonogh, 1992 Conservation and evolution of transcriptional mechanisms in eukaryotes. Trends Gen. **8:** 27-32.

Hawley, D. K., A. D. Johnson and W. R. McClure, 1985 Functional and physical characterization of transcription initiation complexes in the bacteriophage lambda O_R region. J. Biol. Chem. **260:** 8618-8626.

Hengartner, C. J., C. M. Thompson, J. Zhang, D. M. Chao, S.-M. Liao, A. J. Koleske, S. Okamura and R. A. Young, 1995 Association of an activator with an RNA polymerase II holoenzyme. Genes Dev. **9:** 897-910.

Herschbach, B., and A. D. Johnson, 1993 The yeast α2 protein can repress transcription by RNA polymerase I and II but not III. Mol. Cell. Biol. **13:** 4015-4025.

Herschbach, B. M., M. B. Arnaud and A. D. Johnson, 1994 Transcriptional repression directed by the yeast α2 protein *in vitro*. Nature **370:** 309-311.

Herskowitz, I., J. Rine and J. Strathern, 1992 Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*, pp. 583-656 in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*, Vol. II, edited by E. W. Jones, J. R. Pringle and J. R. Broach. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Hoey, T., and M. Levine, 1988 Divergent homeo box proteins recognize similar DNA sequences in *Drosophila*. Nature **332:** 858-861.

Jiang, Y. W., and D. J. Stillman, 1992 Involvement of the SIN4 global transcriptional regulator in the chromatin structure of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 4503-4514.

Jiang, Y. W., and D. J. Stillman, 1995 Regulation of *HIS4* expression by the *Saccharomyces cerevisiae* SIN4 transcriptional regulator. Genetics **140:** 103-114.

Johnson, A. D., and I. Herskowitz, 1985 A repressor (*MAT*α2 product) and its operator control expression of a set of cell type specific genes in yeast. Cell **42:** 237-247.

Johnson, F. B. and M. A. Krasnow, 1992 Differential regulation of transcription preinitiation complex assembly by activator and repressor homeo domain proteins. Genes Dev. **6:** 2177-2189.

Kang, M. E., and M. E. Dahmus, 1993 RNA polymerases IIA and IIO have distinct roles during transcription from the TATA-less murine dihydrofolate reductase promoter. J. Biol. Chem. **268:** 25033-25040.

Keleher, C. A., C. Goutte and A. D. Johnson, 1988 The yeast cell-type-specific repressor α2 acts cooperatively with a non-cell-type-specific protein. Cell **53:** 927-936.

Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson and A. D. Johnson, 1992 SSN6-TUP1 is a general repressor of transcription in yeast. Cell **68:** 927-937.

Kim, Y.-J., S. Björklund, Y. Li, M. H. Sayre and R. D. Kornberg, 1994 A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77:** 599-608.

Koleske, A. J., and R. A. Young, 1994 An RNA polymerase II holoenzyme responsive to activators. Nature **368:** 466-469.

Komachi, K., M. J. Redd and A. D. Johnson, 1994 The WD repeats of TUP1 interact with the homeodomain protein α2. Genes Dev. **8:** 2857-2867.

Krishna, V., K. Chatterjee, J.-K. Lee, A. Rentoumis and J. L. Jameson, 1989 Negative regulation of the thyroid-stimulating hormone α gene by thyroid hormone: receptor interaction adjacent to the TATA box. Proc. Natl. Acad. Sci. USA **86:** 9114-9118.

Laybourn, P. J., and M. E. Dahmus, 1989 Transcription-dependent structural changes in the C-terminal domain of mammalian RNA polymerase subunit IIa/o J. Biol. Chem. **264:** 6693-6698.

Laybourn, P. J., and M. E. Dahmus, 1990 Phosphorylation of RNA polymerase II occurs subsequent to interaction with the promoter and before initiation of transcription. J. Biol. Chem. **265:** 13165-13173.

Lee, J., and A. Goldfarb, 1991 *lac* repressor acts by modifying the inital transcribing complex so that it cannot leave the promoter. Cell **66:** 793-798.

Lemontt, J. F., D. R. Fugit and V. L. MacKay, 1980 Pleiotropic mutations at the *TUP1* locus that affect the expression of mating-type-dependent functions in *Saccharomyces cerevisiae*. Genetics **94:** 899-920.

Liao, S.-M., J. Zhang, D. A. Jeffery, A. J. Koleske, C. M. Thompson *et al.*, 1995 A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature **374:** 193-196.

Lin, Y.-S., I. Ha, E. Maldonado, D. Reinberg and M. R. Green, 1991 Binding of general transcription factor TFIIB to an acidic activating region. Nature **353:** 569-571.

Littlewood, T. D., B. Amati, H. Land, and G. I. Evan, 1992 MAX and c-MYC/MAX DNA-binding activities in cell extracts. Oncogene **7:** 1783-1792.

Lu, H., O. Flores, R. Weinman, and D. Reinberg, 1991 The nonphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. Proc. Natl. Acad. Sci. USA **88:** 10004-10008.

McClure, W. R., 1985 Mechanism and control of transcription initation in prokaryotes. Ann. Rev. Biochem. **54:** 171-204.

McKnight, S. L., and K. R. Yamamoto, 1992 *Transcriptional Regulation*, Vol. I & Vol. II (Cold Spring Harbor Laboratory Press, Plainview, NY).

Morse, R. H., S. Y. Roth and R. T. Simpson, 1992 A transcriptionally active tRNA gene interferes with nucleosome positioning *in vivo*. Mol. Cell. Biol. **12:** 4015-4025.

Mukai, Y., S. Harashima and Y. Oshima, 1991 AAR1/TUP1 protein, with a structure similar to that of the β subunit of G proteins, is required for a1-α2 and α2 repression in cell type control of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:** 3773-3779.

Nonet, M., D. Sweetser, and R. A. Young, 1987 Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. Cell **50:** 909-915.

O'Brien, T., S. Hardin, A. Greenleaf, and J. T. Lis, 1994 Phosphorylation of RNA polymerase II C-terminal domain and transcriptional elongation. Nature **370:** 75-77.

Parvin, J. D., and P. A. Sharp, 1993 DNA topology and a minimal set of basal factors for transcription by RNA polymerase II. Cell **73:** 533-540.

Patterton, H.-G., and R. T. Simpson, 1994 Nucleosomal location of the *STE6* TATA box and Matα2p-mediated repression. Mol. Cell. Biol. **14:** 4002-4010.

Rio, D., A. Robbins, R. Myers and R. Tjian, 1980 Regulation of simian virus 40 early transcription *in vitro* by a purified tumor antigen. Proc. Natl. Acad. Sci. USA **77:** 5706-5710.

Roberts, S. G. E., I. Ha, E. Maldonado, D. Reinberg and M. R. Green, 1993 Interaction between an acidic activator and transcription factor TFIIB is required for transcriptional activation. Nature **363:** 741-744.

Roth, S. Y., M. Shimizu, L. Johnson, M. Grunstein and R. T. Simpson, 1992 Stable nucleosome positioning and complete repression by the yeast α2

repressor are disrupted by amino-terminal mutations in histone H4. Genes Dev. **6:** 411-425.

Rothstein, R. J., and F. Sherman, 1980 Genes affecting the expression of cytochrome c in yeast: genetic mapping and genetic interactions. Genetics **94:** 871-889.

Rudolph, H., and A. Hinnen, 1987 The yeast *PHO5* promoter: phosphate-control elements and sequences mediating mRNA start-site selection. Proc. Natl. Acad. Sci. USA **84:** 1340-1344.

Sauer, F., J. D. Fondell, Y. Ohkuma, R. G. Roeder, and H. Jackle, 1995 Control of transcription by Krüppel through interactions with TFIIB and TFIIE beta. Nature **375:** 162-164.

Schamhart, D. H. J., A. M. A. Ten Berge and K. W. Van De Poll, 1975 Isolation of a catabolite repression mutant of yeast as a revertant of a strain that is maltose negative in respiratory deficient state. J. Bacteriol. **121:** 747-752.

Schreck, R., M. F. Carey, and I. Grummt, 1989 Transcriptional enhancement by upstream activators is brought about by different molecular mechanisms for class I and II RNA polymerase genes. EMBO J. **8:** 3011-3017.

Schultz, J., L. Marshall-Carlson and M. Carlson, 1990 The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 4744-4756.

Schultz, J., and M. Carlson, 1987 Molecular analysis of *SSN6*, a gene functionally related to the SNF1 protein kinase of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:** 3637-3645.

Serizawa, H., J. W. Conaway, and R. C. Conaway, 1993 Phosphorylation of C-terminal domain of RNA polymerase II is not required in basal transcription. Nature **363:** 371-374.

Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson, 1991 Nucleosomes are positioned with base pair precision adjacent to the α2 operator in *Saccharomyces cerevisiae*. EMBO J. **10:** 3033-3041.

Svaren, J., J. Schmitz, and W. Hörz, 1994 The transactivation domain of PHO4 is required for nucleosome disruption at the *PHO5* promoter. EMBO J. **13:** 4856-4862.

Thompson, C. M., A. J. Koleske, D. M. Chao, and R. A. Young, 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73:** 1361-1375.

Triezenberg, S. J., 1995 Structure and function of transcriptional activation domains. Cur. Opin. Gen. Dev. **5:** 190-196.

Trumbly, R. J., 1992 Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **6:** 15-21.

Tzamarias, D., and K. Struhl, 1994 Functional dissection of the yeast CYC8-TUP1 transcriptional co-repressor complex. Nature **369:** 758-761.

Venter, U., J. Svaren, J. Schmitz, A. Schmid, and W. Hörz, 1994 A nucleosome precludes binding of the transcription factor PHO4 *in vivo* to a critical target site in the *PHO5* promoter. EMBO J. **13:** 4848-4855.

Vogel, K., W. Hörz, and A. Hinnen, 1989 The two positively acting regulatory proteins PHO2 and PHO4 physically interact with *PHO5* upstream activation regions. Mol. Cell. Biol. **9:** 2050-2057.

von Hippel, P. H., D. G. Bear, W. D. Morgan, and J. A. McSwiggen, 1984 Protein-nucleic acid interactions in transcription: a molecular analysis. Annu. Rev. Biochem. **53:** 389-446.

Wahi, M., and A. D. Johnson, 1995 Identification of genes required for α2 repression in *Saccharomyces cerevisiae*. Genetics **140:** 79-90.

Wang, W., M. Carey, and J. D. Gralla, 1992 Polymerase II promoter activation: closed complex formation and ATP-driven start site opening. Science **255:** 450-453.

Wickner, R. B., 1974 Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine-5'-monophosphate in deoxyribonucleic acid *in vivo*. J. Bacteriol. **117:** 252-260.

Williams, R. E., U. Varanasi and R. J. Trumbly, 1991 The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. Mol. Cell. Biol. **11:** 3307-3316.

Wolffe, A., 1994 Transcription: in tune with the histones. Cell **77:** 13-16.

Xiao, H., A. Pearson, B. Coulombe, R. Truant, S. Zhang et al., 1994 Binding of basal factor TFIIH to the acidic activation domains of VP16 and p53. Mol. Cell. Biol. **14:** 7013-7024.

Zervos, A. S., J. Gyuris, and R. Brent, 1993 MXI1, a protein that specifically interacts with MAX to bind MYC-MAX recognition sites. Cell **72:** 223-232.

Zitomer, R. S., and C. V. Lowry, 1992 Regulation of gene expression by oxygen in *Saccharomyces cerevisiae.* Microbiol. Reviews **56:** 1-11.

Chapter 2

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

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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.

INTRODUCTION

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 (CARLSON et al. 1984; LEMONTT et al. 1980; ROTHSTEIN and SHERMAN 1980; WICKNER 1974). 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 (ROTHSTEIN and SHERMAN 1980; SCHAMHART et al. 1975; SCHULTZ et al. 1990; TRUMBLY 1986).

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 α2 repression. We have recently learned that *ARE1* is identical to a component of the RNA polymerase II holoenzyme (see DISCUSSION), suggesting that α2 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°C and in either rich YEPD medium or in synthetic SD drop-out medium. Clumpy cells were dispersed by adding EDTA to 25mM prior to 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 *E. 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 *are MFA2* were recovered from crosses between *are MFA2::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 *MATa* 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 γδ 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 (utilizing 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*II, 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*II 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 one to two 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 minutes) 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 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.

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 seconds 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 one hour 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 the standard deviation 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 50mM 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 mg 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 second exposure to UV irradiation in a Stratagene UV Stratalinker. Probes were radioactively labelled with ^{32}P by random priming using an Amersham Megaprime DNA labelling 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 UCSF, 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°C 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°C 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°C for two hours 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 polymerase chain reaction (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 *MATa* 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 which 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 *MATa* 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 which consists of 63.5 µl H_2O, 10 µl 10X PCR buffer (100mM Tris HCl pH 8.3, 500mM KCl, 15mM $MgCl_2$), 16 µl 1.25mM each dNTP, 5 µl 2 µM primer 1, and 5 µl 2µM primer 2. Samples are boiled 5 min and then spun down briefly. 0.5 µl Taq polymerase (2.5 U) is added and reactions are overlayed with oil. Amplification is for 30 cycles of 94°C 1 min/ 42°C 2 min/ 65°C 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°C for five 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 400X 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 which 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 approximately 8,800 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 *MATa* strain (for testing linkage to *MATα*) or a *tup1*, *ssn6*, or *sin4* deletion mutant was used (see MATERIALS AND METHODS). Following 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, 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: 1) all of the mutants could mate despite being defective for α2 repression, and 2) 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 to 15 asci tested per cross) following 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 *MATa* 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 following 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). (For analysis of the *are1 are2* double mutant, see Ch. 3.)

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; that is, 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 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 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 about 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 (3-fold), 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, that is, 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 (2- to 8-fold and 3- to 6-fold, respectively), resembling the effect of the *tup1* mutation (1- to 6-fold). 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°C, but none are temperature-sensitive for growth at 37°C. 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 seen in both **a** and α haploid cells (**a**/α diploids were not examined) 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 for complementation of the *MFA2::lacZ* derepression. One plasmid, pMW15, was isolated which 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 which disrupted the complementation activity mapped to a region of approximately 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 contains 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) following 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; that is, 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 7-fold decrease in repression caused by the *are1* disruption is not significantly different from that exhibited by the *are1-5* or *are1-41* mutants (7- to 9-fold).

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* (SERGEI KUCHIN and MARIAN 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 et al. 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, that is, transcription in the absence of known UAS elements (Figure 3). SIN4, ARE3, and ARE4 belong to the class which 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 together as transcriptional repressors (KELEHER et al. 1992; reviewed in JOHNSTON and CARLSON 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 (CARLSON et al 1984; LEMONTT et al. 1980; ROTHSTEIN and SHERMAN 1980; WICKNER 1974). 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 (KIM et al. 1994; KOLESKE and YOUNG 1994; THOMPSON et al. 1993), 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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LITERATURE CITED

BURKHOLDER, A. C., and L. H. HARTWELL, 1985 The yeast α-factor receptor: structural properties deduced from the sequence of the *STE2* gene. Nucleic Acids Research **13:** 8463-8475.

CARLSON, M., and D. BOTSTEIN, 1982 Two differentially regulated mRNAs with different 5' ends encode secreted and intracellular forms of yeast invertase. Cell **28:** 145-154.

CARLSON, M., B. C. OSMOND, L. NEIGEBORN and D. BOTSTEIN, 1984 A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107:** 19-32.

CHEN, S., R. W. WEST, JR., S. L. JOHNSON, H. GANS, B. KRUGER and J. MA, 1993a *TSF3*, a global regulatory protein that silences transcription of yeast *GAL* genes, also mediates repression by α2 repressor and is identical to *SIN4*. Mol. Cell. Biol. **13:** 831-840.

CHEN, S., R. W. WEST, JR., J. MA, S. L. JOHNSON, H. GANS and G. WOLDEHAWARIAT, 1993b *TSF1* to *TSF6*, required for silencing the *Saccharomyces cerevisiae GAL* genes, are global regulatory genes. Genetics **134:** 701-716.

CHURCH, G. M., and W. GILBERT, 1984 Genomic sequencing. Proc. Natl. Acad. Sci. USA **81:** 1991-1995.

COOPER, J. P., S. Y. ROTH and R. T. SIMPSON, 1994 The global transcriptional regulators, SSN6 and TUP1, play distinct roles in the establishment of a repressive chromatin structure. Genes Dev. **8:** 1400-1410.

ELBLE, R., and B. TYE, 1991 Both activation and repression of a-mating-type-specific genes in yeast require transcription factor MCM1. Proc. Natl. Acad. Sci. USA **88:** 10966-10970.

GUARENTE, L., and E. HOAR, 1984 Upstream activation sites of the *CYC1* gene of *Saccharomyces cerevisiae* are active when inverted but not when placed downstream of the TATA box. Proc. Natl. Acad. Sci. USA **81:** 7860-7864.

GUYER, M., 1978 The γδ sequence of F is an insertion sequence. J. Mol. Biol. 126: 347-365.

HALL, M. N., and A. D. JOHNSON, 1987 Homeo Domain of the yeast repressor α2 is a sequence-specific DNA-binding domain but is not sufficient for repression. Science **237:** 1007-1012.

HERSKOWITZ, I., J. RINE and J. STRATHERN, 1992 Mating-type determination and mating-type interconversion in *Saccharomyces cerevisiae*, pp. 583-656 in *Volume II. The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*. edited by E. W. Jones, J. R. Pringle and J. R. Broach. Cold Spring Harbor Laboratory Press, New York.

