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The mechanism of Ssn6 and Tup1 repression of transcription in the yeast Saccharomyces cerevisiae

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The mechanism of Ssn6 and Tup1 repression of transcription in the yeast *Saccharomyces cerevisiae*

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Michael Redd

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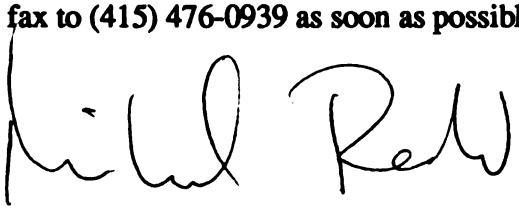

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To Jody and Nadja

PREFACE AND ACKNOWLEDGMENTS

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The mechanism of Ssn6 and Tup1 repression of transcription in the yeast *Saccharomyces cerevisiae*

Michael Redd

Abstract

Gene regulation is central to the ability of cells to adapt to their surroundings. Genes are turned off or on depending upon whether the protein encoded by that gene is required. This thesis concerns the mechanism of repression of transcription by Ssn6 and Tup1 in the yeast *Saccharomyces cerevisiae*. Ssn6 and Tup1 are required for the repression of at least eight sets of genes that are regulated by a variety of cues, including the determination of yeast cell type. The fusion of either Ssn6 or Tup1 to lexA DNA binding domain results in a fusion protein capable of repression of transcription from a promoter containing a lexA DNA binding site. Chapter one presents evidence that the α2 homeodomain protein involved in mating type determination, binds directly to the Tup1 protein. This result provides evidence that α2 directs the repression of genes by recruiting the Ssn6/Tup1 repression machine which then brings about repression and suggests that other gene sets may be similarly regulated by specific DNA binding proteins able to recruit Ssn6 and Tup1. A general model has been proposed in which Ssn6 and Tup1 repress transcription by organizing chromatin over promoters preventing accessibility to factors involved in activation. Chapter two concerns a simple experiment designed to test this model. Evidence is presented contradicting the chromatin model. Ssn6 and Tup1 are associated in a large complex in yeast extracts. The third chapter outlines the purification and characterization of the Ssn6/Tup1 complex as well as evidence that this purified complex is able to mediate repression of transcription *in vitro*.

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Chapter 1

The Repression of Transcription by Ssn6 and Tup1

Introduction

One of the most important ways cells respond to their environments is by changing patterns of gene expression. Gene regulation involves a plethora of factors and obscure terminology that to many biologists seems only distantly related to the biology of the cell. In this review two proteins, Ssn6 and Tup1, comprising a transcriptional repression machine in the yeast *Saccharomyces cerevisiae* will be discussed with an emphasis on the basic biological processes affected by these proteins. Ssn6 and Tup1 repress at least eight genes sets involved in a variety of processes, including the determination of yeast cell type, maintenance of homeostasis in a changing environment, and the repair of damage to DNA. The recurring motif in this review is that each gene set is regulated by a specific DNA binding protein whose function is to recruit Ssn6 and Tup1 to target genes in order to bring about the repression of their transcription. Derepression is effected by the inactivation of the DNA binding proteins allowing the repression machine to drift from the target genes and the activation machinery to take its place.

The identification of Tup1 and Ssn6/Cyc8

Both *TUP1* and *SSN6* (*CYC8*) have been identified by a variety of laboratories. Wickner (1974) first identified the *TUP1* gene in a screen for mutations that allowed thymidine uptake (125). Since then the *TUP1* gene has been rediscovered in an extraordinary number of genetic experiments: *flk1* as a mutation that abolished glucose repression (95), *umr7* for resistance to UV-induced mutation of *CAN1* to *can1* (59), *cyc9* for increased expression of iso-2-cytochrome c (CYC7) (90), *amm1* for mutations that stabilized a plasmid bearing a mutant ARS (114), *aer2* for aerobic expression of *CYC7* (133), in screens for mutants defective for regulation of the a-specific and haploid-specific mating type genes (122, 72), and *crt4* in a screen for mutations that express high levels of the inducible ribonucleotide

reductase *RNR3* (135). Likewise mutations in the *SSN6* gene has been identified multiple times: as *cyc8* involved in the repression of *CYC7* (90), *ssn6* as a suppressor of *snf1* mutations (10), and as *crt8* as a repressor of *RNR3* (135). Strains bearing mutations in either *SSN6* or *TUP1* share similar pleiotropic phenotypes: mutant cells are extremely flocculent (clumpy) in liquid culture, temperature sensitive for growth at 37°C, and insensitive to glucose repression. Diploid cells homozygous for either *ssn6* or *tup1* are defective for sporulation and display an altered budding pattern-- polar budding instead of medial as in wild type diploids. *MATα* tup1 or *ssn6* strains are defective for mating and exhibit a pear shaped cell morphology (schmoo), normally seen only when haploid cells are treated with mating pheromone (60). Finally, *ssn6* and *tup1* mutants constitutively express a variety of genes: *SUC2* coding for invertase and other glucose repressed enzymes, the a-specific genes, *BAR1* and the genes encoding a-factor in *MATα* cells (10) (60). Originally the pleiotropic nature of *SSN6* and *TUP1* mutations led geneticists to propose that these mutations altered a fundamental cellular structure, the cell membrane for example, in order to produce such a variety of defects (90). An alternative view held that Tup1 and Ssn6 were regulators of the expression of a large number of genes (60). Ssn6 and Tup1 are now known function as the transcription repressors of at least eight sets of target genes: the haploid-specific and a-specific genes involved in mating type determination, the glucose repressed genes and the glucose induced genes involved in the catabolism of various carbon sources, the DNA damage inducible genes involved in DNA repair, the hypoxic genes involved in growth in low oxygen, the mid-late sporulation-specific genes involved in spore formation, and the flocculin genes whose function is unknown.

A model for Ssn6 and Tup1 function.

Keleher et al. (1992) found that fusion of the bacterial lexA DNA binding domain to Ssn6 resulted in a fusion protein capable of repressing transcription in yeast of a promoter

containing a lexA binding site. Repression by lexA-Ssn6 is dependent on Tup1. Furthermore, lexA-Tup1 is also capable of repression of a test promoter but has only a partial dependence upon the presence of Ssn6 (120). These results show that Ssn6 and Tup1 can directly repress transcription of promoters in yeast and that Tup1 may function downstream of Ssn6 in repression. How then are Ssn6 and Tup1 targeted to the sets of genes they are known to repress? Keleher et al.(1992) proposed that Ssn6 and Tup1 are recruited to various gene sets by specific DNA binding proteins each of which is the subject of a specific pathway of regulation (Fig. 1). This model is now supported by many experimental facts. The DNA binding proteins responsible for directing the repression of many of the gene sets repressed by Ssn6/Tup1 have been identified: α2 for the mating type specific genes (47), Mig1 for the glucose repressed genes (77), Rgt1 for the glucose induced genes (82), Rox1 for the hypoxic genes (2), and possibly Sfl1 for the flocculin genes. In the case of the mating type genes, α2 was shown to interact directly with both Tup1 and Ssn6 in vitro (see below).

TUP1 and SSN6 encode proteins containing repeated domains

The SSN6 and TUP1 gene products belong to two different protein families whose principal characteristic is that they contain repeated domains. The 713 amino acid Tup1 protein contains seven repeated domains, termed WD repeats, originally found in the β–subunit of transducin, a G-protein (75). WD repeat domains are approximately 40 amino acids long and usually end in the tryptophan aspartate or WD motif. The WD repeats are found in proteins of a variety of functions including signal transduction, RNA splicing, secretion and regulation of gene expression (19). Recently the crystal structure of β-transducin has been solved, revealing a donut shaped protein in which the WD repeats form wedge shaped components of the ring (123). Proteins containing WD repeats are often found in multiprotein complexes, which led to the suggestion that these repeats may

mediate protein-protein interactions. This prediction was confirmed by the demonstration that the WD repeat portion of Tup1 interacts with α2 in vitro (54). These results suggest that α2 recruits Ssn6 and Tup1 by interaction with WD repeats of Tup1. However, the WD repeats are not required for repression of other gene sets. For example, the amino terminal 200 residues of TUP1 deleted for all seven WD repeats is sufficient for the repression of the glucose repressed genes and the hypoxic genes. Deletion analysis of lexA-Tup1 identified two domains able to mediate repression, one within the first 200 amino acids and the other near the first WD repeat (120). The amino terminus of Tup1 is also known to mediate Tup1 multimerization.

The Ssn6 protein contains a 34 amino acid repeat termed the tetratricopeptide repeat (TPR). TPRs are also found in proteins of many functions including protein import to the mitochondria and peroxisome, proteolysis of cyclins, RNA splicing and regulation of gene expression (for a review see 57). Structural modeling suggest TPR domains have a high probability of forming amphipathic α-helices leading to the "snap helix" model in which the TPR is composed of two domains one forming a hydrophobic pocket, the other forming a hydrophobic knob that may snap together (40). Like the WD repeats the TPRs are able to mediate protein-protein interactions. A lexA-Ssn6 fusion containing the amino terminus of Ssn6 and only the first three TPRs is capable of interacting with Tup1 *in vivo* and *in vitro* while other combinations of TPRs are not. Moreover, distinct TPRs are required for repression of different sets of genes in vivo (119). TPRs 1-3 , the Tup1 interaction domain, are sufficient for repression of the mating type genes. TPRs 4-7 are required for hypoxic gene repression, and TPRs 8-10 are required for glucose repression. The simple model that specific groups of TPRs are required for interaction with specific DNA binding proteins is complicated by the fact that most of the Ssn6 TPRs are able to bind to α2 in vitro (100). Although many of the TPRs are able to bind to α2 in vitro, it remains possible that TPRs 1-3 bind with higher affinity and mediate the binding in vivo.

In yeast extracts, Ssn6 and Tup1 are found associated in a large protein complex (126). This complex has been purified and found to be composed entirely of Tup1 and Ssn6 at a stoichiometry of four Tup1s to one Ssn6 (121, 84). A complex made of four Tup1 subunits and one Ssn6 with each of the protein components containing multiple repeated domains able to mediate protein-protein interactions seems ideally suited for interacting with the variety of proteins thought to recruit Ssn6 and Tup1. Recruitment to gene sets by specific DNA binding proteins can apparently occur by interaction with either the Ssn6 or Tup1 or by interaction with both proteins. The remainder of the review will focus on the regulation of the gene sets known to be repressed by Ssn6/Tup1.