HOEKSTRA, M. F., A. J. DEMAGGIO and N. DHILLON, 1991 Genetically identified protein kinases in yeast. Trends in Genetics **7:** 293-297.

HUXLEY, C., E. D. GREEN and I. DUNHAM, 1990 Rapid assessment of *Saccharomyces cerevisiae* mating type by PCR. Trends in Genetics **6:** 236.

ITO, H., K. FUKUDA, K. MURATA and A. KIMURA, 1983 Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153:** 163-168.

JIANG, Y. W., and D. J. STILLMAN, 1992 Involvement of the SIN4 global transcriptional regulator in the chromatin structure of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 4503-4514.

JOHNSON, A. D., and I. HERSKOWITZ, 1985 A repressor (*MATα2* product) and its operator control expression of a set of cell type specific genes in yeast. Cell **42:** 237-247.

JOHNSTON, M., and M. CARLSON, 1992 Regulation of carbon and phosphate utilization, pp. 193-281 in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression.* edited by Jones, E. W., J. R. Pringle, and J. R. Broach. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

KELEHER, C. A., M. J. REDD, J. SCHULTZ, M. CARLSON and A. D. JOHNSON, 1992 SSN6-TUP1 is a general repressor of transcription in yeast. Cell **68:** 927-937.

KELEHER, C. A., C. GOUTTE and A. D. JOHNSON, 1988 The yeast cell-type-specific repressor α2 acts cooperatively with a non-cell-type-specific protein. Cell **53:** 927-936.

KIM, J., S. BJORKLUND, Y. LI, M. H. SAYRE and R. D. KORNBERG, 1994 A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77:** 599-608.

KOLESKE, A. J., and R. A. YOUNG, 1994 An RNA polymerase II holoenzyme responsive to activators. Nature 368: 466-469.

KOMACHI, K., M. J. REDD and A. D. JOHNSON, 1994 The WD repeats of TUP1 interact with the homeodomain protein α2. Genes Dev. **8**: 2857-2867.

LAWRENCE, C. W., 1991 Classical mutagenesis techniques pp. 273-281 in *Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G.

R. FINK. Academic Press, San Diego.

LEMONTT, J. F., D. R. FUGIT and V. L. MACKAY, 1980 Pleiotropic mutations at the *TUP1* locus that affect the expression of mating-type-dependent functions in *Saccharomyces cerevisiae*. Genetics **94:** 899-920.

MAKAY, V. L., S. K. WELCH, M. Y. INSLEY, T. R. MANNEY, J. HOLLY, G. C. SAARI and M. L. PARKER, 1988 The *Saccharomyces cerevisiae BAR1* gene encodes an exported protein with homology to pepsin. Proc. Natl. Acad. Sci. **85:** 55-59.

MANIATIS, T., E. F. FRITSCH and J. SAMBROOK, 1982 *Molecular Cloning: a Laboratory Manual*.. Cold Spring Harbor Laboratory, N.Y.

MILLER, J. H., 1972 *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

MORTIMER, R. K., and D. C. HAWTHORNE, 1969 Yeast genetics, pp. 385-460 in *The Yeasts*, edited by A. H. ROSE and J. S. HARRISON. Academic Press, New York.

NASMYTH, K., 1983 Molecular analysis of a cell lineage. Nature **302:** 670-676.

NONET, M. L., and R. A. YOUNG, 1989 Intragenic and extragenic suppressors

of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. Genetics **123:** 715-724.

ROTHSTEIN, R. J., and F. SHERMAN, 1980 Genes affecting the expression of cytochrome *c* in yeast: genetic mapping and genetic interactions. Genetics **94:** 871-889.

SANCAR, A., and W. D. RUPP, 1979 Cloning of *uvrA*, *lexC*, and *ssb* genes of *Escherichia coli*. Biochemical and Biophysical Research Communications **90:** 123-129.

SCHAMHART, D. H. J., A. M. A. Ten Berge and K. W. Van De Poll, 1975. Isolation of a catabolite repression mutant of yeast as a revertant of a strain that is maltose negative in a respiratory deficient state. J. Bacteriol. **121:** 747-752.

SCHENA, M., L. P. FREEDMAN and K. R. YAMAMOTO, 1989 Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Genes Dev. **3:** 1590-1601.

SCHULTZ, J., and M. CARLSON, 1987 Molecular analysis of *SSN6*, a gene functionally related to the *SNF1* protein kinase of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:** 3637-3645.

SCHULTZ, J., L. MARSHALL-CARLSON and M. CARLSON, 1990 The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 4744-4756.

SHERMAN, F., G. R. FINK and C. W. LAWRENCE, 1979 *Methods in Yeast Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.

SHIMIZU, M., S. Y. ROTH, C. SZENT-GYORGYI and R. T. SIMPSON, 1991 Nucleosomes are positioned with base pair precision adjacent to the α2 operator in *Saccharomyces cerevisiae*. EMBO 10: 3033-3041.

SIKORSKI, R. S., and J. D. BOEKE, 1991 *In vitro* mutagenesis and plasmid shuffling: from cloned gene to mutant yeast pp. 302-318 in *Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G. R. FINK. Academic Press, San Diego.

SILICIANO, P. G., and K. TATCHELL, 1984 Transcription and regulatory signals at the mating type locus in yeast. Cell **37:** 969-978.

SPRAGUE, G. F., JR., 1991 Assay of yeast mating reaction pp. 77-93 in *Guide to Yeast Genetics and Molecular Biology*, edited by C. GUTHRIE and G. R. FINK. Academic Press, San Diego.

STRICH, R., M. R. SLATER and R. E. ESPOSITO, 1989 Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. Proc. Natl. Acad. Sci. USA **86:** 10018-10022.

SUROSKY, R. T., R. STRICH, and R. E. ESPOSITO, 1994 The yeast *UME5* gene regulates the stability of meiotic mRNAs in response to glucose. Mol. Cell. Biol. **14:** 3446-3458.

THOMPSON, C. M., A. J. KOLESKE, D. M. CHAO and R. A. YOUNG, 1993 A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73:** 1361-1375.

TRUMBLY, R. J., 1992 Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **6:** 15-21.

TRUMBLY, R. J., 1986 Isolation of *Saccharomyces cerevisae* mutants constitutive for invertase
synthesis. J. Bacteriol. **166:** 1123-1127.

VALLIER, L. G., and M. CARLSON, 1994 Synergistic release from glucose repression by *mig1* and *ssn* mutations in *Saccharomyces cerevisiae*. Genetics **137:** 49-54.

VINH, D. B. N. et al., 1993 Genetic evidence for functional interactions between actin noncomplementing (*ANC*) gene products and actin cytoskeletal proteins in *Saccharomyces cerevisiae*. Genetics **135:** 275-286.

WICKNER, R. B., 1974 Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine-5'-monophosphate in deoxyribonucleic acid *in vivo*. J. of Bacteriol. **117:** 252-260.

WILLIAMS, F. E., and R. J. TRUMBLY, 1990 Characterization of *TUP1*, a mediator of glucose repression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 6500-6511.

WILLIAMS, F. E., U. VARANASI and R. J. TRUMBLY, 1991 The CYC8 and TUP1 proteins involved in glucose represssion in *Saccharomyces cerevisiae* are associated in a protein complex. Mol. Cel. Biol. **11:** 3307-3316.

FIGURE LEGENDS

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 standard deviation was typically <20% of the mean. Transformants were grown in glucose medium under uracil selection.

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 standard deviation was typically <20% of the mean. Transformants were grown in glucose medium under uracil selection.

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, that is, 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).

TABLE 1

***S. cerevisiae* strains**

Strain	Genotype
Haploids	
SM1196	*MATα MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
SM1179	*MATa MFA2::lacZ can1gal2 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*

TABLE 1

Continued

Strain	Genotype
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*

TABLE 1

Continued

Strain	Genotype
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*

TABLE 2

Plasmids

Name	Description	Source
pVZ-1	Bluescript vector pBS+ from Stratagene 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 TRUMBLY 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
2μML (pAJ169)[a]	3.4-kb *Bam*HI-*Xho*I fragment containing *MCM1* in YEp13	EBLE and TYE 1991
2μMU (pAJ170)	3.4-kb *Bam*HI-*Xho*I fragment containing *MCM1* in YEp24	EBLE and TYE 1991

TABLE 2

Continued

Name	Description	Source
pLGΔ-312S (pAJ1)	*CYC1-lacZ* reporter, *URA3*, 2μ origin	GUARENTE and HOAR 1984
pAJ3	pAJ1 with an α2-MCM1 site inserted into the unique *SalI* 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

TABLE 2

Continued

Name	Description	Source
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	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.

TABLE 3

Isolation of genes required for α2 repression

Mutation	No. of 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).

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	+	+	+	+	+	+	−	+
are4-87	+	+	+	+	+	+	+	−

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

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. Following 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		
	MATa	*MATα*	Repression
wild-type	140	0.7	200
tup1Δ::LEU2	110	110	1.0 (200x)
sin4Δ::LEU2	90	17	5.3 (38x)
are1-5	200	13	15 (13x)
are1-41	220	8.1	27 (7.4x)
are2-13	100	12	8.3 (24x)
are2-30	80	3.8	21 (9.5x)
are2-40	120	7.1	17 (12x)
are2-59	110	8.5	13 (15x)
are3-57	150	11	14 (14x)
are4-87	180	9.0	20 (10x)

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 3 independent colonies on different days. The standard deviation 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.

TABLE 7

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

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

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 standard deviation is <15% of the mean. Numbers in parentheses indicate fold decrease in repression relative to the wild-type strains SM1179 (*MATa*) and SM1196 (*MATα*). All strains were grown in YEPD medium.

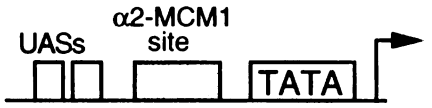
Genotype	β-Galactosidase Activity		Repression
	 pAJ1	 pAJ3	(pAJ1/pAJ3)
α *wild-type*	180	1.3	140
α *tup1Δ::LEU2*	180	120	1.5 (93x)
α *sin4Δ::LEU2*	600	37	16 (8.7x)
α *are1-41*	93	3.2	29 (4.8x)
α *are2-13*	200	29	6.9 (20x)
α *are3-57*	190	20	9.5 (15x)
α *are4-87*	470	15	31 (4.5x)

Figure 1

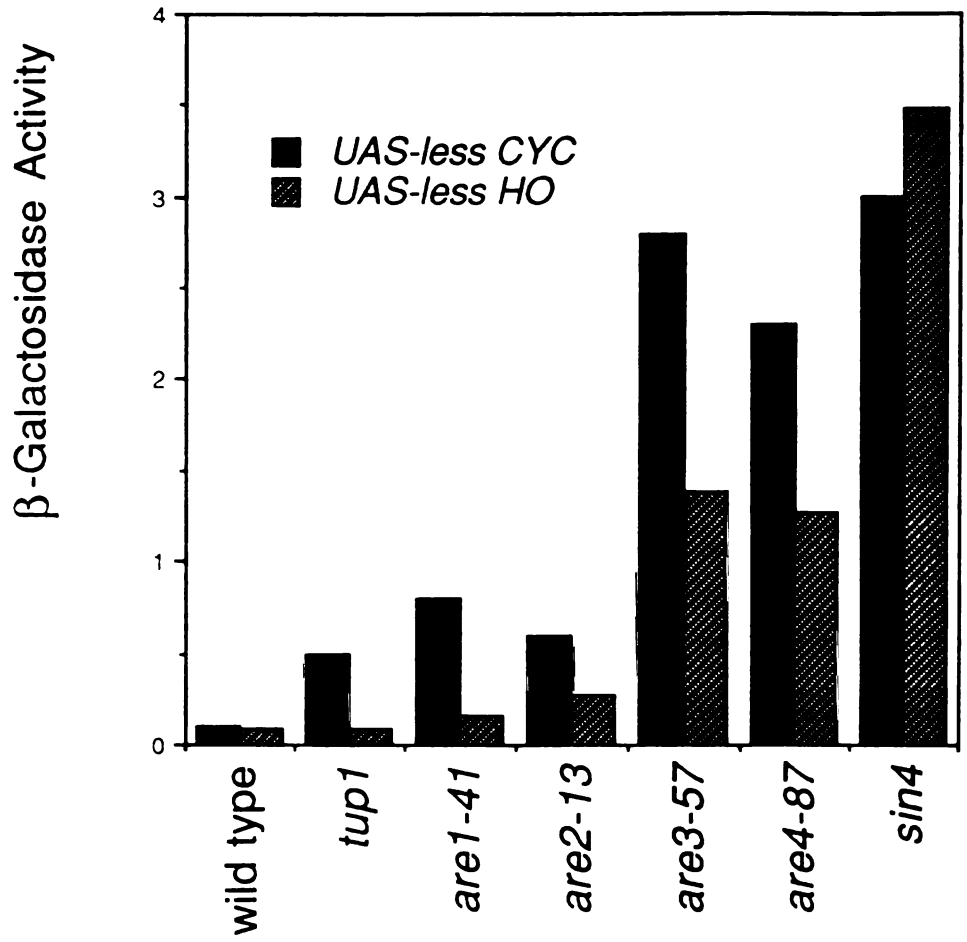

β-Galactosidase Activity
4
3
2
1
0
UAS-less CYC
UAS-less HO
wild type
tup1
are1-41
are2-13
are3-57
are4-87
sin4

Figure 2

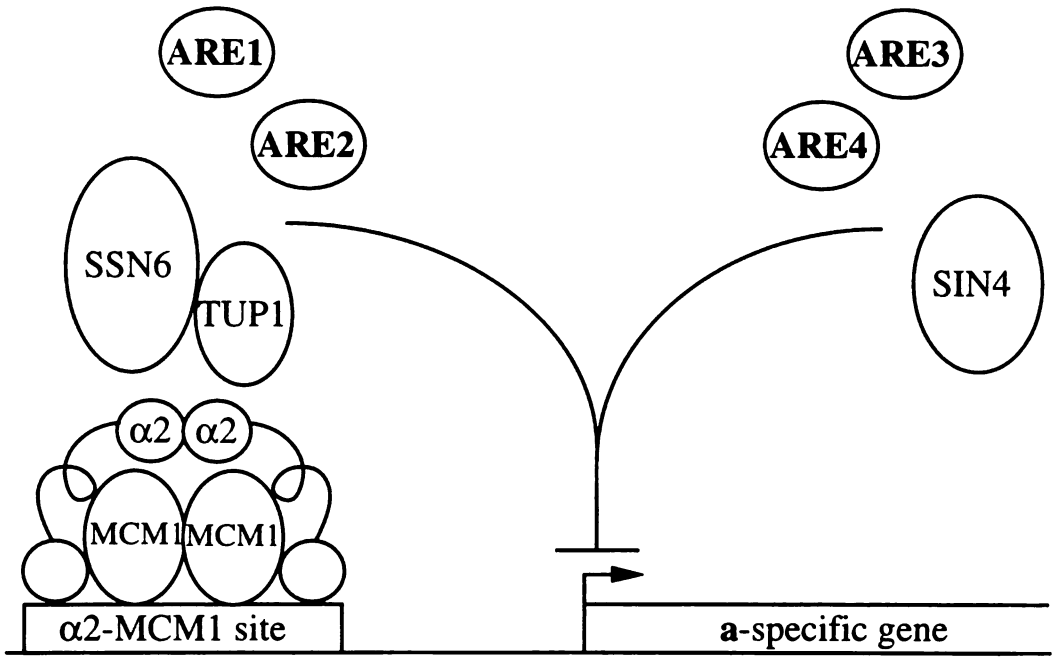

ARE1
ARE2
ARE3
ARE4
SSN6
TUP1
SIN4
α2
α2
MCM1
MCM1
α2-MCM1 site
a-specific gene

Figure 3

Supplement to Chapter 2

Other genes tested for identity to the *ARE* genes: I tested whether *ARE2*, *ARE3*, and *ARE4* might encode other known *S. cerevisiae* general negative regulators. I focused on *SIN1*, *SIN2*, *SIN3*, *SIN5*, the gene encoding histone H4 (*HHF*), and *RGR1* because they were identified in the same screen(s) as, or interact with, *SIN4*. The *SIN* (*SWI* -independent) genes were identified as suppressors of mutations in the *SWI1* and *SWI5* genes, which encode transcriptional activators of the *HO* gene (Stern et al., 1984; Sternberg et al., 1987). SIN1 may be a chromatin component (Kruger and Herskowitz, 1991; Lefebvre and Smith, 1993); SIN2 is identical to histone H3 (Herskowitz et al., 1992; Kruger et al., 1995). The *SIN3* gene was also identified in the screen for negative regulators of early meiosis-specific genes; i.e., in the same screen in which *UME5/ARE1/SSN3/SRB10* was isolated (Strich et al., 1989). *SIN5* has not been cloned. Mutations in two other genes not identified in the original *SIN* screen were later shown to suppress *swi* mutations: *HHF* (Herskowitz et al., 1992; Kruger et al., 1995), which encodes histone H4, and *RGR1* (Stillman et al., 1994). *RGR1* was originally identified in a screen for negative regulators of the *SUC2* gene (Sakai et al., 1988) and was subsequently shown to reside in the same genetic pathway as *SIN4* (Stillman et al., 1994). Furthermore, biochemical evidence suggests that SIN4 and RGR1 proteins physically interact (Jiang et al., 1995). In addition, *rgr1* mutants, like the *are* mutants, are clumpy (Sakai et al., 1990). Neither *sin1*, *sin2*, *sin3*, *sin5*, or *hhf* mutations have been reported to cause a clumpy phenotype; however, it is possible that this difference is due to differences in strain backgrounds.