The role of Ssn6/Tup1 in mating type determination --the a-specific genes and α2

S. cerevisiae exists as three differentiated cell types: **a** and α haploid cells, and the product of haploid cell mating, **a**/α diploid cells (for review see 46). Haploid cell type is determined by the allele present at the MAT locus-- **a**-cells contain *MAT**a*** and α-cells *MATα*. The *MATα* locus encodes genes for the α1 and α2 proteins which regulate cell type specific genes in order to determine the α-cell type. *α1* encodes the transcriptional activator of a set of genes required for expression of α-cell characteristics, the α-specific genes. *α2* encodes a repressor of another cell type specific gene set that is normally expressed in **a**-cells, the **a**-specific genes (asg) (Table I). Thus the **a**-cell type is achieved by the action two regulatory proteins α1 and α2 which turn on the α-specific genes and turn off the **a**-specific genes respectively. Examples of α-specific genes include the α-factor mating pheromone and the receptor for the **a**-factor mating pheromone. The **a**-cell is determined by the expression of the asg. Although *MAT**a*** encodes genes for the a1 and a2 proteins, neither of these proteins is required for the expression of the asg genes and **a**-cell determination. The a1 protein functions in diploid cells (see following section) and the

function of a2 remains a mystery. Thus the a-cell represents a default state with respect to cell differentiation, upon which the products of the *MATα* locus act to determine an α-cell. Examples of a-specific genes include those encoding a-factor mating pheromone *MFa1* and *MFa2* ,and the cell surface receptor of the α cell pheromone α-factor, encoded by the *STE2* gene (Table I).

The binding of α2 to the asg requires an additional protein, Mcm1. α2, a homeodomain protein, binds cooperatively with Mcm1, a MADS box protein, to a 30 base pair DNA sequence or operator found upstream of each asg. After DNA binding, α2 (and possibly Mcm1) recruits the general repression machine composed of Ssn6 and Tup1 in order to carry out repression (Fig. 1). This model is supported by the following observations: in strain deleted for SSN6, α2 is capable of binding to an asg operator but unable to repress transcription (50). α2 binds to both Ssn6 and Tup1 in vitro (54, 100). Ssn6 binds to the homeodomain located at the C-terminus of α2 while Tup1 interacts with the amino terminus. Mutations within the amino terminus abolish both repression in vivo as well as Tup1 binding in vitro. Mutations in Tup1 that abolish binding to α2 have been isolated in six of the seven WD repeats of Tup1. Mapping these TUP1 mutations onto the known structure of the WD repeat protein β-transducin, places all of the mutations on a single binding surface of Tup1. Remarkably, α2 binds to the same surface of Tup1 as does the G-protein alpha subunit to β-transducin, indicating that the same basic protein architecture is involved in both transcription and signal transduction.

The role of α2/Mcm1/Ssn6/Tup1 in regulating mating type switching

Ssn6 and Tup1 have recently been demonstrated to play a role in the directionality of mating type switching in yeast. In homothallic yeast, mating type switching occurs in a regulated fashion such that a single cell gives rise to four granddaughter cells, two having switched mating type and two of the of the original mating type, allowing mating to occur.

Since diploid cells grow faster, are more resistant to mutagens, and are better able to withstand starvation, the ability of yeast to switch mating type and mate incestuously could prove to be a great advantage in a competitive environment. Mating type switching is initiated by HO endonuclease cutting at the MAT locus. The subsequent double strand break is repaired by gene conversion with mating type information at the transcriptionally silent cassettes *HMRa* and *HMRα* located at either end of chromosome III. The directionality of switching is regulated so that *MATa* is replaced by information from *HMRα* 80% of the time and *MATα* is replaced by information at *HMLa* with greater than 95% efficiency, ensuring that mating type conversion takes place (Fig. 2).

How does the MAT locus know which end of chromosome to reach to? Wu and Haber (1996) have located an enhancer of recombination near HML that is capable of activating recombination over 40kb of the left arm of chromosome 3 in an a-cell specific manner. In α cells the enhancer is repressed by the action of α2, Ssn6 and Tup1, lowering the recombination rate of 175 kb of the left side of chromosome 3, and preventing *MATα* from recombining with *HMLα*. In fact, in a strain deleted for *HMRa*, HO cleavage of *MATα* is lethal for 30% of cells indicating that HML is not readily available for repair of the gap (129). In the converse experiment, HO cleavage of *MATa* in a strain harboring a deletion of HML does not result in lethality suggesting that MATa can be repaired by information at HMR. The recombination enhancer has been localized to a 700 base pair portion of the left arm of chromosome III (130). The enhancer is able to promote recombination when placed at other loci and contains two consensus α2/Mcm1 operators. Two models have been proposed for the role of α2/Ssn6/Tup1 in the regulation of the recombination enhancer. One model is that α2 directs the repression of an a-specific gene required for the activation of the enhancer. However, this model does not explain why the left arm of chromosome 3 is inactive for recombination in α cells. The favored model is that α2/Mcm1 binding at the operators found within the recombination enhancer directs the Ssn6/Tup1 dependent repression of the enhancer, possibly by organizing the left arm of

chromosome 3 into a higher order structure (Fig. 2). In any case, the regulation of the directionality of mating type switching by α2/Ssn6/Tup1 is a remarkable example of how cells are able to activate or repress recombination over large regions of a chromosome.

The regulation of α2 turnover.

Given that α2/Mcm1/Ssn6/Tup1 repress the asg, when is this repression lifted? When cells switch from α to a, the a-specific genes are expressed within a single generation, implying that the α2 protein is rapidly degraded. Hochstrasser and Varshavsky (1990) demonstrated that α2 does indeed have a short half life of approximately 4 minutes in vivo. Two signals within the α2 sequence confer instability on heterologous proteins by targeting the fusion proteins to two separate degradation pathways. Both pathways involve ubiquitination and subsequent degradation by the proteosome (41). One of the degradation signals, termed *Deg*1, is located in the 67 amino terminal residues of α2. *Deg*1 mediated degradation requires the *UBC6* and *UBC7* genes encoding ubiquitin conujugating enzymes that are localized in the nuclear and endoplasmic reticulum membrane (11). *Deg*2. the other degradation signal of α2, is located within the carboxyl-terminus and requires another set of ubiquitinating enzymes, *UBC4* and *UBC5*, for degradation (11). Abolishing both degradation pathways by deletion of UBC6 and UBC4 increases the half life of α2 from 4 minutes to one hour (11). Thus two pathways of ubiquitin-dependent degradation assure that the α2 protein is short lived. Both α2 and Rox1, another Ssn6/Tup1 dependent regulatory protein, are unstable proteins. Presumably this instability enables yeast to quickly respond to changes in cell type or environmental cues. The instability of α2 allows cells switching mating type from a to α, to express the asg within a single generation.

The role of a1/α2/Ssn6/Tup1 in the repression of the haploid specific genes

As described above, the mating of a and α haploid yeast yields a new cell type, the a/α diploid. The diploid cell is highly differentiated (see above section) from haploid yeast, enabling diploid cells to undergo meiosis and sporulation. The diploid cell type is determined by the products of the *MATa* and *MATα* loci (for review see 46). In diploid a/α cells as well as in α–cells, the α2 protein directs repression of the asg. However, in combination with the a1 protein, α2 is directs the repression of another set of genes normally expressed in haploid cells, the haploid-specific genes (hsg) (Table II). One of the haploid specific genes repressed by a1/α2 is the α1 gene which results in the inactivation of the αsg in diploids. Thus in diploid cells, α2 in combination with Mcm1 directs the repression of the asg while in combination with the a1 protein directs the repression of a second set of genes the hsg. a1 is a also a homeodomain protein and has been demonstrated to interact with α2 in vitro, binding cooperatively to a DNA element found upstream of the hsg (hsg operator) (33). The hsg operator differs in sequence from the asg operator bound by α2/Mcm1, yet it functions in an analogous manner, mediating the repression of heterologous genes if placed upstream. Furthermore, a1/α2 is thought to recruit the Ssn6/Tup1 proteins in order to repress the hsg (Fig. 3).

The Role of Ssn6/Tup1 in Glucose Repression; Glucose repression and Carbon utilization in yeast

Much like a hormone, glucose has a series of dramatic effects on yeast cells. The volume and activity of organelles such as mitochondria and peroxisomes are dramatically reduced in the presence of glucose. Since glucose is the preferred carbon source, the catabolism of alternate carbon sources such as galactose, maltose, sucrose, ethanol and lactate is repressed when glucose is present. Finally, glucose represses the processes of oxidative phosphorylation, gluconeogenesis and the glyoxylate cycle.

The catabolism of glucose in S. cerevisiae is highly specialized in a manner that has proved very useful for baking and brewing (for review see 127). Unlike most organisms, S. cerevisiae ferments glucose to ethanol and CO_2 even in the presence of oxygen. This behavior, also termed the Crabtree effect, is shared by approximately half of the yeast species tested. On the surface, aerobic fermentation seems an inefficient strategy, resulting in fewer ATP than if the sugar was fully metabolized through oxidative phosphorylation. The conversion of fermentable sugars to ethanol may be a way of sequestering these carbon sources from competing organisms that are unable to metabolize ethanol. Additionally, high ethanol concentrations tolerated by yeast may kill competing organisms.

Aerobic catabolism of fermentable carbon sources other than glucose proceeds by both fermentation as well as respiration. The switch between fermentation and growth on nonfermentable carbon sources--ethanol, acetate, glycerol or lactate-- is termed the diauxic shift. The diauxic shift requires a period of time for the re-tooling of basic metabolic pathways as well as for the biogenesis of more mitochondria and peroxisomes. Since hexose phosphate is required for biosynthesis, the enzymes responsible for gluconeogenesis and the glyoxylate cycle are derepressed in nonfermentable carbon sources.

The effect of glucose on yeast is known to be mediated by a variety of regulatory mechanisms: repression of transcription, induction of transcription, alteration of RNA stability, and the post transcriptional modulation of protein activity. Glucose repression has been defined as the reduction of gene expression at the transcriptional level as opposed to glucose inactivation which occurs post-transcriptionally (for reviews on glucose repression see 48, 86, 116). While Ssn6 and Tup1 are required for the repression of many genes in response to glucose, other glucose repression pathways independent of Ssn6/Tup1 also exist.