I transformed α *MFA2::lacZ*-bearing *are2*, *are3*, and *are4* mutants (MWY16, MWY25, and MWY28) with plasmids containing these genes or with control plasmids (Table 1), and then tested transformants for complementation of the *MFA2::lacZ* derepression and clumpy phenotypes. No complementation was observed in any case. I gave the *are4* mutant to the D. Stillman laboratory to test whether the *ARE4* mutation is linked to *SIN5* (this linkage analysis is still in progress). The *sin5* allelism test was not performed with the *are2* and *are3* mutants; instead, both *ARE2* and *ARE3* were cloned (see Chapter 3 and Discussion).

Levels of *MATα2* and *TUP1* expression in *α are* mutants: As discussed in Chapter 2, the derepression phenotype of the *are* mutants could be due to lower expression levels of some of the known repression components. It is possible that the *ARE* gene products are transcriptional activators of genes encoding known α2 repression components. Consistent with this view, some of the *are* mutations are partially suppressed by overexpression of known components: both alleles of *are1* by *MATα*, one allele of *are2* by *TUP1*, and the one isolated allele of *are4* by *SIN4*.

In Chapter 2 (Genetics **140:** 79-90), I described the effects of *are* mutations on *MATα2* and *TUP1* RNA levels; however, the data were not shown. Here I show the Northern blots and data for the quantitation of RNA levels (see Materials and Methods in Chapter 2). The Northern blot in Figure 1A shows

that the level of *MATα2* expression in the α *are2*, *are3*, and *are4* mutants is not significantly lower than that in the isogenic *ARE* strain (90%, 93%, and 140% of wild-type levels in the α *are2*, *are3*, and *are4* mutants, respectively). In the α *are1* mutant, however, levels of *MATα2* expression are only 64% of that in the wild-type strain. Thus, it is possible that the repression defect in the *are1/srb10* mutant is due to lower levels of α2 protein resulting from the decreased expression of *MATα2*. The Northern blot in Figure 1B shows that the level of *TUP1* expression is not significantly lower in any of the α *are* mutants compared to that in the isogenic wild-type strain (99%, 93%, 112%, and 120% of wild-type levels in the α *are1*, *are2*, *are3*, and *are4* mutants, respectively).

Evidence suggests that the catalytic activity of SRB10 protein kinase is required for α2 repression: Given the model that SSN6-TUP1 brings about repression by interacting with the pol II holoenzyme, a further question is whether the kinase activity of SRB10 is required for repression. It could be that the kinase activity is not required for repression, but instead, SRB10 simply serves to recruit another factor to the holoenzyme. This factor then interacts with SSN6-TUP1. If the kinase activity of SRB10 is not required, then an *srb10* allele whose gene product lacks kinase activity but is still incorporated into the holoenzyme might restore repression to an α *are1*

deletion mutant. The *srb10-3* allele contains a point mutation which inactivates the kinase activity of SRB10 (Liao et al., 1995). However, the defective protein is stably incorporated into the holoenzyme (Liao et al., 1995).

I tested whether this mutant allele restores α2 repression to an *α are1* deletion mutant. A plasmid (pSL220; a kind gift of Sha-Mei Liao and R. A. Young, see Table 1) bearing the *srb10-3* allele or a control plasmid (pRS316) was transformed into the **a** and *α are1Δ::LEU2 MFA2::lacZ* strains (MWY14 and MWY13) and into isogenic **a** and α *ARE1* wild-type strains. Quantitative β-galactosidase assays were performed and *MFA2::lacZ* repression was quantitated as before (Materials and Methods, Ch. 2). As shown in Table 1, the *srb10-3* allele does not restore repression in the *are1* deletion mutant (repression is down 3.6-fold in the absence and 4.5-fold in the presence of the *srb10-3* allele). This result suggests that the catalytic activity of SRB10 is required for its role in α2 repression, be that role direct through an interaction with SSN6-TUP1 or indirect through its effect on levels of *MATα2* expression.

An alternative explanation for the lack of complementation is that the mutant protein encoded by *srb10-3* alters the normal composition of the holoenzyme such that a factor which is required for α2 repression is missing. Liao et al. (1995) did determine by Western analysis that the largest subunit of pol II, SRB2, SRB5, and the defective SRB10 are present in their usual stoichiometric amounts. They did not test, however, the over 15 other

holoenzyme components. However, for reason of simplicity, I favor the former explanation, i. e., that the catalytic activity of SRB10 is required for α2 repression. Furthermore, there is indirect evidence that SRB10 phosphorylates SSN6-TUP1 (M. J. Redd and A. D. Johnson, unpublished observations; discussed in Chapter 4)

Derepression of endogenous *a*-specific genes in the *α are* mutants: In order to ensure that the *are* repression defect lies at the transcriptional level, I analyzed the expression of two endogenous **a**-specific genes, *STE2* and *BAR1*, in the *α are* mutants by Northern analysis. Figure 2A reveals that *STE2* is expressed in both the *α are1* and *are2* mutants; it is unclear whether *STE2* is expressed in the *α are3* and *are4* mutants; the sensitivity of the Northern analysis may be insufficient to detect derepression of *STE2* in these two strains. Figure 2B indicates that *BAR1* is expressed in the *α are1, are2,* and *are3* mutants but is not detectable by Northern analysis in the *α are4* mutant. In cases where expression of *BAR1* or *STE2* is observed, the expression is significantly lower than that observed in an *α tup1* deletion mutant, consistent with the fact that the *are* mutants have only a partial effect on repression, whereas repression is eliminated in the *tup1* mutant (Keleher et al., 1992). Because I decided to focus on *ARE1* and *ARE2* (see Chapter 3), the Northern analysis was not repeated for the *α are3* and *are4* mutants.

Restriction map and DNA sequence of the *ARE1* locus: Because restriction maps which include restriction sites upstream and downstream of published DNA sequences can be useful for future work, I include here the restriction map of the genomic insert of pMW15 (Figure 3), which contains *ARE1* (see Table 2, Ch. 2). The locations of transposon insertions which disrupt the ability of this plasmid to complement the *are1* repression defects are also mapped (Materials and Methods, Ch. 2).

In addition, I present the DNA sequence and deduced amino acid sequence of the *ARE1* locus because, although published elsewhere as *UME5* (Surosky et al., 1994) and *SRB10* (Liao et al., 1995), I independently sequenced this gene and found the same sequence (Figure 4).

REFERENCES

Herskowitz, I., B. Andrews, W. Kruger, J. Ogas, A. Sil, C. Coburn, and C. Peterson, 1992 Integration of multiple regulatory inputs in the control of *HO* expression in yeast in *Transcriptional Regulation*, pp. 949-974. edited by S. L. McKnight and K. R. Yamamoto. Cold Spring Harbor Laboratory Press, Plainview, NY.

Jiang, Y. W., P. R. Dohrman, and D. J. Stillman, 1995 Genetic and physical interactions between yeast RGR1 and SIN4 in chromatin organization and transcriptional regulation. Genetics **140:** 47-54.

Kruger, W., and I. Herskowitz, 1991 A negative regulator of *HO* transcription, *SIN1* (*SPT2*), is a nonspecific DNA-binding protein related to HMG1. Mol. Cell. Biol. **11:** 4135-4146.

Kruger, W., C. L. Peterson, A. Sil, C. Coburn, E. N. Moudrianakis, and I. Herskowitz, 1995 Amino acid substitutions in the structured domains of histones H3 and H4 partially relieve the requirement of the yeast SWI/SNF complex for transcription. Genes Dev. **9:** 2770-2779.

Lefebvre, L., and M. Smith, 1993 Mutational and functional analysis of dominant *SPT2* (*SIN1*) suppressor alleles in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:** 5393-5407.

Liao, S.-M., J. Zhang, D. A. Jeffery, A. J. Koleske, C. M. Thompson *et al.*, 1995 A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature **374:** 193-196.

Parent, S. A., C. M. Fenimore, and K. A. Bostian, 1985 Vector systems for studying DNA sequences. Yeast **1:** 83-138.

Sakai, A., Y. Shimizu, and F. Hishinuma, 1988 Isolation and characterization of mutants which show an oversecretion phenotype *in Saccharomyces cerevisiae.* Genetics **119:** 499-506.

Sakai, A., Y. Shimizu, S. Kondou, T. Chibazakura, and F. Hishinuma, 1990 Structure and molecular analysis of *RGR1,* a gene required for glucose repression of *Saccharomyces cerevisiae.* Mol. Cell. Biol. **10:** 4130-4138.

Sikorski, R. S., and P. Hieter, 1989 A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae.* Genetics **122:** 19-27.

Stern, M., Jensen, R. E., and I. Herskowitz, 1984 Five *SWI* genes are required for the expression of the *HO* gene in yeast. J. Mol. Biol. **178:** 853-868.

Sternberg, P. W., M. J. Stern, I. Clark, and I. Herskowitz, 1987 Activation of the yeast *HO* gene by release from multiple negative controls. Cell **48:** 567-577.

Stillman, D. J., S. Dorland, and Y. Yu, 1994 Epistasis analysis of suppressor mutations that allow HO expression in the absence of the yeast SWI5 transcriptional activator. Genetics **136:** 781-788.

Strich, R., M. R. Slater, and R. E. Esposito, 1989 Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. Proc. Natl. Acad. Sci. USA **86:** 10018-10022.

Surosky, R. T., R. Strich, and R. E. Esposito, 1994 The yeast *UME5* gene regulates the stability of meiotic mRNAs in response to glucose. Mol. Cell. Biol. **14:** 3446-3458.

FIGURE LEGENDS

Figure 1. RNA levels of *MATα2* and *TUP1* in the *α are* mutants.

(A) A Northern blot was hybridized with a *MATα2* probe and a *LYS2* probe as an internal control. (B) A Northern blot was hybridized with a *TUP1* probe and a *LYS2* probe as an internal control. In both (A) and (B), RNA was prepared from the following isogenic strains (Table 1, Ch. 2): EG123 (***a*** *wild-type*), 246-1-1 (*α wild-type*), MWY10 (*α are1-41*), MWY15 (*α are2-13*), MWY24 (*α are3-57*), MWY27 (*α are4-87*), AJY166 (*α tup1Δ::LEU2*). See Materials and Methods in Ch. 2.

Quantitation: RNA transcripts were quantitated using an Applied Biosystems Phosphor Imager using ImageQuant software (see Materials and Methods, Ch. 2). In each case, the amount of transcript from either *MATα2* or *TUP1* was normalized to the amount of control transcript from *LYS2*. The normalized values for the blot probed with a *MATα2* and *LYS2* fragment are: 0.89 (***a*** *wild-type*), 2.08 (*α wild-type*), 1.34 (*α are1-41*), 1.88 (*α are2-13*), 1.94 (*α are3-57*), 2.93 (*α are4-87*), 1.24 (*α tup1Δ::LEU2*). The raw data used for these calculations, with the values for *MATα2* first and *LYS2* second are: 1308, 1461 (***a*** *wild-type*); 3186, 1530 (*α wild-type*); 4430, 3305 (*α are1-41*); 4178, 2224 (*α are2-*

13); 5232, 2693 (α *are3-57*); 6291, 2144 (α *are4-87*); 5768, 4643 (α *tup1Δ::LEU2*). The normalized values for the blot probed with a *TUP1* and *LYS2* fragment are: 2.65 (***a*** *wild-type*), 2.57 (α *wild-type*), 2.55 (α *are1-41*), 2.38 (α *are2-13*), 2.88 (α *are3-57*), 3.09 (α *are4-87*), 0.37 (α *tup1Δ::LEU2*). The raw data used for these calculations, with the values for *TUP1* first and *LYS2* second are: 6267, 2367 (***a*** *wild-type*); 6473, 2518 (α *wild-type*); 13406, 5252 (α *are1-41*); 9362, 3933 (α *are2-13*); 8402, 2915 (α *are3-57*); 11043, 3577 (α *are4-87*); 3121, 8347 (α *tup1Δ::LEU2*).

Figure 2. Derepression of endogenous **a**-specific genes in the α *are* mutants.

(A) A Northern blot was hybridized with a *STE2* probe and a *LYS2* probe as an internal control. (B) A Northern blot was hybridized with a *BAR1* probe and a *LYS2* probe as an internal control. In both (A) and (B), RNA was prepared from the following isogenic strains (Table 1, Ch. 2): EG123 (***a*** *wild-type*), 246-1-1 (α *wild-type*), MWY10 (α *are1-41*), MWY15 (α *are2-13*), MWY24 (α *are3-57*), MWY27 (α *are4-87*), AJY166 (α *tup1Δ::LEU2*). See Materials and Methods in Ch. 2.

Figure 3. Restriction map of the *ARE1* gene.

A restriction map of the genomic insert contained in the complementing plasmid pMW15 is shown. To determine the location of *ARE1* on this insert, the plasmid was subjected to transposon mutagenesis (see Materials and

Methods, Ch. 2). Transposon insertions which disrupt the complementation activity were mapped by restriction analysis and are indicated by the short arrows. Most of the insertions cluster in a 3-kb region. It is not known why the plasmid containing the most leftward insertion lacks complementation activity. The *ARE1* open reading frame, deduced from the DNA sequence, is indicated by the arrow. Restriction sites: B, *Bgl*II; P, *Pvu*II; S, *Sph*I; Sc, *Sac*II.

Figure 4. DNA sequence and deduced amino acid sequence of the *ARE1* gene.

Nucleotides are numbered at the right with +1 designating the first base of the putative initiation codon. Amino acids are also numbered at right. This deduced open reading frame is 1,165 nucleotides and encodes a 555 amino-acid CDC28-related protein kinase identical to UME5/SSN3/SRB10. Unpublished primer extension analysis from the R. A. Young lab (Liao et al., 1995), however, indicates that the *ARE1/UME5/SSN3/SRB10* transcript is initiated at position +12 in this open reading frame, and that the amino acid sequence therefore lacks the 14 N-terminal amino acids reported here and reported for the UME5 amino acid sequence (Surosky et al., 1994). I have not performed an independent primer extension analysis.

TABLE 1

Plasmids

Name	Description	Source
pRS315	*LEU2, CEN6, ARSH4*	SIKORSKI and HIETER 1989
pRS316	*URA3, CEN6, ARSH4*	SIKORSKI and HIETER 1989
YEp24	*URA3*, 2μ origin	PARENT et al. 1985
M2597	5.8-kb *Sal*I-*Hin*dIII fragment containing *RGR1* cloned into *Sal*I-*Hin*dIII cleaved YEplac195, a *URA3* vector	D. J. STILLMAN LAB
WB39	*SIN1, CEN, URA3*	I. HERSKOWITZ LAB
pCP206	*HTH-2/SIN2, URA3, CEN, ARS1*	CRAIG PETERSON
pCP204	*HHF-2, URA3, CEN, ARS1*	CRAIG PETERSON
M723	5.9-kb *Xho*I-*Bam*HI fragment containing *SIN3* cloned into *Sal*I-*Bam*HI cleaved YEp352, a *URA3* vector (Yeast 2: 163)	D. J. STILLMAN LAB
pSL220	*srb10-3, URA3, CEN, ARS*	R. A. YOUNG LAB

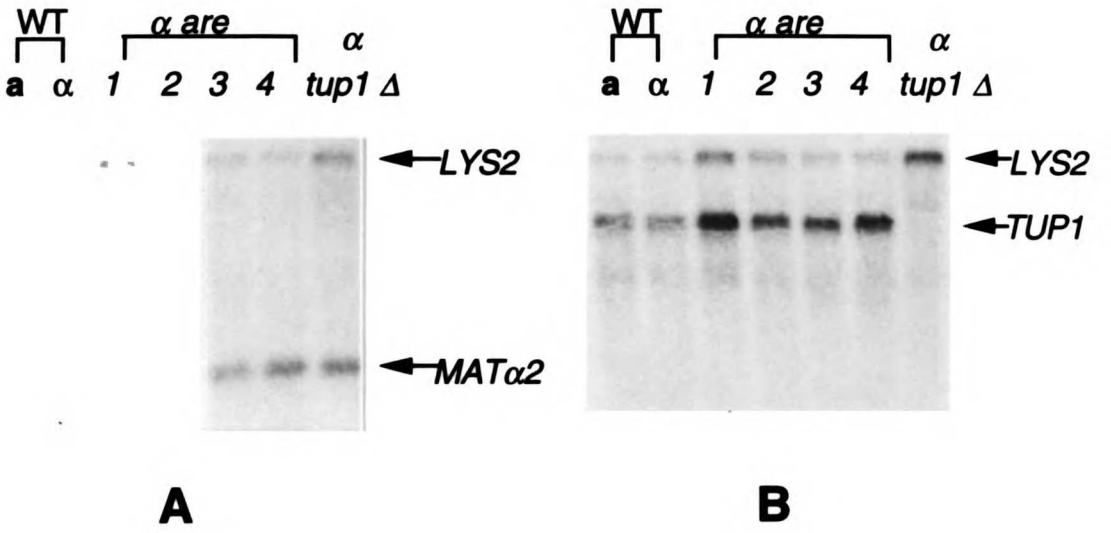
TABLE 2

Effect of the *srb10-3* allele on repression in an *are1* deletion mutant

Genotype	Units of β-galactosidase activity		
	MATa	*MATα*	Repression
ARE⁺/pRS316	160 ± 50	2.6 ± 0.1	62
are1Δ::LEU2/pRS316	170 ± 10	9.7 ± 0.5	17 (3.6×)
ARE⁺/pSL220	180 ± 30	2.7 ± 0.4	67
are1Δ::LEU2/pSL220	180 ± 20	12 ± 0.6	15 (4.5×)

Numbers represent units of β-galactosidase activity in *MFA2::lacZ* strains of the indicated genotype (Table 1, Chapter 2) transformed with a control plasmid (pRS316) or with a plasmid bearing the *srb10-3* allele (pSL220). Repression is defined as the ratio of β-galactosidase activity in an **a** strain to that in the isogenic α strain. Numbers in parentheses indicate fold decrease in repression in the *are1Δ* mutant bearing either pRS316 or pSL220 relative to the wild-type strain bearing the same plasmid. Results are averages of assays performed in triplicate. Two independent transformants for each strain were assayed. All strains were grown in glucose medium under uracil selection.