Three groups of glucose repressed genes.

The genes that are subject to glucose repression can be organized into three groups based on the stringency of their regulation. In the first group are the regulated genes required for gluconeogenesis including fructose bisphosphatase (*FBP1*) and PEP carboxykinase (*PCK1*), and the gene encoding the glyoxylate cycle enzyme isocitrate lyase (*ICL1*). This group of genes is strictly repressed by all fermentable carbon sources in order to avoid futile cycling between glycolysis and gluconeogenesis. The second group includes the genes involved in the Krebs cycle, respiration, mitochondrial and peroxisome biogenesis and in the utilization of nonfermentable carbon sources. The genes in the second group are repressed at an intermediate level; they are off in glucose, and partially derepressed in fermentable carbon sources. The third group includes genes involved in the utilization of fermentable carbon sources such as galactose, sucrose and maltose. The GAL, MAL, and SUC genes are derepressed in the absence of glucose and in the case of the GAL and MAL genes are further induced by the presence of their respective sugars, galactose and maltose.

The Mig1 protein

The Mig1 protein has been identified as the DNA binding protein that directs Ssn6/Tup1 repression of a number of genes (Table 3) (77). Mig1 contains an amino terminal Cys_2His_2 zinc finger DNA binding domain related to the Wilms tumor protein Wt, Sp1, and early growth Egr/Krox proteins. The Mig1 zinc fingers bind to a GC box (5 'GCGGGG 3 ') located upstream of the genes it regulates. An A/T rich region 5 ' to the GC box is required for DNA binding, possibly due to the bendability of this sequence (64).

LexA-Mig1 represses transcription of an heterologous promoter containing lexA operators in a glucose dependent manner (115). Deletion analysis identified a 24 amino acid domain found at the extreme carboxyl terminus necessary for repression (79). Repression by LexAMig1 requires both Ssn6 and Tup1. Whether the interaction between

Ssn6 and Mig1 is mediated by the 24 amino acid repression domain has not been established. However, Mig1 interacts in vivo with Ssn6 by two hybrid analysis, and this region seems the likely interaction surface (115).

Surprisingly, in the absence of Ssn6, LexAMig1 is a potent activator (115). This result suggests that Ssn6 in addition to bringing Tup1 along is able to mask a Mig1 activation domain. Although no genes have been identified that are activated by Mig1, the ability to repress and activate transcription is also shared by the related Wilms tumor protein and the Egr/Krox proteins.

The regulation of Mig1.

In the absence of glucose or in low glucose, Mig1 directed repression is abolished. Two sequences from the middle portion of Mig1 are required for regulation in the absence of glucose. Deletion of these regulatory sequences converts Mig1 into a constitutive repressor (79). A model for the inactivation of Mig1 in the absence of glucose has been proposed in which phosphorylation of the regulatory domain(s) prevents Mig1 nuclear localization (Fig. 4)(79). This model is supported by three observations. (1). Mig1 is under the control of the Snf1/Snf4 kinase required for activation of most glucose repressed genes. In the absence of Snf1, glucose repressed genes cannot be derepressed. (2). Mig1 is hyperphosphorylated and inactive (115). (3). LexAMig1 is an activator in the absence of Ssn6 when cells are grown in glucose. However, LexAMig1 does not activate in ssn6Δ cells grown in galactose (115). Moreover, a Mig1-VP16 fusion protein activates transcription in glucose but is inactivated in galactose in a Snf1 dependent manner demonstrating that the Mig1 protein is able to inactivate a heterologous protein domain in the absence of glucose (79). The inactivation of Mig1-VP16 requires the two regulatory domains described above. Whether Mig1 nuclear localization is regulated by

phosphorylation remains to be proven. This kind of regulation is reminiscent of the regulation of Swi5 by Cdc28 and Pho4 by Pho85 (71, 78).

In the presence of glucose, the Snf1/Snf4 kinase is counteracted by a glucose sensing and transduction pathway that culminates in a protein phosphatase, Glc1/Reg1. Glc/Reg1 phosphatase may act directly upon the Snf1/Snf4 kinase or may compete with Snf1/Snf4 for substrates like Mig1 (117).

Many of the genes regulated by Mig1 are subject to dual lock repression, that is, Mig1 directs the repression of the activator of the gene as well as the gene itself. The best characterized example of this kind of regulation is the glucose repression of the GAL genes (76). Mig1 binding sites are found upstream of not only the galactokinase gene (GAL1) but also the activator of the GAL genes, GAL4. Mig1/Ssn6/Tup1 are responsible for approximately 3x repression of both the GAL4 and the GAL1 genes. However, the 3x reduction in Gal4 leads to a 30x reduction in GAL1, probably due to the cooperative binding of Gal4 to the UAS_{GAL1}. Together these mechanisms lead to 90x repression of GAL1 (49). Repression of the MAL genes also involves a double lock and the suggestion has been made that the gluconeogenic genes are similarly regulated (36, 132).