Figure 1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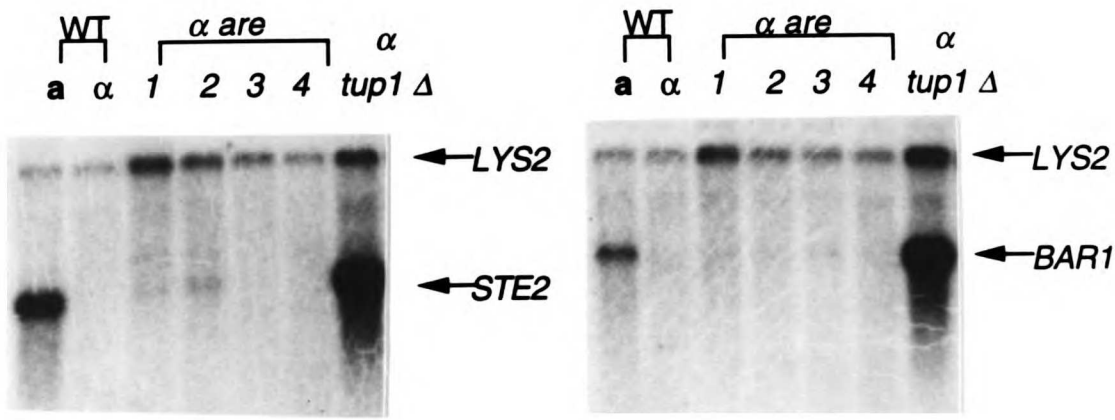
α are
α
a α 1 2 3 4 tup1 Δ
LYS2
MATα2
A
WT
α are
α
a α 1 2 3 4 tup1 Δ
LYS2
TUP1
B

Figure 2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α
a
α
1
2
3
4
tup1 Δ
LYS2
STE2
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a
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2
3
4
tup1 Δ
LYS2
BAR1

A B

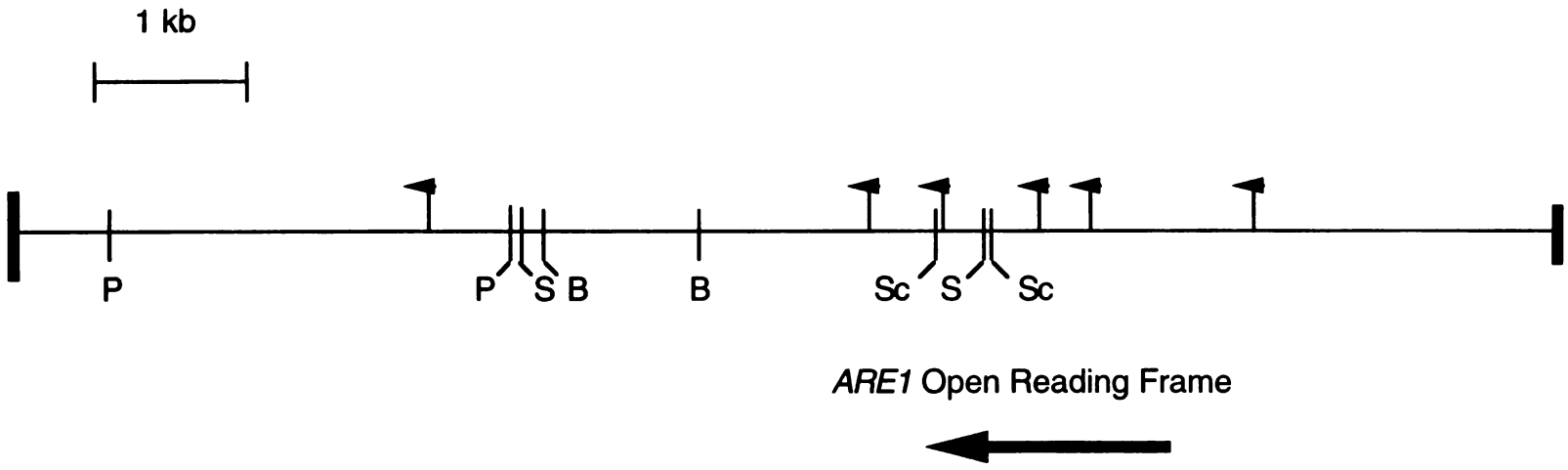

Figure 3

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TCTGGCACAGTAGCCTACGTTACAGTAAAATTACTATATCCGGTACGCATTTTCATTAGTATCTGGGGAGCACCATATTTTGGGAAATGG  -456
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TTTTCAGTTGAAGTTGCGCACTCGGCATATGATTTATAGATTCCCAATATATTGTACTTCGTTATATATGTGTTACGAATATTTTTGATT  -276
TCGTTTTAGAGAGTTTTGATTAGAGGAAATTATAGCTTTTTTTAACAGTGAAATAAATATCATACATCAAAAGTCTTCAAGAATTACGTG  -186
GTGTGGCTTAAGTTGCGTTTCATTTTCCCGCTTCAATACTTGAAAGTTATCCCACAATCACTGCTGACAAAAAGGATACAAGAAAGGTTT   -96
ATAGGAAAGAAAAAAGGCGGAAGGGTATACTGAAGTTAGTAATTTTGCTTCCCAATTGAATTAAGGCCGCCTAGTTTTGACGGGAGGAGA    -6
GAGAA                                                                                          -1

ATGTATAATGGCAAGGATAGAGCACAAAACTCCTATCAGCCAATGTACCAAAGGCCTATGCAGGTACAAGGACAACAGCAAGCTCAATCG    90
M  Y  N  G  K  D  R  A  Q  N  S  Y  Q  P  M  Y  Q  R  P  M  Q  V  Q  G  Q  Q  Q  A  Q  S     30

TTCGTTGGAAAGAAAAACACAATCGGAAGTGTGCATGGAAAAGCCCCGATGCTAATGGCCAATAATGATGTTTTTACTATTGGACCTTAT   180
F  V  G  K  K  N  T  I  G  S  V  H  G  K  A  P  M  L  M  A  N  N  D  V  F  T  I  G  P  Y     60

AGGGCAAGAAAAGATAGAATGCGGGTATCTGTCTTAGAAAAGTACGAAGTTATTGGCTACATTGCTGCGGGCACATATGGTAAAGTTTAC   270
R  A  R  K  D  R  M  R  V  S  V  L  E  K  Y  E  V  I  G  Y  I  A  A  G  T  Y  G  K  V  Y     90

AAAGCGAAAAGACAAATCAACTCCGGTACCAATTCCGCTAATGGTTCTAGTCTGAATGGTACCAATGCGAAAATTCCGCAGTTTGACAGC   360
K  A  K  R  Q  I  N  S  G  T  N  S  A  N  G  S  S  L  N  G  T  N  A  K  I  P  Q  F  D  S    120

ACGCAACCAAAATCAAGCTCTTCAATGGACATGCAGGCAAATACAAACGCATTAAGAAGAAACTTGTTAAAGGATGAAGGAGTGACCCCC   450
T  Q  P  K  S  S  S  S  M  D  M  Q  A  N  T  N  A  L  R  R  N  L  L  K  D  E  G  V  T  P    150

GGAAGAATACGAACTACGAGGGAAGATGTATCCCCGCACTATAATTCCCAAAAACAAACCCTCATTAAAAAACCGCTGACGGTATTTTAT   540
G  R  I  R  T  T  R  E  D  V  S  P  H  Y  N  S  Q  K  Q  T  L  I  K  K  P  L  T  V  F  Y    180

GCCATTAAAAAGTTCAAGACAGAGAAGGATGGCGTCGAACAATTGCATTATACGGGAATATCTCAGAGTGCCTGTAGAGAAATGGCATTA   630
A  I  K  K  F  K  T  E  K  D  G  V  E  Q  L  H  Y  T  G  I  S  Q  S  A  C  R  E  M  A  L    210

TGTCGAGAATTGCACAACAAGCATTTAACCACATTAGTGGAAATTTTTTTGGAAAGGAAATGTGTCCATATGGTATACGAATATGCGGAG   720
C  R  E  L  H  N  K  H  L  T  T  L  V  E  I  F  L  E  R  K  C  V  H  M  V  Y  E  Y  A  E    240

CATGATCTGCTACAAATTATCCACTTCCATTCCCATCCCGAAAAAAGGATGATACCACCAAGAATGGTTCGGTCTATTATGTGGCAGCTT   810
H  D  L  L  Q  I  I  H  F  H  S  H  P  E  K  R  M  I  P  P  R  M  V  R  S  I  M  W  Q  L    270

TTAGACGGCGTATCGTATCTTCATCAAAATTGGGTGCTTCATCGAGATTTGAAACCCGCAAATATAATGGTGACCATAGATGGATGTGTT   900
L  D  G  V  S  Y  L  H  Q  N  W  V  L  H  R  D  L  K  P  A  N  I  M  V  T  I  D  G  C  V    300

AAAATTGGTGATTTAGGTTTGGCCAGAAAATTTCATAATATGCTGCAAACCCTCTATACTGGGGATAAAGTGGTTGTCACTATATGGTAC   990
K  I  G  D  L  G  L  A  R  K  F  H  N  M  L  Q  T  L  Y  T  G  D  K  V  V  V  T  I  W  Y    330

CGTGCACCTGAGTTGCTATTGGGAGCACGGCACTATACCCCTGCGGTTGATTTATGGTCCGTTGGCTGCATTTTTGCAGAACTGATAGGA  1080
R  A  P  E  L  L  L  G  A  R  H  Y  T  P  A  V  D  L  W  S  V  G  C  I  F  A  E  L  I  G    360

TTACAGCCCATATTTAAAGGTGAAGAAGCTAAACTAGACTCTAAAAAGACTGTTCCATTTCAAGTGAATCAACTACAGAGAATTTTGGAA  1170
L  Q  P  I  F  K  G  E  E  A  K  L  D  S  K  K  T  V  P  F  Q  V  N  Q  L  Q  R  I  L  E    390

GTTCTTGGCACTCCCGATCAAAAAATTTGGCCTTATTTGGAGAAGTATCCAGAATATGATCAAATTACGAAGTTTCCAAAGTATAGGGAT  1260
V  L  G  T  P  D  Q  K  I  W  P  Y  L  E  K  Y  P  E  Y  D  Q  I  T  K  F  P  K  Y  R  D    420

AACCTTGCTACATGGTATCATTCCGCGGGAGGAAGGGACAAGCATGCTTTAAGCTTACTTTACCACTTGTTAAATTATGATCCAATTAAA  1350
N  L  A  T  W  Y  H  S  A  G  G  R  D  K  H  A  L  S  L  L  Y  H  L  L  N  Y  D  P  I  K    450

AGAATAGATGCATTTAATGCGTTGGAACATAAGTACTTCACAGAAAGTGATATTCCTGTTAGTGAAAATGTATTTGAAGGTCTAACTTAC  1440
R  I  D  A  F  N  A  L  E  H  K  Y  F  T  E  S  D  I  P  V  S  E  N  V  F  E  G  L  T  Y    480

AAATACCCGGCAAGAAGAATTCACACGAACGATAATGACATCATGAATCTTGGATCAAGAACGAAAAACAATACACAAGCTTCAGGAATC  1530
K  Y  P  A  R  R  I  H  T  N  D  N  D  I  M  N  L  G  S  R  T  K  N  N  T  Q  A  S  G  I    510

ACCGCAGGTGCCGCTGCAAATGCGTTAGGTGGGCTTGGTGTTAACCGTAGAATTCTGGCCGCGGCAGCAGCAGCCGCTGCTGCGGTGTCA  1620
T  A  G  A  A  A  N  A  L  G  G  L  G  V  N  R  R  I  L  A  A  A  A  A  A  A  A  A  V  S    540

GGAAACAATGCATCAGATGAGCCATCTCGAAAGAAAAACAGAAGATAG                                             1668
G  N  N  A  S  D  E  P  S  R  K  K  N  R  R  *                                              555