Two glucose dependent repressor proteins, Mig2 and Rgm1, containing zinc fingers related to Mig1, have been identified. Mig2 was identified in a screen for proteins other than Mig1 responsible for glucose repression of the *SUC2* gene coding for invertase (65). The Mig2 protein contains zinc fingers that are very similar to those of Mig1, and is able to bind to the same GC element bound by Mig1. However, Mig2 and Mig1 differ in their affinity for various Mig1 sites. Mig2 repression of transcription also requires Ssn6 and Tup1. Other than Suc2, genes that are regulated by Mig2 have not been identified. Rgm1 was cloned by homology to Msn2, a zinc finger containing activator protein involved in the stress response in yeast (26). Overexpression of Rgm1 impairs cell growth. Replacement of the Rgm1 zinc fingers with those of Mig1 results in a fusion

protein that behaves like Mig1, suggesting that Rgm1 is a glucose dependent repressor that recruits Ssn6 and Tup1. Genes regulated by Rgm1 have not been identified.

The role of Rgt1/Ssn6/Tup1 in repression of the Glucose induced genes

Recently, a group of genes that are induced in the presence of glucose were shown to be repressed by Ssn6/Tup1 in the absence of glucose. The glucose induced genes include the hexose transporter genes HXT1-4 (see Table IV). HXT2 and HXT4 encode high affinity glucose transporters and are induced in low glucose. At high levels of glucose, HXT2 and HXT4 are repressed by Mig1/Ssn6/Tup1 (81). HXT3 encodes a transporter that is induced at low glucose and partially repressed by Mig1/Ssn6/Tup1 in high glucose. HXT1 encodes a low affinity glucose transporter that is only expressed in high glucose. In the absence of glucose, HXT1-4 are repressed by yet another pathway of repression directed by the DNA binding protein RGT1 and requiring Ssn6/Tup1 (82). Rgt1 is a Cys_6Zn_2 zinc cluster protein in the Gal4 family. Rgt1 binds to DNA elements found upstream of HXT1, 2, 3, and 4, containing a single CGG triplet. LexA-Rgt1 represses transcription of a CYC1 promoter possessing lexA operators upstream of the UAS_{cyc1} in the absence of glucose. Finally, repression by lexA-Rgt1 is dependent upon Ssn6 and Tup1 (Fig. 5) (82).

Rgt1 however plays another interesting role in the regulation of the hexose transporter genes; at high levels of glucose Rgt1 is converted to an activator of transcription. The activation function of Rgt1 is required for the activation of the low affinity glucose transporter HXT1 in high glucose. Mig1 repression prevents Rgt1 from activating HXT2, 3, and 4 in high glucose. However, a deletion of Mig1 does not fully derepress HXT2 in high glucose suggesting that another glucose repressor like Mig2 may also play a role at the HXT2 gene (81).

The regulation of RGT1.

LexA-Rgt1 activates transcription in high glucose, represses transcription in the absence of glucose and is inactive in low glucose (82). The low glucose sensor Snf3 and the Grr1 protein are required for the inactivation of Rgt1 in low glucose. Rgt1 is converted to an activator in high glucose by the action of the high glucose sensor Rgt2 and Grr1 (see Fig. 5). The mechanism of Rgt1 regulation by Grr1 is not clear. Grr contains 12 leucine repeat domains that may mediate protein-protein interactions with targets such as Rgt1 (29). The conversion of Rgt1 from a repressor to an activator in high glucose remains an interesting question. The combinatorial regulation of the hexose transporter genes by both Mig1 and Rgt1 apparently allows yeast to finely tune expression of these genes in response to the level of glucose in the environment.

The role of Ssn6 and Tup1 in regulation of the mid-late sporulation-specific genes DIT1 and DIT2

Sporulation is pathway of differentiation that diploid yeast undergo in response to nitrogen and carbon source limitation that leads to the induction of a set of sporulation-specific genes. These induced genes can be separated into groups according to the timing of induction: the early, middle, mid-late, and late genes (for review see 69) . The Ume6 protein and URS1, the DNA element to which it binds, are required for the repression of many early sporulation-specific genes during vegetative growth. Recently, Ssn6 and Tup1 have been implicated in repression of two divergently transcribed mid-late sporulation-specific genes, DIT1 and DIT2, encoding enzymes that produce dityrosine involved in spore wall formation (see Table V) (30). A negative regulatory element (NRE^{DIT}) between DIT1 and DIT2 directs repression of these genes during vegetative growth. The NRE^{DIT} confers 1000x repression upon the CYC1 promoter and requires Ssn6 and Tup1; however, this fusion promoter is not derepressed under sporulation conditions. The DIT promoter

contains at least two other elements that are required for derepression which may be required in cis for the inactivation of the NRE^{DIT} upon sporulation. Another curiosity of DIT gene repression is that deletion of TUP1 has a greater effect than deletion of SSN6, suggesting that Tup1 may play the primary role of both interacting with DNA bound proteins at the DIT promoter as well as carrying out the repression function much like repression of the a-specific genes directed by α2. The simplest model is that Ssn6 and Tup1 interact with the protein(s) that binds to the NRE^{DIT}; however, it is formally possible that the effect of Tup1 and Ssn6 on DIT expression is indirect. No other sporulation-specific genes have been identified that are regulated by Ssn6/Tup1.

The Role of Ssn6/Tup1 in the repression of the hypoxic genes.