GCTTCTATTTTTATATATATTTGGAATTTTTCATTCCACAGCACTGTCACTATTATATTCATTAAACTTTTTTTTATCTTTATAGTATTT  1758
AAATCGGCATACAGTTTCAATTTTTCGCTTTAGAGGCACTAAGAATGCAAGTCTGCAACATTCAGGTAAAATAATGGGTTGATTTTAGGT  1848
CGAGCTAAAACCCTGTTCTCCGCAGATGTATGCGAATTTCGTCATAATTCATCTCAACTAATGGGGCTTTAAAACATATGAATATCTCAT  1938
GCATTCCC                                                                                     1946
```

Figure 4

Chapter 3

Further Links between α2 Repression and the RNA Polymerase II Holoenzyme: *SRB8* is Required for Repression and Interacts Genetically with *TUP1*

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ABSTRACT Transcriptional repression of the **a**-specific genes in *Saccharomyces cerevisiae* α cells is known to require several proteins. The homeodomain protein, α2, binds cooperatively with Mcm1p to an operator site located upstream of each **a**-specific gene. Operator-bound α2-Mcm1p recruits a general repressor complex, comprised of Ssn6p and Tup1p, to the DNA. The Ssn6p-Tup1p complex brings about transcriptional repression of the **a**-specific genes (referred to as α2 repression) by an unknown mechanism. We recently reported the identification of mutations in four genes, *ARE1*, *ARE2*, *ARE3*, and *ARE4*, which cause partial loss of α2 repression. *ARE1* codes for a component of the RNA polymerase II holoenzyme, so we proposed that α2 repression involves an interaction between Ssn6p-Tup1p and the holoenzyme. Here we report that *ARE2* is identical to *SRB8*, which encodes another holoenzyme component. In addition, we analyze the phenotype of an *srb8* deletion allele and present genetic evidence for an interaction between Tup1p and Srb8p. These findings support the view that Ssn6p-Tup1p exerts repression by acting on the pol II holoenzyme.

Transcription of genes transcribed by RNA polymerase II in yeast is typically controlled by regulatory proteins that bind to specific sequences located upstream of their target genes. Transcriptional activation is generally thought to involve interactions between specific DNA-binding activator proteins and components of the general transcription machinery (reviewed in 23). These components include general transcription factors, RNA polymerase II (pol II), and other proteins associated with pol II, variously termed SRB proteins or mediators (8, 15, 17, 22, 43). In contrast to transcriptional activation, relatively little is known about how specific DNA-binding repressor proteins turn off transcription.

One well-studied transcriptional repressor is the *Saccharomyces cerevisiae* homeodomain protein, α2, which is present in only one of the two *S. cerevisiae* haploid cell types, the α cell type. In α cells, α2 turns off transcription of genes specifically expressed in **a** cells, the other haploid cell type (reviewed in 9). α2 repression of these so-called **a**-specific genes requires the concerted action of several proteins. α2 binds cooperatively with Mcm1p to a conserved DNA sequence, called the **a**-specific gene (**asg**) operator, located upstream of each **a**-specific gene (12, 13). Operator-bound α2-Mcm1p recruits a general repressor complex, composed of Ssn6p and Tup1p (51), to the DNA. Once recruited, Ssn6p-Tup1p brings about transcriptional repression (14, 18, 46).

Like α2-Mcm1p, Ssn6p-Tup1p is esssential for repression of the **a**-specific genes (14). Unlike α2-Mcm1p, however, Ssn6p-Tup1p is required for repression

of many other genes, including the haploid-specific genes in **a**/α diploids (25), the glucose-repressible genes (35, reviewed in 45), the hypoxic genes (reviewed in 52), and the DNA-damage inducible genes (reviewed in 6). Consequently, *ssn6* and *tup1* mutants have pleiotropic phenotypes, including sporulation deficiency, slow growth, and clumpiness (4, 20, 31, 33, 36, 49).

How Ssn6p-Tup1p mediates repression by α2 is unknown. To learn more about this process, we recently carried out a genetic screen for mutants defective in α2 repression, and we reported the identification of mutations in four genes designated *ARE1*, *ARE2*, *ARE3*, and *ARE4* (for alpha2 repression) (47). The *are* mutations lead to partial loss of α2 repression and cause growth defects similar to those resulting from *ssn6* or *tup1* mutations: they grow slowly and are clumpy.

The sequence of *ARE1* revealed that it encodes a serine/threonine protein kinase belonging to the CDC28 family of protein kinases (47). Interestingly, *ARE1* was also identified as *UME5* (42), *SSN3* (19), and *SRB10* (22). *UME5* was identified in a screen for mutants defective in repression of early meiosis-specific genes during vegetative growth (41), and *SSN3* was identified in a screen for mutants defective in glucose repression (4), a Tup1p-Ssn6p-dependent process (45). The *SRB* (suppressor of RNA polymerase B) genes were identified because mutations in these genes suppress a growth defect caused by partial truncation of the carboxy-terminal domain (CTD) of the largest subunit of pol II (8, 22, 27, 43). The CTD is involved in transcriptional

regulation although its exact role(s) is not well understood (reviewed in 5). Biochemical studies have shown that the SRB proteins are associated with each other, along with pol II and several general transcription factors, in a complex termed the pol II holoenzyme (8, 15, 17, 22, 43). The finding that *ARE1* is identical to *SRB10* provided the first genetic link between α2 repression and the general transcription machinery.

In this paper we report that *ARE2* is identical to *SRB8*, which encodes another component of the pol II holoenzyme. We analyze the phenotype caused by an *srb8* deletion and present genetic evidence for an interaction between Tup1p and the pol II holoenzyme. The results presented here provide independent evidence for a connection between α2 repression and the general transcription machinery.

MATERIALS AND METHODS

Media, growth conditions, and genetic methods: Liquid and solid media have been described (37). 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°C in either rich (YEPD) or synthetic (SD) drop-out media. Standard genetic methods for mating, sporulation, tetrad analysis, and curing plasmids were used (24, 39). Yeast cells were transformed by the lithium acetate method (11).

Yeast strains and plasmids: *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 *MAT* (40). Strains SM1196 and SM1179 have been described (7). The **a** and α *srb8Δ::LEU2* strains MWY44 and MWY45 were created as follows. The *srb8Δ::LEU2* allele, which encodes only the N-terminal 406 amino acids of the 1226-amino-acid Srb8p protein, was first introduced into diploid MWY33, generating MWY43, by transforming with a 3.9-kb *Bam*HI-*Pst*I fragment isolated from pMW26 cut with *Bam*HI and *Pst*I and selecting for Leu^+ transformants. This *LEU2* disruption also deletes the first 62 nucleotides of a 384 nucleotide open reading frame, YCR82W, which is directly downstream of *SRB8* (28). YCR82W does not encode *ARE2* since pMW21, which contains the YCR82W open reading frame and 3904 nucleotides upstream and 258 nucleotides downstream, fails to complement the *are2* defect (data not shown). Correct integration was confirmed by PCR analysis (see below). MWY43 was then sporulated and an **a** (MWY44) and an α (MWY45) Leu^+ segregant were recovered from dissected tetrads. The **a** and α *are1Δ::LEU2 are2-13* strains (MWY40 and MWY39) were created as follows. The *are1Δ::LEU2* allele (47) was first introduced into the homozygous *are2-13* diploid strain MWY47 as described in ref. 47. The resulting *are2/are2 are1/ARE1* diploid was then sporulated and **a** and α Leu^+ segregants were recovered from dissected tetrads. MWY2, MWY16, and MWY17 have been described (47).

Plasmid pMW18, containing the *ARE2* gene, was isolated from a YCp50-based yeast genomic library (30). Plasmid pMW19 was constructed by deleting a ~ 5-kb *Hin*dIII fragment containing *TUP1* from pMW18. Plasmid pMW22 was

derived from pMW19 by deleting a 1.9-kb *Pvu*II fragment from within the *SRB8* locus. Plasmid pMW26, which was used to disrupt the *SRB8* locus, was constructed in two steps. First, a 4.5-kb *Eco*NI-*Nhe*I fragment (the ends filled in using Klenow fragment) from pMW19 was subcloned into the *Sma*I site of pRS316, generating pMW21. (The fragment is oriented with the *Eco*NI end towards the *Bam*HI site in the pRS316 polylinker.) pMW21 was then cut with *Pac*I and *Bgl*II, deleting a 2.8-kb fragment encoding the C-terminal 819 amino acids of Srb8p, and then ligated to a 2.2-kb *LEU2* PCR fragment with PCR-introduced *Pac*I and *Bgl*II ends. The high-copy *TUP1*-bearing plasmid pFW28 has been described (50). The standard yeast vectors, YEp24 and pRS316, have also been described (3, 38).

Cloning of *ARE2*: MWY16 (*α are2-13 MFA2::lacZ*) was transformed with a YCp50-based yeast genomic library (30). Transformants were plated at ~65 colonies per SD -Ura plate, and an X-Gal filter assay (see below) was used to screen for white (*MFA2::lacZ* repressed) transformants. As a secondary screen, transformants that appeared white were tested for clumpiness in liquid SD -Ura medium. Of ~4000 transformants screened by X-Gal filter assay, two transformants, 13-30 and 13-38, were both white on X-Gal and nonclumpy in liquid medium. Both mutant phenotypes were restored upon curing the plasmids. The library plasmid (pMW18) was isolated from transformant 13-38 and analyzed further.

Linkage analysis: To test whether *are2-13* was an allele of *TUP1*, an allelism test was performed between MWY16 (*α are2-13 MFA2::lacZ*) and MWY2 (*a*

tup1Δ::LEU2 MFA2::lacZ). Upon sporulating diploids derived from crossing these two strains, tetrads were dissected (a total of 13 asci from two independent diploids) and the clumpy phenotype of segregants scored. Surprisingly, we found that while *ARE2* is not allelic to *TUP1*, it is closely linked; one of the thirteen dissected tetrads had a nonclumpy, Leu^- segregant. To further test whether the *are2-13* mutation was in *TUP1*, we replaced the *TUP1* locus in the MWY16 with a known wild-type *TUP1* gene and showed that this replacement did not rescue the *are2-13* defect (data not shown).

β-Galactosidase assays: The X-Gal filter assay has been described previously (34). In brief, colonies or patches of cells are replicated onto Whatman filter paper overlaying an appropriate medium plate. After growing the cells on the filter overnight, the filter is dipped into liquid nitrogen for 10 sec to permeabilize the cells and then placed onto a Whatman filter paper saturated with Z buffer (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, 0.027% β-mercaptoethanol, and 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 air-drying.

Quantitative liquid β-galactosidase assays were performed as described in ref. 47. For each strain, two to four independent colonies were assayed in triplicate on different days. Clumpy cells were dispersed by adding EDTA to 25 mM before measuring optical density. Numbers represent averages; standard deviations are shown in Table 2, 3, and 4.

PCR assays: The PCR amplification protocol and three primers used to determine which *MAT* allele is present have been described (10). Two modifications that improve 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 *MAT*α. To confirm integration of *srb8Δ::LEU2*, a PCR amplification protocol described in ref. 47 was used.

RESULTS

Cloning of *ARE2*. Characterization of the *are* mutants suggested that *TUP1*, *ARE1*, and *ARE2* might be more functionally related to each other than to either *ARE3* or *ARE4*. First, unlike *are3* or *are4* mutations, *tup1*, *are1*, and *are2* mutations do not cause an increase in basal transcription (i.e., transcription in the absence of an upstream activating sequence) *in vivo* (47). Second, alleles of *ARE1* display unlinked noncomplementation with several alleles of *ARE2*, suggesting a possible physical interaction between the Are1p and Are2p proteins (47). Finally, *TUP1* overexpression partially suppresses one of the *are2* alleles, *are2-13*, identified in our screen. *TUP1* overexpression does not suppress any of the *are1*, *are3*, or *are4* alleles isolated in our screen (data not shown).

To clone *ARE2*, we used an α *are2* strain containing a chromosomal *MFA2::lacZ* reporter. Because *MFA2* is an **a**-specific gene, the *MFA2::lacZ*

reporter is subject to α2 repression in α cells. Since this reporter is strongly repressed in wild-type α cells, yeast colonies derived from these cells remain white in an X-Gal filter assay (see Materials and Methods). Because *are2* mutants are defective in α2 repression, β-galactosidase is produced and the mutant colonies appear blue. We transformed the α *are2-13 MFA2::lacZ* strain MWY16 with a low-copy yeast genomic library (30) and then screened for white transformants on X-Gal, i.e., for complementation of the *MFA2::lacZ* derepression phenotype.

One plasmid (pMW18) was isolated that complements both the *MFA2::lacZ* derepression and the clumpy phenotype caused by the *are2-13* mutation (data not shown). Since we knew that *TUP1* carried on a high-copy plasmid partially suppresses the repression defect in the *are2-13* strain (47), we had to consider the possibility that pMW18 carried *TUP1* and not *ARE2*. Restriction map analysis indicated that *TUP1* was, in fact, located on the approximate 11-kb genomic insert of pMW18 (data not shown). However, there were still reasons to believe that *ARE2* also resided on the insert. First, pMW18 complements both the *MFA2::lacZ* derepression and the clumpy phenotype of the *are2* mutant whereas *TUP1* overexpression suppresses only the *MFA2::lacZ* derepression. Second, linkage analysis had previously indicated that *ARE2* resides near *TUP1* (data not shown, see Materials and Methods), making the presence of *TUP1* on the genomic insert less surprising. To test whether *ARE2* also resides on the insert, a *Hin*dIII fragment containing

the entire *TUP1* locus was deleted from pMW18, and the resulting plasmid (pMW19) was tested for ability to complement the *are2* defect. The *TUP1*-deleted plasmid complements both phenotypes, strongly suggesting that *ARE2* is located on the genomic insert (data not shown).

***ARE2* is identical to *SRB8*.** As noted above, we previously observed that alleles of *are1* display unlinked noncomplementation with alleles of *are2*, suggesting a physical interaction between the Are1p and Are2p proteins. Given that *ARE1* is identical to *SRB10* (47) and that the SRB proteins are associated together in a large multiprotein complex (8, 15, 17, 22, 43), it seemed possible that *ARE2* might be another *SRB* gene. We learned that *SRB8* is approximately 2-kb proximal to *TUP1* (personal communication, S.-M. Liao & R. A. Young); subsequent restriction map analysis indicated that *SRB8* is located on the genomic insert of pMW19 (data not shown). The sequence of *SRB8* , which is identical to the open reading frame YCR81W (28), has been reported previously (8). It consists of a 3678 nucleotide open reading frame which encodes a predicted protein of 1226 amino acids; the deduced protein sequence bears no obvious homologies to other proteins. To test whether *SRB8* is the complementing gene, we deleted an internal 1.9-kb *Pvu*II fragment from *SRB8* carried on pMW19 and then tested this plasmid (pMW22) for ability to complement the *are2* defect. Neither *MFA2::lacZ* derepression nor the clumpy phenotype was complemented by pMW22, indicating that *SRB8* is *ARE2* (data not shown).

As a final test to determine whether *ARE2* and *SRB8* are identical, an

allelism test was performed between *srb8Δ* and *are2* strains. A *srb8Δ::LEU2* allele was introduced into the wild-type **a**/α *MFA2::lacZ leu2* homozygous diploid MWY33. This disruption removes 67 percent of the *SRB8* coding sequence, leaving a segment that encodes the N-terminal 406 amino acids. Following sporulation and tetrad dissection of the heterozygous diploid (MWY43), an ***a*** *srb8Δ::LEU2 MFA2::lacZ* segregant (MWY44) was isolated and crossed to the α *are2-13 MFA2::lacZ* strain MWY16. The resulting diploid sporulated inefficiently unless transformed with a plasmid bearing *SRB8* (pMW19), indicating that the *are2-13* and *srb8* mutants fail to complement. Following tetrad dissection of the sporulated diploid, the segregants were cured of the plasmid and the mutant phenotypes then analyzed. Tetrad analysis (a total of 18 asci tested from two independent diploids) indicated that the *are2* and *srb8* mutations are tightly linked. All segregants of either mating-type were clumpy and all α segregants were derepressed for *MFA2*::lacZ (data not shown). Taken together, these results indicate that *ARE2* is identical to *SRB8*.

Growth phenotype of the *srb8* deletion. While the *srb8* deletion is not lethal, *srb8* deletion mutants obtained by sporulation and tetrad dissection of an *srb8Δ::LEU2/SRB8* diploid (MWY43) are clumpy and grow more slowly than the wild-type *SRB8* spore colonies. The mutant spore colonies readily acquire mutations that partially suppress the clumpy growth; sectors of faster growing cells within the *srb8Δ* spore colony appear at high frequency (2 to 4 sectors per spore colony). Two distinct colony types arise upon streaking out the mutant

spore colonies, partially clumpy (apparently arising from cells in the faster growing sectors) and smaller, very clumpy colonies. As described in ref. 47, this same sectoring phenomenon was observed in *srb10* mutant spore colonies obtained by sporulation and tetrad dissection of an *srb10Δ::LEU2/SRB10* diploid. Despite the slow growth phenotype, the *srb8Δ::LEU2* strain is not able to grow at 37°C.