Many excellent reviews have been written on the subject of the effect of oxygen upon gene regulation in yeast (8, 136). This review will be primarily concerned with the Rox1 repressor of the heme regulated hypoxic genes. A variety of processes in yeast require oxygen as an electron acceptor. These include respiration and the biosynthesis of heme, sterols and fatty acids. Hence, yeast are able to grow anaerobically only in the presence of fermentable carbon sources and with the addition of sterols and fatty acids. A group of genes, termed the hypoxic genes, are derepressed in response to oxygen limitation. The genes have been assigned to two groups. The first group includes genes that are expressed at low levels aerobically and are fully derepressed anaerobically (see Table VI). Genes in this group include proteins required for the synthesis of heme, sterol and fatty acids as well as sterol uptake. The second group of hypoxic genes is organized in pairs of genes-- one being expressed aerobically and the other expressed anaerobically (Table VI). These genes encode proteins involved in electron transport, sterol synthesis, and a translation initiation factor of unknown function. Presumably, the genes expressed anaerobically enable yeast to cope with limiting oxygen. The anaerobically expressed Cox5b and Cyc7 enzymes have

a higher turnover rate than their aerobic counterparts Cox5a and Cyc1 respectively, presumably enabling them to use oxygen more efficiently.

Rox1

The Rox1 protein is responsible for directing the repression of the hypoxic genes in combination with Ssn6 and Tup1 (2). Rox1 contains an amino terminal HMG DNA binding domain which binds to a consensus site (5 ' $P_yP_yP_yATTGTTCTC$ 3 ') located upstream of each hypoxic gene. The Rox1 domain responsible for interaction with Ssn6 and/or Tup1 has not been identified. The carboxyl terminal two thirds of Rox1 contains redundant sequences necessary for repression but a direct interaction with Ssn6 or Tup1 has yet to be demonstrated (15).

Regulation of Rox1 activity

The levels of *ROX1* RNA are regulated by at least two proteins, Hap1 and Rox1 itself. Hap1 is a heme dependent activator of transcription that activates *ROX1* transcription in the presence of oxygen (14). Two steps in synthesis of heme require oxygen. Thus when oxygen is limiting, heme synthesis decreases which in turn decreases the capacity of Hap1 to activate transcription. As *ROX1* RNA and protein levels drop, the hypoxic genes are derepressed (Fig. 6). Addition of heme even in the absence of oxygen results in the repression of the hypoxic genes supporting the idea that heme levels are the signal for repression. Like α2, the Rox1 protein has a short half life, allowing cells to quickly respond to changes in oxygen.

Recently it has been established that Rox1 represses its own expression through several binding sites located upstream of the *ROX1* promoter (14). Rox1 autoregulation is thought to prevent the accumulation of high Rox1 levels resulting in the repression of the

hypoxic genes essential for growth. Rox1 overexpression has been shown to inhibit cell growth. The ability of Rox1 to repress transcription of the hypoxic genes as well as its own expression is apparently due to the strength of the Rox1 binding sites located upstream of each gene. The stringently repressed genes have strong binding sites while the partially repressed genes have weaker sites. The weak Rox1 binding sites upstream of the *ROX1* gene act as a governor preventing over-accumulation of Rox1 in aerobic conditions. In summary the *ROX1* gene is activated by Hap1 in reponse to oxygen, as Rox1 protein levels rise, Rox1 represses the hypoxic genes as well as the *ROX1* gene. In the absence of oxygen, transcription of the *ROX1* gene declines and the remaining Rox1 protein is proteolysed--the hypoxic genes are derepressed (Fig. 5).

The Role of Ssn6/Tup1 in repression of the DNA damage inducible genes

From bacteria to humans, the response to DNA damage seems universal, leading to cell cycle arrest and the induction of enzymes involved in the repair of DNA (for review see 24). Around 20 damage inducible genes have been identified in yeast. At least two of these genes, *RNR2*, and *RNR3*, are repressed by an Ssn6/Tup1 dependent pathway in the absence of DNA damage (Table VII) (22, 23). *RNR2* and *RNR3* encode components of ribonucleotide reductase (RNR), the enzyme which converts ribonucleotides to deoxyribonucleotides. RNR catalyzes the limiting step in the production of dNTP providing a control point for regulating dNTP pools critical for the synthesis of new DNA during repair and replication. A genetic screen to identify genes responsible for the repression of the RNR genes identified alleles of *SSN6* and *TUP1* as well as the *CRT1* gene (135). The *CRT1* gene has yet to be identified, but a simple model would have *CRT1* encode a DNA binding protein that directs the repression of the RNR genes by recruiting Ssn6/Tup1 (Fig. 6).

The regulation of the RNR genes.

RNR gene expression is complex. *RNR2* is cell cycle regulated, being induced at S phase as well in response to DNA damage (21). *RNR3* is induced 100-500 fold by DNA damage but is not cell cycle regulated (23). DNA damage induction does not require passage through S phase and can occur in other points of the cell cycle. Although mutations in *CRT1*, *SSN6* and *TUP1* derepress the RNR genes, further induction takes place in the presence of the DNA damaging agent MMS. The DUN genes (damage uninducible) have been identified genetically as activators of the RNR genes in response to DNA damage (134). In addition to DNA damage, replication blockers or low dNTP pools induce the RNR genes. The pathway for signal generation and transduction includes DNA polymerase epsilon (DNA Pol II) as well as the yeast Mec1 and Tel1 kinases homologous to the gene product of the human ataxia telangiectsia (ATM) gene (74, 94). At the end of the kinase cascade is the Dun1 kinase (Fig. 6) (134). The transcription activators that are at the bottom of the signal transduction pathway have not been identified. The activator of the DNA damage inducible genes in yeast functions in the same pathway as the tumor suppressor protein p53 in humans. The importance of the DNA damage induction cascade is underscored by the fact that mutations in p53 or the ATM kinase predispose humans to cancer.