Level of *MFA2::lacZ* derepression in an *srb8* deletion mutant. To determine whether the *srb8* deletion has a greater effect on α2 repression than does the *are2-13* mutation (the strongest *are2* allele isolated in our screen), the small, very clumpy **a** and α *srb8Δ::LEU2* segregants were isolated and the levels of *MFA2::lacZ* expression were quantitated using a β-galactosidase assay (see Materials and Methods). We define repression as the ratio of β-galactosidase activity in an **a** cell to that in the isogenic α cell. As indicated in Table 2, the decrease in repression caused by the *srb8* deletion is not significantly different from that resulting from the *are2-13* mutation. In the former case, repression is decreased by 87%, and in the latter case, by 92%.

A genetic interaction between *TUP1* and *SRB8*. Although both the *are2-13* and *srb8Δ::LEU2* mutations cause similar decreases in α2 repression of *MFA2::lacZ* , they are differentially suppressed by *TUP1*. Overexpression of *TUP1* suppresses the *MFA2::lacZ* derepression in the *are2-13* strain but not in the *srb8Δ::LEU2* deletion strain (Fig. 1). The suppression by *TUP1*

overexpression is quantitated in Table 3. *TUP1* overexpression in the α *are2-13* strain reduces the level of *MFA2::lacZ* expression from 5 units of β-galactosidase activity to 0.9 units, the level seen in the wild-type α strain. In contrast, *TUP1* overexpression does not significantly restore repression of *MFA2::lacZ* in the α *srb8Δ::LEU2* strain (9.9 units of β-galactosidase activity in the absence of *TUP1* overexpression and 6.4 units in its presence). From this result, it appears that *SRB8* is not simply a transcriptional activator of *TUP1*, since if *SRB8* stimulated transcription of *TUP1*, then *TUP1* overexpression should suppress both alleles. Consistent with this conclusion, levels of *TUP1* transcript are not significantly reduced in an α *are2* strain compared to levels in an isogenic wild-type strain (data not shown). Furthermore, this genetic interaction supports the view that Ssn6p-Tup1p interacts with the holoenzyme (see Discussion).

Level of derepression in an *srb8 srb10* double mutant. Ssn6p-Tup1p may exert repression by interacting with several different SRB proteins. Interaction with more than one SRB protein would explain why a null *are1/srb10* allele (47) and the *are2/srb8* alleles examined here and in ref. 47 cause only partial loss of repression whereas repression is eliminated by null *ssn6* and *tup1* alleles (14, 47). If multiple contacts between Ssn6p-Tup1p and different SRB proteins do exist, then *srb* double mutants might cause greater loss of repression than either mutant alone. An *srb10 srb11* double mutant, however, has no greater effect on glucose repression than does either single mutant (19). This is not an unexpected result since *SRB11* appears to encode the cyclin partner of the

Srb10p protein kinase (19, 22). We tested whether or not an *srb8 srb10* double mutant has a greater effect on α2 repression than either single mutant by comparing the level of *MFA2::lacZ* repression in strains carrying either one or both mutations. As indicated in Table 4, repression in either single mutant is more than twice that in the double mutant (14 or 25 in the single mutants vs. 6.7 in the double mutant). These observations are consistent with the view that Ssn6p-Tup1p brings about repression by interacting with several different SRB proteins.

DISCUSSION

We have demonstrated here that *ARE2* is identical to *SRB8* and have shown elsewhere that *ARE1* is identical to *SRB10* (47). The *SRB* genes (suppressor of RNA polymerase B) were isolated as suppressors of a cold-sensitive growth defect caused by partial truncation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (pol II) (15, 22, 27, 43). The CTD plays a role in transcriptional regulation since, in *S. cerevisiae*, for example, partial deletions of the CTD cause defects in inducible gene expression (29, 32) and also enhance the effect of certain deletion mutations in the Gal4p transcriptional activator (1); the exact role the CTD plays in transcriptional regulation is not well understood. Biochemical studies have shown that the SRB proteins are associated with pol II, several general transcription factors, and other proteins, in a complex termed the pol II holoenzyme (8, 15, 17, 22, 43). The pol II holoenzyme appears to be a form of

pol II that is required for general transcription (44).

Based on the finding that *ARE1* is identical to *SRB10*, we proposed that Ssn6p-Tup1p brings about α2 repression via a physical interaction with the SRB proteins (47). Despite the appeal of this model, there is an alternative and less interesting explanation for the involvement of Srb10p in α2 repression. The effect of the *srb10* mutation on repression could be indirect and result from defects in transcriptional activation of *MATα2*. For example, Northern blot analysis demonstrated that levels of *MATα2* transcript are 35% lower in an *α are1/srb10* mutant compared with levels in an isogenic wild-type strain (data not shown). Furthermore, *MATα2* overexpression suppresses the *MFA2::lacZ* derepression in *α are1* mutants (data not shown). It is possible that the derepression caused by the *are1/srb10* mutation is simply due to lower levels of α2 protein.

The identification of *ARE2* as *SRB8*, however, has provided additional support for a direct role of the *SRB* proteins in α2 repression. Levels of *MATα2* are not significantly lower in an *α are2* mutant compared to that in the isogenic wild-type strain, and *MATα2* overexpression does not suppress the *are2* defect (data not shown). Thus, Srb8p is not simply a positive regulator of *MATα2*. Second, the allele-specific suppression of *srb8* by overexpression of *TUP1* indicates that Srb8p is also not a positive regulator of *TUP1*.

The identity of both *ARE1* and *ARE2* to *SRB* genes strengthens the notion

that Ssn6p-Tup1 brings about repression by interacting with the holoenzyme. Perhaps some of the SRB proteins inhibit a step in transcription initiation in response to interaction(s) with Ssn6p-Tup1p. Another possibility is that Ssn6p-Tup1p binds tightly to the holoenzyme through multiple contacts with SRB proteins. This tight association could anchor the holoenzyme to the promoter, preventing it from beginning elongation. In either case, multiple contacts between Ssn6p-Tup1p and the SRB proteins could explain why single *srb* mutations (*srb8* or *srb10*) cause only partial loss of α2 repression (47). Consistent with this view, we found that there is greater loss of repression in an *srb8 srb10* double mutant than in either the *srb8* or *srb10* single mutant. In addition, the allele-specific interaction between *TUP1* and *SRB8* could suggest a physical interaction between Tup1p and Srb8p. One possibility is that the protein encoded by *are2-13* binds to Tup1p with lower affinity than does the wild-type protein and that *TUP1* overexpression drives this weakened interaction forward.

Ssn6p-Tup1p may interact with four (out of the nine) SRB proteins, namely, Srb8p, Srb9p, Srb10p, and Srb11p (Figure 2). Recessive mutations in the genes encoding these four SRB proteins cause phenotypes similar to some of those resulting from mutations in *SSN6* and *TUP1*: slow growth, clumpiness, and defects in α2 and glucose repression (8, 19, 22, 47, 48). In fact, mutations in these four *SRB* genes were independently identified in a screen for defects in glucose repression (4, 19, 48). The independent isolation of genes that encode components of the pol II holoenzyme in genetic screens for defects in repression

requiring Ssn6p-Tup1p implies an important role for the SRB proteins in transcriptional repression.

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REFERENCES

1. **Allison, L. A., and C. J. Ingles.** 1989. Mutations in RNA polymerase II enhance or suppress mutations in *GAL4*. Proc. Natl. Acad. Sci. USA **86**:2794-2798.

2. **Allison, L. A., J. K.-C. Wong, V. D. Fitzpatrick, M. Moyle, and C. J. Ingles.** 1988. The C-terminal domain of the largest subunit of RNA polymerase II of *Saccharomyces cerevisiae, Drosophila melanogaster,* and mammals: a conserved structure with an essential function. Mol. Cell. Biol. **8**:321-329.

3. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8**:17-24.

4. **Carlson, M., B. C. Osmond, L. Neigeborn, and D. Botstein.** 1984. A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107**:19-32.

5. **Dahmus, M. E.** 1995. Phosphorylation of the C-terminal domain of RNA polymerase II. Biochem. Biophys. Acta **1261**:171-182.

6. **Elledge, S. J., Z. Zhou, J. B. Allen, and T. A. Navas.** 1993. DNA damage and cell cycle regulation of ribonucleotide reductase. Bioessays **15**:333-339.

7. **Hall, M. N. and A. D. Johnson.** 1987. Homeo domain of the yeast repressor α2 is a sequence-specific DNA-binding domain but is not sufficient for repression. Science **237**:1007-1012.

8. **Hengartner, C. J., C. M. Thompson, J. Zhang, D. M. Chao, S.-M. Liao, A. J. Koleske, S. Okamura, and R. A. Young.** 1995. Association of an activator with an RNA polymerase II holoenzyme. Genes Dev. **9**:897-910.

9. **Herskowitz, I., J. Rine, and J. Strathern.** 1992. Gene expression, p. 583-656. *In* E. W. Jones, J. R. Pringle, and J. R. Broach (ed.), The molecular and cellular biology of the yeast *Saccharomyces*, Volume II. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.

10. **Huxley, C., E. D. Green, and I. Dunham.** 1990. Rapid asssessment of *S. cerevisiae* mating type by PCR. Trends Genet. **6**:236.

11. **Ito, H., K. Fukada, K. Murata, and A. Kimura.** 1983. Transformation of intact yeast cells treated with alkali cations. J. Bacteriol. **153**:163-168.

12. **Johnson, A. D. and I. Herskowitz.** 1985. A repressor (*MATα2* product) and its operator control expression of a set of cell type specific genes in yeast. Cell **42**:237-247.

13. **Keleher, C. A., C. Goutte, and A. D. Johnson.** 1988. The yeast cell-type-specific repressor α2 acts cooperatively with a non-cell-type-specific protein. Cell **53**:927-936.

14. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. SSN6-TUP1 is a general repressor of transcription in yeast. Cell **68**:927-937.

15. **Kim, Y.-J., S. Björklund, Y. Li, M. H. Sayre, and R. D. Kornberg.** 1994. A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77**:599-608.

16. **Koleske, A. J., S. Buratowski, M. Nonet, and R. A. Young.** 1992. A novel transcription factor reveals a functional link between the RNA polymerase II CTD and TFIID. Cell **69**:883-894.

17. **Koleske, A. J. and R. A. Young.** 1994. An RNA polymerase II holoenzyme responsive to activators. Nature **368**:466-469.

18. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD repeats of TUP1 interact with the homeodomain protein α2. Genes Dev. **8**:2857-2867.

19. **Kuchin, S. V., P. Yeghiayan, and M. Carlson.** 1995. SSN8-SSN3, a cyclin and cyclin-dependent kinase pair that contribute to transcriptional control in yeast. Proc. Natl. Acad. Sci. USA **92**:4006-4010.

20. **Lemontt, J. F., D. R. Fugit, and V. L. MacKay.** 1980. Pleiotropic mutations at the *TUP1* locus that affect the expression of mating-type-dependent functions in *Saccharomyces cerevisiae*. Genetics **94**:899-920.

21. **Liao, S.-M., I. C. Taylor, R. E. Kingston, and R. A. Young.** 1991. RNA polymerase II carboxy-terminal domain contributes to the response to multiple acidic activators *in vitro*. Genes Dev. **5**:2431-2440.

22. **Liao, S.-M., J. Zhang, D. A. Jeffery, A. J. Koleske, C. M. Thompson, D. M. Chao, M. Viljoen, H. J. J. Van Vuuren, and R. A. Young.** 1995. A

kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature **374:**193-196.

23. **McKnight, S. L. and K. R. Yamamoto.** 1992. Transcriptional regulation, Vol. I and II. Cold Spring Harbor Laboratory Press, Plainview, NY.

24. **Mortimer, R. K. and D. C. Hawthorne.** 1969. Yeast genetics, p. 385-460. *In* A. H. Rose and J. S. Harrison (ed.), The yeasts. Academic Press, NY.

25. **Mukai, Y., S. Harashima, and Y. Oshima.** 1991. AAR1/TUP1 protein, with a structure similar to that of the β subunit of G proteins, is required for a1-α2 and α2 repression in cell type control of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **11:**3773-3779.

26. **Nonet, M., D. Sweetser, and R. A. Young.** 1987. Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. Cell **50:**909-915.

27. **Nonet, M. L. and R. A. Young.** 1989. Intragenic and extragenic supressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. Genetics **123:**715-724.

28. **Oliver, S. G., Q. J. M. van der Aart, M. L. Agostoni-Carbone, M. Aigle, et al**. 1992. The complete DNA sequence of yeast chromosome III. Nature **357:**38-46.

29. **Peterson, C. L., W. Kruger, and I. Herskowitz.** 1991. A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. Cell **64:**1135-1143.

30. **Rose, M. D., P. Novick, J. H. Thomas, D. Botstein, and G. R. Fink.** 1987. A *Saccharomyces cerevisiae* genomic plasmid bank based on a centromere-containing shuttle vector. Gene **60:** 237-243.

31. **Rothstein, R. J. and F. Sherman.** 1980. Genes affecting the expression of cytochrome c in yeast: genetic mapping and genetic interactions. Genetics **94:**871-889.

32. **Scafe, C., D. Chao, J. Lopes, J. P. Hirsch, S. Henry, and R. A. Young.** 1990. RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. Nature **347:**491-494.

33. **Schamhart, D. H. J., A. M. A. Ten Berge, and K. W. Van De Poll.** 1975. Isolation of a catabolite repression mutant of yeast as a revertant of a strain that is maltose negative in respiratory deficient state. J. Bacteriol. **121:**747-752.

34. **Schena, M., L. P. Freedman, and K. R. Yamamoto.** 1989. Mutations in the glucocorticoid receptor zinc finger region that distinguish interdigitated DNA binding and transcriptional enhancement activities. Genes Dev. **3:**1590-1601.

35 **Schultz, J. and M. Carlson.** 1987. Molecular analysis of *SSN6*, a gene functionally related to the SNF1 protein kinase of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **7:**3637-3645.

36. **Schultz, J., L. Marshall-Carlson, and M. Carlson.** 1990. The N-terminal TPR region is the functional domain of SSN6, a nuclear

phosphoprotein of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**4744-4756.

37. **Sherman, F., G. R. Fink, and C. W. Lawrence.** 1979. Methods in yeast genetics. Cold Spring Harbor Laboraory, Cold Spring Harbor, NY.

38. **Sikorski, R. S. and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19-27.

39. **Sikorski, R. S. and J. D. Boeke.** 1991. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*, p. 302-318. *In* C. Guthrie and G. R. Fink (ed.), Guide to yeast genetics and molecular biology. Academic Press, San Diego.

40. **Siliciano, P. G. and K. Tatchell.** 1984. Transcription and regulatory signals at the mating type locus in yeast. Cell **37:**969-978.

41. **Strich, R., M. R. Slater, and R. E. Esposito.** 1989. Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. Proc. Natl. Acad. Sci. USA **86:**10018-10022.

42. **Surosky, R. T., R. Strich, and R. E. Esposito.** 1994. The yeast *UME5* gene regulates the stability of meiotic mRNAs in response to glucose. Mol. Cell. Biol. **14:**3446-3458.

43. **Thompson, C. M., A. J. Koleske, D. M. Chao, and R. A. Young.** 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73:**1361-1375.

44. **Thompson, C. M. and R. A. Young.** 1995. General requirement for RNA polymerase II holoenzymes *in vivo*. Proc. Natl. Acad. Sci. USA **92:**4587-4590.

45. **Trumbly, R. J.** 1992. Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **6:**15-21.

46. **Tzamarias, D. and K. Struhl.** 1994. Functional dissection of the yeast CYC8-TUP1 transcriptional co-repressor complex. Nature **369:** 758-761.

47. **Wahi, M. and A. D. Johnson.** 1995. Identification of genes required for α2 repression in *Saccharomyces cerevisiae*. Genetics **140:**79-90.

48. **Wenjie, S., I. Treich, N. Qian, S. Kuchin, and M. Carlson.** Mol. Cell. Biol. in press.

49. **Wickner, R. B.** 1974. Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine-5'monophosphate in deoxyribonucleic acid *in vivo*. J. of Bacteriol. **117:**252-260.

50. **Williams, F. E. and R. J. Trumbly.** 1990. Characterization of *TUP1*, a mediator of glucose repression in *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:**6500-6511.

51. **Williams, F. E., U. Varanasi, and R. J. Trumbly.** 1991. The CYC8 and TUP1 proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. Mol. Cell. Biol. **11:**3307-3316.

52. **Zitomer, R. S. and C. V. Lowry.** 1992. Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. Microbiol. Rev. **56:**1-11.

Figure legends

Figure 1. Allele-specific suppression of *are2/srb8* by *TUP1* overexpression. *MFA2::lacZ* strains containing the indicated *are2/srb8* allele were transformed with either the high-copy *URA3* plasmid, YEp24, or a YEp24 vector bearing *TUP1* (pFW28). Transformants were patched on an SD plate lacking uracil, and then an X-Gal filter assay was performed (see Materials and Methods). Patches of strains expressing *MFA2::lacZ* turn blue (grey in photo) on X-Gal, whereas those repressing *MFA2::lacZ* remain white. For comparison, isogenic wild-type **a** and α *MFA2::lacZ* strains (SM1179 and SM1196, respectively) carrying YEp24 were also assayed.

Figure 2. Model for α2 repression of the **a**-specific genes. α2-Mcm1p recruits Ssn6p-Tup1p to the upstream control region of the **a**-specific genes. Ssn6p-Tup1p then interacts with the RNA polymerase (pol II) holoenzyme, which is comprised of pol II, several general transcription factors, and other proteins, including those termed mediators or SRB proteins. The interaction between Ssn6p-Tup1p and the holoenzyme may involve multiple contacts with a subset of the SRB proteins (see text). In response to this interaction, the SRB proteins might inhibit transcription initiation. Another possibility is that through these multiple contacts, Ssn6-Tup1 anchors the holoenzyme to the promoter, thereby preventing pol II from leaving the promoter to begin elongation. Biochemical evidence indicates that Srbp10 and Srb11p, a cyclin C

homologue, physically interact (19, 22). It is not yet known whether these two SRB proteins interact directly with Srb8p and Srb9p.

Table 1. *S. cerevisiae* strains

Strain	Genotype
Haploids	
246-1-1	*MATα can1 gal2 his4 leu2 suc2Δ trp1 ura3*
EG123	*MATa can1 gal2 his4 leu2 suc2Δ trp1 ura3*
SM1196	*MATα MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
SM1179	*MATa MFA2::lacZ can1gal2 his4 leu2 suc2Δ trp1 ura3*
MWY2	*MATa tup1Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY16	*MATα are2-13 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY44	*MATa srb8Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
MWY45	*MATα srb8Δ::LEU2 MFA2::lacZ can1 gal2 his4 leu2 suc2Δ trp1 ura3*
Diploids	
MWY33	SM1179 × SM1196
MWY43	$\frac{MAT\alpha}{MATa}$ $\frac{srb8\Delta::LEU2}{SRB8}$ $\frac{MFA2::lacZ}{MFA2::lacZ}$ $\frac{can1}{can1}$ $\frac{gal2}{gal2}$ $\frac{his4}{his4}$ $\frac{leu2}{leu2}$ $\frac{suc2\Delta}{suc2\Delta}$ $\frac{trp1}{trp1}$ $\frac{ura3}{ura3}$

Table 2. Expression of *MFA2::lacZ* in wild-type and *are2/srb8* strains

	Units of β-galactosidase activity		
Genotype	*MATa*	*MATα*	Repression
ARE$^+$	130 ± 20	1.1 ± 0.3	120
are2-13	97 ± 25	9.4 ± 2.2	10 (92%)
srb8Δ::LEU2	120 ± 20	7.7 ± 0.7	16 (87%)

Numbers represent units of β-galactosidase activity in *MFA2::lacZ* strains of the indicated genotype. Results are averages of assays performed in triplicate. At least three independent colonies for each strain were assayed. Numbers in parentheses indicate the percent decrease in repression relative to that in the wild-type strains SM1179 (*MATa*) and SM1196 (*MATα*). All strains were grown in YEPD.

Table 3. Effect of *TUP1* overexpression on *MFA2::lacZ* expression in *are2/srb8* strains.

Genotype	Plasmid	Units of β-galactosidase activity
a ARE$^+$	YEp24	120 ± 14
α ARE$^+$	YEp24	0.9 ± 0.03
α are2-13	YEp24	5.0 ± 1.5
α are2-13	YEp24-*TUP1*	0.9 ± 0.2
α srb8Δ::LEU2	YEp24	9.9 ± 0.7
α srb8Δ::LEU2	YEp24-*TUP1*	6.4 ± 1.4

Numbers represent units of β-galactosidase activity in *MFA2::lacZ* strains of the indicated genotype (see Table 1). Strains carry either the control plasmid YEp24 or YEp24 containing *TUP1* (pFW28). Results are averages of assays performed in triplicate. Two independent colonies for each strain were assayed. All strains were grown in SD medium lacking uracil.

Table 4. Double mutant analysis

Units of β-galactosidase activity			
Genotype	*MATa*	*MATα*	Repression
ARE$^+$	180 ± 30	1.1 ± 0.1	160
are2-13	100 ± 10	7.0 ± 2.9	14
are1Δ::LEU2	160 ± 10	6.3 ± 3.5	25
are1Δ::LEU2 are2-13	140 ± 5	21 ± 4	6.7

Numbers represent units of *b*-galactosidase activity in *MFA2::lacZ* strains of the indicated genotype (see Table 1). Results are averages of assays performed in triplicate. Two independent colonies for each strain were assayed. All strains were grown in YEPD.

β-Galactosidase Activity

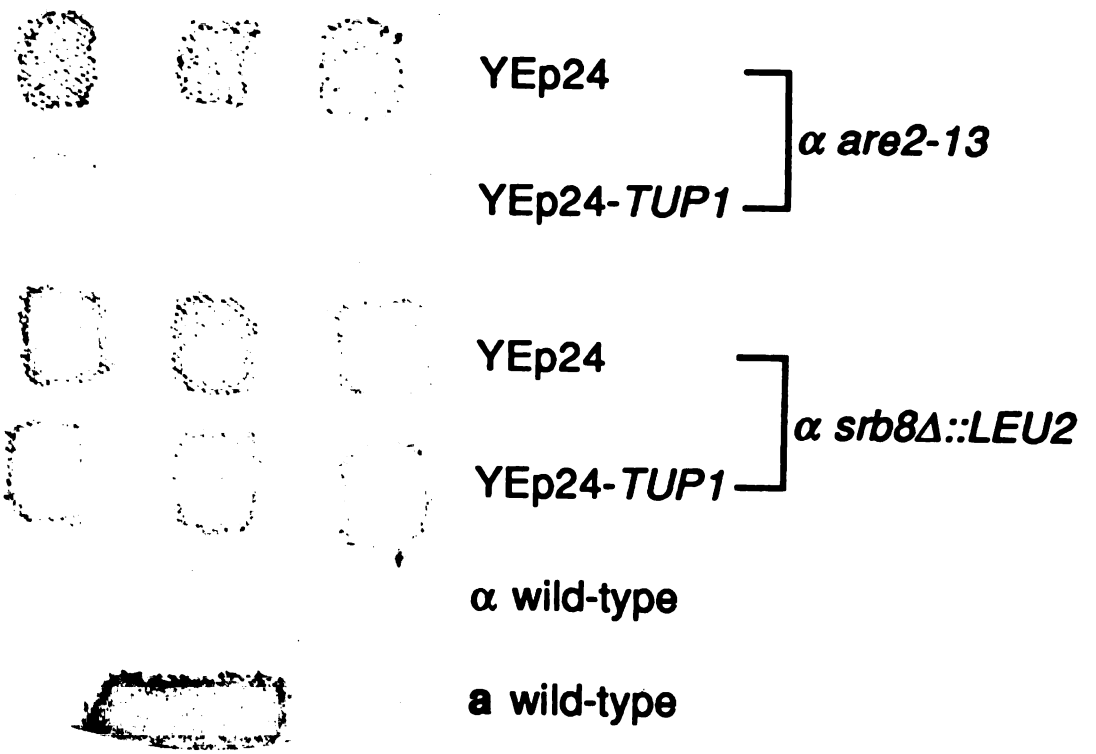

Figure 1

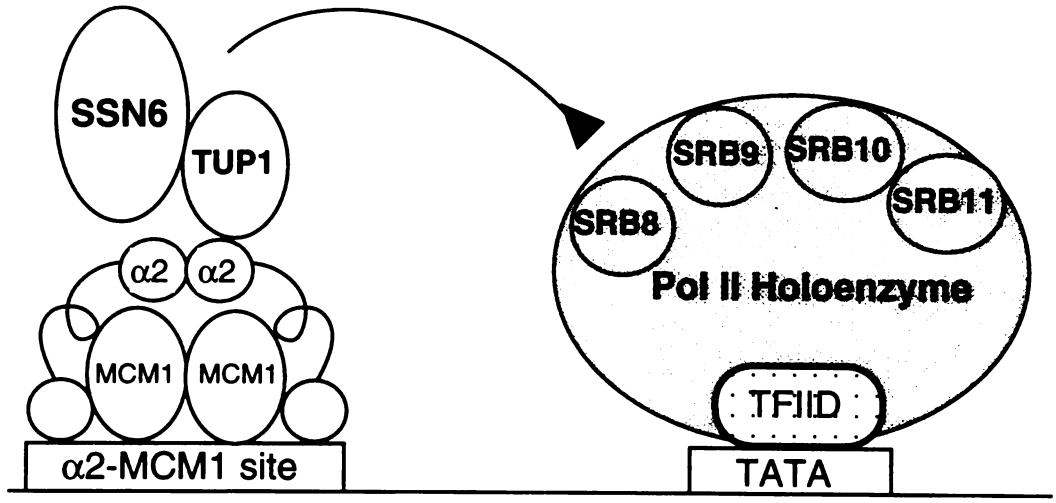
SSN6
TUP1
α2
α2
MCM1
MCM1
α2-MCM1 site
SRB9
SRB10
SRB11
SRB8
Pol II Holoenzyme
TFIID
TATA

Figure 2

Chapter 4

Conclusion

I have isolated mutants with defects in repression of the a-specific genes (α2 repression) in *S. cerevisiae* α cells. As expected, I recovered mutations in genes previously known to be required for α2 repression, namely, *MATα2*, *SSN6*, *TUP1* and *SIN4*. In addition, I recovered mutations in four other genes, designated the *ARE* genes for alpha2 repression. Like mutations in *SSN6*, *TUP1*, and *SIN4*, the *are* mutations cause pleiotropic phenotypes including clumpiness, slow growth, decreased sporulation efficiency, and abnormal cell morphology (Wickner, 1974; Schamhart et al., 1975; Lemontt et al., 1980; Rothstein and Sherman, 1980; Carlson et al., 1984; Jiang and Stillman, 1992; Chen et al., 1993). This similarity suggests a functional relationship between the *ARE* gene products and the previously identified general negative regulators required for α2 repression.

I found that these general negative regulators and the *ARE* gene products can be divided into two classes based on their ability to repress basal transcription, i.e., transcription in the absence of known upstream activating sequences (UASs). SIN4, ARE3, and ARE4 belong to the class which represses basal transcription. Mutations in *SIN4* strongly derepress at least four different UAS-less reporter genes (Jiang and Stillman, 1992; Chen et al., 1993). Mutations in both *ARE3* and *ARE4* strongly derepress basal transcription of the two UAS-less reporter genes tested in this study. Two additional observations are consistent with grouping these gene products together: first, both the *sin4* and *are4-87* mutations significantly increase activated

transcription of the *CYC1-lacZ* test gene, unlike mutations in *TUP1* or the other *ARE* genes; second, overexpression of *SIN4* partially suppresses the *are4-87* mutation.

SSN6, TUP1, ARE1, and ARE2 belong to the other class. Mutations in *TUP1*, *ARE1*, and *ARE2* do not significantly derepress at least two different UAS-less reporter genes. Furthermore, genetic interactions I observed are consistent with this grouping: *TUP1* overexpression suppresses a mutation in *ARE2*; and alleles of *ARE1* and *ARE2* display unlinked noncomplementation, suggesting a possible physical interaction between the ARE1 and ARE2 proteins (Vinh et al., 1993). Because SSN6 and TUP1 function together as transcriptional repressors (Keleher et al., 1992; reviewed in Johnston and Carlson, 1992; Trumbly, 1992), SSN6, like TUP1, is not expected to repress basal transcription, although this was not tested. It is not clear how SSN6-TUP1-ARE1-ARE2 cooperates with ARE3-ARE4-SIN4 to bring about full levels of α2 repression.

The molecular characterization of the *ARE* genes should help elucidate their role in repression. I decided to focus on *ARE1* and *ARE2* since they seem more closely related to *SSN6* and *TUP1* than do *ARE3* and *ARE4*. Cloning and sequencing *ARE1* revealed that it encodes a CDC28-related protein kinase, and has been previously identified as *UME5* (Surosky et al., 1994), *SSN3* (Kuchin et al., 1995), and *SRB10* (Liao et al., 1995). The *UME* genes were identified in a screen for mutants defective in repression of early meiosis-specific genes during vegetative growth (Strich et al., 1994). The *SSN*

genes were identified in a screen for mutants defective in glucose repression (Carlson et al., 1984), a TUP1-SSN6-dependent process (Trumbly, 1992). The fact that *ARE1* is also required for glucose repression supports the view that *ARE1* is important for SSN6-TUP1-mediated repression. Finally, the *SRB* (suppressor of RNA polymerase B) genes were identified because mutations in these genes suppress a growth defect caused by partial truncation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (pol II) (Nonet et al., 1989; Thompson et al., 1993; Liao et al., 1995; Hengartner et al., 1995). The cloning of *ARE2* revealed it to be identical to another *SRB* gene, *SRB8*. Unlike the SRB10 protein, SRB8 has no obvious homologies to other proteins in databases. Because the identity of *ARE1* and *ARE2* to *SRB* genes provides a clue to the mechanism of α2 repression, the *SRB* genes and the CTD deserve further attention.

The CTD consists of a series of highly conserved heptapeptide repeats; in *S. cerevisiae* there are 26 or 27 repeats, in *Drosophila melanogaster* 45, and in mouse 52 (reviewed in Dahmus, 1995). In *S. cerevisiae*, CTD deletions leaving less than ten repeats are lethal, while partial deletions of the CTD result in growth defects such as cold- and temperature-sensitivity (Nonet et al., 1987; Allison et al., 1988). The CTD plays a role in transcriptional regulation since, for example, these partial deletions cause defects in inducible gene expression at some promoters (Scafe et al., 1990; Peterson et al., 1991), and also enhance the effect of a crippled GAL4 transcriptional activator on activation (Allison and Ingles, 1989). The exact role of the CTD in

transcriptional regulation is not well understood.

Biochemical studies have recently shown that the SRB proteins are associated together, along with pol II, TFIIB, TFIIF, TFIIH, and several other proteins, in a complex termed the pol II holoenzyme (Thompson et al., 1993; Koleske and Young, 1994; Kim et al., 1994; Liao et al., 1995; Hengartner et al., 1995). The model of how the general transcription machinery assembles at promoters has recently been revised to take into account these findings (Koleske and Young, 1994). At least in yeast, it is now thought that first, TFIID binds the TATA box, then the pol II holoenzyme binds the TATA-TFIID complex, and finally, TFIIE binds the TATA-TFIID-holoenzyme.

The pol II holoenzyme is a form of polymerase that is required for transcription, since a temperature-sensitive *srb4* allele prevents expression of a wide spectrum of genes, including the a-specific gene, *STE2*, at the restrictive temperature (Thompson and Young, 1995). Furthermore, the dramatic decrease in general transcription upon shifting the *srb4* mutant to the restrictive temperature mimics that which is caused by a temperature-sensitive allele of *RPB1*, which encodes the largest subunit of pol II (Thompson and Young, 1995). The holoenzyme contains most of the SRB protein in the cell, whereas it contains only about 6% of pol II (Koleske and Young, 1994). The SRB-free pol II is presumably the elongating form of pol II as well as pol II that has finished elongating but has not yet reassociated with the SRB proteins.

The finding that *ARE1* is identical to *SRB10* leads to a specific model for

the mechanism of α2 repression. Genetic evidence suggests that SRB10 plays a role in both transcriptional activation and repression. However, the exact role of SRB10 in transcriptional regulation is unclear. Induction of a *GAL* promoter by galactose *in vivo* is significantly reduced in *srb10* mutants (Liao et al., 1995), suggesting that SRB10 is a positive regulator of transcription. On the other hand, SRB10 was identified in three independent genetic screens for negative regulators (Carlson et al., 1984; Surosky et al., 1994; Kuchin et al., 1995; and this study). The role of SRB10 in either repression or activation may be indirect. However, as described below, it is conceivable that SRB10 is directly involved in both repression and activation through phosphorylation of the CTD.

Two forms of pol II exist *in vivo*: one which contains an unphosphorylated CTD (pol IIA), and one in which the CTD is extensively phosphorylated (pol IIO) (reviewed in Dahmus, 1995). The state of CTD phosphorylation changes during transcription. Pol IIA is incorporated into the general transcription machinery preferentially over pol IIO (Laybourn and Dahmus, 1989; Lu et al., 1991; Chesnut et al., 1992; reviewed in Dahmus, 1995). Later during transcription initiation or shortly thereafter, pol IIA becomes extensively phosphorylated, generating pol IIO, the elongating form of pol II (Laybourn and Dahmus, 1990; reviewed in Dahmus, 1995). After elongation, the CTD is dephosphorylated and the polymerase can once again become incorporated into the general transcription machinery (reviewed in Dahmus, 1995). SRB10 is believed to phosphorylate the CTD either directly, or

indirectly by activating a CTD kinase. Liao et al. (1995) have shown that CTD phosphorylation is reduced ten-fold in holoenzyme purified from an *srb10* mutant strain compared with a wild-type strain. Furthermore, there is a five-fold reduction in the *in vitro* phosphorylation of recombinant GST-CTD fusion protein by holoenzyme purified from *srb10* cells compared with wild-type cells (Liao et al., 1995).

It is not known at what point in the transcription cycle SRB10 acts. The timing of CTD phosphorylation by SRB10, however, could be central to the role of SRB10 in transcriptional activation and repression. If SRB10 phosphorylates the CTD during transcription initiation, but *after* the general transcription machinery has assembled, the phosphorylation could increase the rate of transcription initiation. The phosphorylation of the CTD is known to cause a conformational change in the CTD (reviewed in Dahmus, 1995). It has been proposed that at some promoters this change in conformation disrupts some of the contacts pol II makes with other components of the transcription machinery complex, and that this disruption facilitates promoter clearance, a final step in transcription initiation in which pol II leaves the promoter to begin the processive elongation phase of transcription (Laybourn and Dahmus, 1989; Dahmus, 1995). A specific DNA-binding activator could activate transcription by stimulating SRB10 to phosphorylate the CTD after the general transcription machinery has assembled

If, however, SRB10 phosphorylates the CTD *before* assembly of the general transcription machinery is complete, the phosphorylation might

decrease the rate of transcription initiation. Biochemical studies have indicated that pol IIA is not well incorporated into the general transcription machinery, although the basis for this inability is unclear (Laybourn and Dahmus, 1990; Lu et al., 1991; Chesnut et al., 1992; reviewed in Dahmus, 1995). One could imagine that premature phosphorylation of the CTD causes the dissociation of some holoenzyme components required for transcription initiation, or perhaps the disassembly of the nascent transcription machinery complex. I propose that once recruited to the upstream control region of the **a**-specific genes by α2-MCM1, SSN6-TUP1 binds to the pol II holoenzyme, before the assembly of the general transcription machinery is complete. After binding to the holoenzyme, SSN6-TUP1 stimulates SRB10 to prematurely phosphorylate the CTD, resulting in a defective transcription machinery complex. Upon phosphorylation of the CTD, SSN6-TUP1 releases the holoenzyme and becomes available for binding another holoenzyme containing an unphosphorylated CTD. Alternatively, SSN6-TUP1 could remain bound to the holoenzyme and anchor the defective complex to the promoter.

I propose that SSN6-TUP1 interacts with the holoenzyme through multiple contacts with SRB proteins, more specifically, through contacts with a subset of the nine SRB proteins: SRB8, SRB9, SRB10, and SRB11. This subset of SRB proteins appears functionally related to SSN6-TUP1. Recessive mutations in this group of *SRB* genes cause phenotypes similar to some of those resulting from mutations in *SSN6* and *TUP1*, namely, slow growth,

clumpiness, and defects in α2 and glucose repression (Liao et al., 1995; Hengartner et al., 1995; Kuchin et al., 1995). Recently, other *SSN* genes have been identified as members of this group: *SSN2* as *SRB9*, *SSN5* as *SRB8/ARE2*, and *SSN8* as *SRB11* (Kuchin et al., 1995; Wenjie et al., in press). As previously mentioned, *SRB10/ARE1* is identical to *SSN3* (Kuchin et al., 1995). Thus, two SSN6-TUP1-dependent repression pathways appear to converge on this particular class of SRB proteins. Furthermore, the allele-specific suppression of *srb8* by *TUP1* suggests a direct interaction between TUP1 and the SRB8 protein, a member of this group. One possibility is that the protein encoded by *are2-13* binds to TUP1 with lower affinity than does the wild-type protein and that TUP1 overexpression drives this weakened interaction forward. The putative interactions between SSN6-TUP1 and these SRB proteins could increase the binding affinity of SSN6-TUP1 for the holoenzyme and could also affect a catalytic function of the holoenzyme in addition to phosphorylation of the CTD.

Of course, SSN6-TUP1 may exert repression independently of the phosphorylation of the CTD. Another way that SSN6-TUP1 could exert repression is by tightly binding to the holoenzyme via multiple interactions with the SRB proteins. Perhaps SSN6-TUP1 stimulates SRB10 to phosphorylate an SRB protein. The phosphorylated SRB protein would bind SSN6-TUP1 with higher affinity than the unphosphorylated form. A strong interaction between SSN6-TUP1 and the holoenzyme would serve to anchor pol II to the promoter, thereby preventing it from leaving the promoter to

begin elongation.

Final proof of a direct interaction between SSN6-TUP1 and the holoenzyme will come from biochemical studies. Convincing evidence of a direct interaction between SSN6-TUP1 and the SRB proteins would include a demonstration that SSN6 or TUP1 copurify with the SRB proteins from yeast. In short, the SRB proteins, particularly SRB8, 9, 10, and 11, should be epitope-tagged, expressed in yeast, and purified. The purified fractions can be analyzed for the presence of TUP1 or SSN6 by separating proteins in the purified fractions in an SDS-polyacrylamide gel, and then probing Western blots of this gel with anti-TUP1 and anti-SSN6 antibodies. If interactions between SSN6-TUP1 and the SRB proteins are too weak to detect in yeast extracts, two other approaches, which rely on affinity chromatography, may have better success. An advantage of affinity chromatography is that it may detect interactions too weak to detect by copurification procedures. First, one could try purifying epitope-tagged SRB proteins expressed in *E. coli* or yeast and then using them to test for interaction with SSN6-TUP1 purified from yeast, a reagent now available in our laboratory (Michael Redd and A. D. Johnson, unpublished results). Second, one could try producing full-length SRB proteins using an *in vitro* ^{35}S-methionine-labelling translation system. The labelled SRB proteins can be tested for interaction with GST-TUP1 or GST-SSN6 (purified from *E. coli*) or with SSN6-TUP1 (purified from yeast) by affinity chromatography.

Assuming that SSN6-TUP1 does interact with SRB proteins, another

question is whether SSN6-TUP1 regulates the kinase activity of SRB10, and more specifically, whether SSN6-TUP1 stimulates SRB10 to phosphorylate the CTD. To pursue this question, components of the holoenzyme purified from *ssn6* or *tup1* mutant cells and from wild-type cells can be separated by SDS-polyacrylamide gel electrophoresis. Components can then be analyzed by Western blotting using antibodies against individual holoenzyme components. The mobility of components from wild-type and mutant cells can be compared to determine whether there are any shifts consistent with a change in the state of phosphorylation. Any components that seem to have shifted mobility can be tested *in vitro* for a direct interaction with SRB10, or for phosphorylation by SRB10 in the presence or absence of SSN6-TUP1. To test more directly whether SSN6-TUP1 stimulates phosphorylation of the CTD, purified holoenzyme can be incubated with γ-^{32}P-ATP in the presence or absence of purified SSN6-TUP1. After incubation, the phosphorylation state of the CTD in holoenzyme incubated with and without SSN6-TUP1 can be compared. Whether SSN6-TUP1 stimulates phosphorylation of the CTD can be tested in a more simple *in vitro* system using purified SSN6-TUP1, epitope-tagged SRB10 and SRB11, which is the cyclin partner of SRB10 (Kuchin et al., 1995; Liao et al., 1995), and recombinant GST-CTD fusion protein.

Preliminary evidence suggests that SSN6-TUP1 itself might be a substrate of SRB10. SSN6 is known to be phosphorylated *in vivo* (Schultz et al., 1990). TUP1 has at least one CDC28 protein kinase recognition site, and

preliminary evidence suggests that TUP1 is phosphorylated *in vivo*. TUP1 purified from yeast migrates as a doublet in SDS polyacrylamide gels (M. J. Redd and A. D. Johnson, unpublished observations). The lower mobility band in this doublet shifts to the higher mobility form when TUP1 is purified from an *srb10* mutant (M. J. Redd and A. D. Johnson, unpublished observations). Furthermore, SSN6 purified from an *srb10* mutant, as compared with from wild-type, completely shifts to a higher mobility form (M. J. Redd and A. D. Johnson, unpublished observations). Finally, the lower mobility band of TUP1 shifts to the higher mobility band when TUP1 purified from a wild-type strain is treated with phosphatase (M. J. Redd and A. D. Johnson, unpublished results). Perhaps the lower mobility forms of SSN6 and TUP1 result from phosphorylation by the SRB10 kinase. Whether or not SSN6-TUP1 is a substrate of SRB10 can be tested *in vitro*. If SRB10 phosphorylates SSN6-TUP1, it will be interesting to study the role of this phosphorylation in repression. Perhaps the phosphorylation of SSN6-TUP1 serves to strengthen the interaction between SSN6-TUP1 and the holoenzyme.

On the surface, the model that SSN6-TUP1 exerts repression by regulating CTD phosphorylation and by interacting with SRB proteins may appear to contradict a finding from our laboratory; namely, that α2-MCM1 can repress RNA polymerase I (pol I)-transcribed genes as well as those of pol II (Hershbach and Johnson, 1993). Pol I does not contain a domain analagous to the CTD. However, SSN6-TUP1 may bring about repression in more than

one way. In fact, the finding that the *srb* mutations cause only partial loss of repression could suggest that SSN6-TUP1 has other ways of exerting repression, some of which could involve components common to both the pol I and pol II transcription machineries or components of chromatin (as discussed in the Introduction).

In this regard, the role of SIN4, ARE3, and ARE4 in repression must not be overlooked. It has been proposed that *SIN4*, which was identified in my screen as well as in the screen for the *SSN* genes as *SSN4* (Wenjie et al., in press), encodes a chromatin component or a regulator of chromatin structure (Jiang and Stillman, 1992). The reason for this view is that phenotypes of *sin4* mutants resemble those resulting from histone depletion experiments (Jiang and Stillman, 1992). For example, in *sin4* mutants, basal transcription is activated and superhelical density of plasmid DNA is decreased (Jiang and Stillman, 1992). Both of these changes can reflect loss of nucleosomes (Worcel et al., 1981; Han et al., 1987; Han et al., 1988). Furthermore, SIN4 affects transcription of a wide array of genes and can have either positive or negative regulatory roles, depending on the gene under its control. This so-called global regulation could be mediated by changes in chromatin structure. Despite the similarities between *sin4* phenotypes and those resulting from changes in chromatin structure, there is no direct evidence linking SIN4 to chromatin.

Recent results from the Kornberg lab shed light on the role of SIN4 in transcription: SIN4, like the SRB proteins, is a component of the pol II

holoenzyme (Li et al., 1995). Therefore, *sin4* mutations may cause phenotypes similar to those resulting from changes in chromatin structure indirectly. As a component of the general transcription machinery, SIN4 can regulate the transcription of many genes.

Exactly how SIN4 functions in the holoenzyme complex remains to be determined. SIN4 was found to exist in a subcomplex within the holoenzyme (Li et al., 1995). This subcomplex consists of three other proteins, two of which have been identified (Li et al., 1995). One is GAL11 (Suzuki et al., 1988; Fassler and Winston, 1989) and the other RGR1 (Sakai et al., 1990). Mutations in *GAL11* cause pleiotropic phenotypes. One of these phenotypes is an α-specific mating defect, suggesting that *gal11* mutants have a defect in α2 repression. *RGR1* is required for glucose repression (Sakai et al., 1988), although it was not identified in the genetic screen for the *SSN* genes (Marian Carlson, personal communication). A safe prediction, which can be tested easily, is that *RGR1* is also required for α2 repression. The fourth component is an as yet unidentified 50 kd protein, termed p50.

It is unclear whether SIN4, GAL11, or this SIN4 subcomplex plays a direct role in α2 repression. Levels of *MATα2* transcript in an *α sin4* mutant are only about 60% of wild-type levels (Wahi and Johnson, unpublished observations) and the partial loss of α2 repression in an *α sin4* mutant can be suppressed by *MATα2* carried on a plasmid (Jiang and Stillman, 1995). Thus the defect in α2 repression in *sin4* mutants could simply be due to lower

levels of α2 protein. Furthermore, mutations in *GAL11* were found by Northern analysis to cause reduced levels of *MATα2* and/or *MATα1* transcript (Fassler and Winston, 1989). The reason for this ambiguity is that in the Northern analysis, *MATα1* and *MATα2* mRNAs comigrated on the Northern gel, and a probe which hybridized to both *MATα1* and *MATα2* mRNA was used (Fassler and Winston, 1989). Demonstration of a direct interaction between the SIN4 subcomplex and SSN6-TUP1 would suggest that the effect of SIN4 (and GAL11) on α2 repression is direct.

I have grouped *ARE3* and *ARE4* with *SIN4* based on genetic observations and on the effect mutations in these genes have on basal transcription. Perhaps the *ARE3* and *ARE4* gene products are also components of the general transcription machinery, possibly even components of the SIN4 complex. However, neither *ARE3* nor *ARE4* is identical to *RGR1*, since mutations in *ARE3* and *ARE4* are not complemented by a plasmid containing *RGR1*. Furthermore, it is unlikely that *ARE3* encodes p50. Recent cloning of *ARE3* (A. Szidon and A. D. Johnson, unpublished results) suggests that it is identical to *ROX3*, an essential gene required to mediate repression of heme-repressed genes, another SSN6-TUP1-dependent process (reviewed in Zitomer and Lowry, 1992; Wenjie et al., in press). It is unlikely that p50 is identical to ROX3, since *ROX3* encodes an ~25 kd protein. (Although, it is possible that ROX3 has an aberrant migration in SDS polyacrylamide gels.) *ARE4*, however, could encode p50. Finally, *ARE4*

–or *ARE3* if it is not *ROX3*–could be identical to *GAL11*. However, it should be noted that mutations in *ARE3* and *ARE4* do not cause a decrease in levels of *MATα2* mRNA as mutations in *GAL11* might. Whether or not ARE3 and ARE4 are components of the holoenzyme or components of the SIN4 subcomplex itself should be tested biochemically. In addition, ARE3, ARE4 (once *ARE4* is cloned), and the SIN4 subcomplex should be tested for a physical interaction with SSN6-TUP1 as described for the SRB proteins.

The physical connection between SSN6-TUP1 and the general transcription machinery remains to be proven, and the exact role of holoenzyme components in repression must be determined. It is interesting to note, however, that there is now precedence for an interaction between a eukaryotic repressor and the general transcription machinery. It was recently discovered that an interaction between the *Drosophila* repressor protein Krüppel and TFIIE results in repression *in vitro* (Sauer et al., 1995). In any case, the independent isolation of genes that encode components of the pol II holoenzyme (*ARE1/SSN3/SRB10*, *SSN8/SRB11*, *ARE2/SSN5/SRB8*, *SIN4/SSN4*, and *SSN7/ROX3*) in genetic screens for defects in repression requiring SSN6-TUP1 implies an important role for holoenzyme components in transcriptional repression. Prior to the identification of the *ARE* and *SSN* genes as holoenzyme components, the holoenzyme was mostly thought of in terms of transcriptional activation. In fact, the holoenzyme was termed "mediator of transcriptional activation" or "mediator" for short by the Kornberg lab (Kim et al., 1994). The genetic evidence described here and by

the Carlson lab (Kuchin et al., 1995; Wenjie et al., in press) suggests that both positive and negative regulators can adjust the rate of transcription initation through interactions with components of the pol II holoenzyme.

References

Allison, L. A., and C. J. Ingles, 1989 Mutations in RNA polymerase II enhance or suppress mutations in *GAL4*. Proc. Natl. Acad. Sci. USA **86:** 2794-2798.

Allison, L. A., J. K.-C. Wong, V. D. Fitzpatrick, M. Moyle, and C. J. Ingles, 1988 The C-terminal domain of the largest subunit of RNA polymerase II of *Saccharomyces cerevisiae, Drosophila melanogaster,* and mammals: a conserved structure with an essential function. Mol. Cell. Biol. **8:** 321-329.

Carlson, M., B. C. Osmond, L. Neigeborn, and D. Botstein, 1984 A suppressor of *snf1* mutations causes constitutive high-level invertase synthesis in yeast. Genetics **107:** 19-32.

Chen, S., R. W. West, Jr., J. Ma, S. L. Johnson, H. Gans *et al.,* 1993 *TSF1* to *TSF6,* required for silencing the *Saccharomyces cerevisiae GAL* genes, are global regulatory genes. Genetics **134:** 701-716.

Chesnut, J. D., J. H. Stephens, and M. E. Dahmus, 1992 The interaction of RNA polymerase II with the adenovirus-2 major late promoter is precluded by phosphorylation of the C-terminal domain of subunit IIa. J. Biol. Chem. **267:** 10500-10506.

Dahmus, M. E., 1995 Phosphorylation of the C-terminal domain of RNA polymerase II. Biochim. Biophys. Acta **1261:** 171-182.

Fassler, J. S., and F. Winston, 1989 The *Saccharomyces cerevisiae SPT13/GAL11* gene has both positive and negative regulatory roles in transcription. Mol. Cell. Biol. **9:** 5602-5609.

Han, M., M. Chang, U. J. Kim, and M. Grunstein, 1987 Histone H2B repression causes cell-cycle-specific arrest in yeast: effects on chromosomal segregation, replication, and transcription. Cell **48:** 589-597.

Han, M., and M. Grunstein, 1988 Nucleosome loss activates yeast downstream promoters *in vivo*. Cell **55:** 1137-1145.

Hengartner, C. J., C. M. Thompson, J. Zhang, D. M. Chao, S.-M. Liao, A. J. Koleske, S. Okamura, and R. A. Young, 1995 Association of an activator with an RNA polymerase II holoenzyme. Genes Dev. **9:** 897-910.

Jiang, Y. W., and D. J. Stillman, 1992 Involvement of the SIN4 global transcriptional regulator in the chromatin structure of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **12:** 4503-4514.

Jiang, Y. W., and D. J. Stillman, 1995 Regulation of *HIS4* expression by the *Saccharomyces cerevisiae* SIN4 transcriptional regulator. Genetics **140:** 103-114.

Johnston, M., and M. Carlson, 1992 Regulation of carbon and phosphate utilization, pp. 193-281 in *The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression*. edited by Jones, E. W., J. R. Pringle, and J. R. Broach. Cold Spring Harbor Laboratory, Cold Spring Harbor, N. Y.

Keleher, C. A., C. Goutte, and A. D. Johnson, 1988 The yeast cell-type-specific repressor α2 acts cooperatively with a non-cell-type-specific protein. Cell **53:** 927-936.

Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson, 1992 SSN6-TUP1 is a general repressor of transcription in yeast. Cell **68:** 927-937.

Kim, Y.-J., S. Björklund, Y. Li, M. H. Sayre, and R. D. Kornberg, 1994 A multiprotein mediator of transcriptional activation and its interaction with the C-terminal repeat domain of RNA polymerase II. Cell **77:** 599-608.

Koleske, A. J., and R. A. Young, 1994 An RNA polymerase II holoenzyme responsive to activators. Nature **368:** 466-469.

Kuchin, S. V., P. Yeghiayan, and M. Carlson, 1995 SSN8-SSN3, a cyclin and cyclin-dependent kinase pair that contributes to transcriptional control in yeast. Proc. Natl. Acad. Sci. USA **92:** 4006-4010.

Laybourn, P. J., and M. E. Dahmus, 1989 Transcription-dependent structural changes in the C-terminal domain of mammalian RNA polymerase subunit IIa/o. J. Biol. Chem. **264:** 6693-6698.

Laybourn, P. J., and M. E. Dahmus, 1990 Phosphorylation of RNA polymerase II occurs subsequent to interaction with the promoter and before initiation of transcription. J. Biol. Chem. **265:** 13165-13173.

Lemontt, J. F., D. R. Fugit, and V. L. MacKay, 1980 Pleiotropic mutations at the *TUP1* locus that affect the expression of mating-type-dependent functions in *Saccharomyces cerevisiae*. Genetics **94:** 899-920.

Liao, S.-M., J. Zhang, D. A. Jeffery, A. J. Koleske, C. M. Thompson *et al.*, 1995 A kinase-cyclin pair in the RNA polymerase II holoenzyme. Nature **374:** 193-196.

Lu, H., O. Flores, R. Weinman, and D. Reinberg, 1991 The unphosphorylated form of RNA polymerase II preferentially associates with the preinitiation complex. Proc. Natl. Acad. Sci. USA **88:** 10004-10008.

Nonet, M., D. Sweetser, and R. A. Young, 1987 Functional redundancy and structural polymorphism in the large subunit of RNA polymerase II. Cell **50:** 909-915.

Nonet, M., and R. A. Young, 1989 Intragenic and extragenic suppressors of mutations in the heptapeptide repeat domain of *Saccharomyces cerevisiae* RNA polymerase II. Genetics **123:** 715-724.

Peterson, C. L., W. Kruger, and I. Herskowitz, 1991 A functional interaction between the C-terminal domain of RNA polymerase II and the negative regulator SIN1. Cell **64:** 1135-1143.

Sakai, A. Y. Shimizu, and F. Hishinuma, 1988 Isolation and characterization of mutants which show an oversecretion phenotype in *Saccharomyces cerevisiae*. Genetics **119:** 499-506.

Sakai, A., Y. Shimizu, S. Kondou, T. Chibazakura, and F. Hishinuma, 1990 Structure and molecular analysis of *RGR1*, a gene required for glucose repression of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 4130-4138.

Sauer, F., J. D. Fondell, Y. Ohkuma, R. G. Roeder, and H. Jackle, 1995 Control of transcription by Krüppel through interactions with TFIIB and TFIIE beta. Nature **375:** 162-164.

Scafe, C., D. Chao, J. Lopes, J. P. Hirsch, S. Henry et al., 1990 RNA polymerase II C-terminal repeat influences response to transcriptional enhancer signals. Nature **347:** 491-494.

Schamhart, D. H. J., A. M. A. Ten Berge, and K. W. Van De Poll, 1975 Isolation of a catabolite repression mutant of yeast as a revertant of a strain that is maltose negative in respiratory deficient state. J. Bacteriol. **121:** 747-752.

Schultz, J., L. Marshall-Carlson, and M. Carlson, 1990 The N-terminal TPR region is the functional domain of SSN6, a nuclear phosphoprotein of *Saccharomyces cerevisiae*. Mol. Cell. Biol. **10:** 4744-4756.

Strich, R., M. R. Slater, and R. E. Esposito, 1989 Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. Proc. Natl. Acad. Sci. USA **86:** 10018-10022.

Surosky, R. T., R. Strich, and R. E. Esposito, 1994 The yeast *UME5* gene regulates the stability of meiotic mRNAs in response to glucose. Mol. Cell. Biol. **14:** 3446-3458.

Suzuki, Y., Y. Nogi, A. Abe, and T. Fukasawa, 1988 GAL11 protein, an auxiliary transcription activator for genes encoding galactose-metabolizing enzymes in *Saccharomyces cerevisae*. Mol. Cell. Biol. **8:** 4911-4999.

Thompson, C. M., A. J. Koleske, D. M. Chao, and R. A. Young, 1993. A multisubunit complex associated with the RNA polymerase II CTD and TATA-binding protein in yeast. Cell **73:** 1361-1375.

Thompson, C. M., and R. A. Young, 1995 General requirement for RNA polymerase II holoenzymes *in vivo*. Proc. Natl. Acad. Sci. USA **92:** 4587-4590.

Trumbly, R. J., 1992 Glucose repression in the yeast *Saccharomyces cerevisiae*. Mol. Microbiol. **6:** 15-21.

Vinh, D. B. N., M. D. Welch, A. K. Corsi, K. F. Wertman, and D. G. Drubin, 1993 Genetic evidence for functional interactions between actin noncomplementing (Anc) gene products and actin cytoskeletal proteins in *Saccharomyces cerevisiae*. Genetics **135:** 275-286.

Wenjie, S., I. Treich, N. Qian, S. Kuchin, and M. Carlson, *SSN* genes that affect transcriptional repression in *Saccharomyces cerevisiae* encode SIN4, ROX3, and SRB proteins associated with RNA polymerase II. Mol. Cell. Biol., in press.

Wickner, R. B., 1974 Mutants of *Saccharomyces cerevisiae* that incorporate deoxythymidine-5'-monophosphate in deoxyribonucleic acid *in vivo*. J. Bacteriol. **117:** 252-260.

Wolffe, A., 1994 Transcription: in tune with the histones. Cell **77:** 13-16.

Worcel, A., S. Strogatz, and D. Riley, 1981 Structure of chromatin and the linking number of DNA. Proc. Natl. Acad. Sci. USA **78:** 1461-1465.

Zitomer, R. S., and C. V. Lowry, 1992 Regulation of gene expression by oxygen in *Saccharomyces cerevisiae*. Microbiol. Reviews **56:** 1-11.