The role of Ssn6/Tup1 in repression of the flocculation genes.

One of the most obvious phenotypes shared by *ssn6* and *tup1* mutants is the growth as clumps or flocs in liquid culture (for review see 108). Yeast strains vary with respect to flocculation. Some wild strains flocculate constitutively, some are inducible for flocculence, and others never flocculate. To the researcher flocculation can be a nuisance, but to commercial brewers the flocculation properties of yeast are extremely important.

Brewing yeast flocculate in response to growth arrest due to oxygen limitation. Flocculent cells settle to the bottom of the brewing chamber, allowing the beer to be separated from the yeast without filtering. The timing of flocculation is important-- too early and fermentation is incomplete, whereas failure to flocculate results in expensive procedures to remove the yeast. Non sexual flocculation resulting from mutations in *SSN6* or *TUP1* is Ca^{2+} dependent and is protease and mannose sensitive. This data led to the model that proteins at the cell surface cause flocculation by binding in a Ca^{2+} dependent manner to mannose residues also present on the cell surface (67, 104). A group of genes have been identified that are responsible for flocculence in yeast. The best characterized are the *FLO1*, *FLO5*, and *FLO8* dominant genes for flocculence. *FLO1* and *FLO5* are a members of a family of four genes in S. cerevisiae called the flocculins (108). The flocculins are highly conserved and contain a variety of repeated elements throughout their sequence. One of the repeated sequences termed the A repeat is 45 amino acids in length and is repeated 18 times in the Flo1 protein. The Flo1 protein localizes at the cell surface in flocculent strains and may be anchored in the plasma membrane. Two other genes *SFL1* and *FLO8* behave as regulators of the FLO genes. Recessive mutations in *sfl1*, *tup1* and *ssn6* lead to constitutive flocculation suggesting that these proteins are repressors of the FLO genes. Two pieces of data support of this model. Mutations of either *TUP1* or *SSN6* lead to derepression of the *FLO1* gene. Furthermore, disruption of the *FLO1* gene reduces the flocculence of *tup1* and *ssn6* strains (110). Northern blots of *flo1* strains probed with a *FLO1* probe indicates that there are at least two other *FLO1* like genes expressed in *ssn6* and *tup1* strains possibly *FLO5* or another flocculin. *SFL1* has homology to the DNA binding domain of the heat-shock transcription factor (31). A simple model is that Sfl1 binds upstream of the FLO genes and recruits Ssn6/Tup1 in order to bring about repression of these genes (Fig. 7). The *FLO8* dominant gene causes constitutive flocculation. The *FLO8* gene encodes a protein with no homology to proteins in the database. Deletion of the *FLO8* gene abolished the flocculence of the dominant *FLO8* strain as well as transcription of the *FLO1* gene (53).

This data suggests that *FLO8* is an activator of *FLO1* (Fig. 7). Flo8 does not contain a recognizable DNA binding domain and could act by interfering with Sfl1. There is no obvious reason why *S. cerevisiae* flocculates. It has been suggested that this characteristic has been selected for during domestication; however, many micro organisms are able to clump for reasons not well understood.

Other factors involved in Ssn6 and TUP1 transcriptional repression.

Genetic screens to isolate genes required for the repression of the glucose repressed genes, the hypoxic genes, and the mating type genes have uncovered genes other than *TUP1* and *SSN6* that are involved in repression. These include *SIN4, ROX3, SRB8, SRB9, SRB10* and *SRB11* . A common link between all of these genes is that the proteins encoded are part of or interact with RNA polymerase II holoenzyme (51, 112). Mutations in any one of the genes in this group result in partial derepression of the Ssn6/Tup1 repressed genes. In contrast, mutations in *TUP1* or *SSN6* completely eliminate repression. Moreover, the mutations in this set of genes affects both activation and repression of many yeast genes, making it difficult to make precise conclusions concerning the role of these proteins.

The SRB genes were isolated as suppressers of the cold sensitive phenotype of a strain carrying a deletion of the heptad repeat of RNA pol II, as well in a variety of other screens (37, 62), including screens for genes involved in repression of a-specific genes (122), and selections for genes defective in glucose repression (3, 101). *SRB10* was also isolated for derepression of the early-sporulation specific genes that are known to be repressed by the *UME6* pathway (106). The fact that *SRB10* mutations arise in screens for the derepression of many genes that are differentially regulated may indicate that the Srb10 protein is a general repressor of RNA pol II that has little to do with specific regulatory circuits. Alternatively, Srb10 could represent a point of confluence between repression

pathways in yeast. *SRB10* and *11* encode a highly conserved cyclin dependent kinase and a cyclin subunit that have been shown to interact in vivo (55, 62). Deletions in *SRB8, 9, 10* and *11* all share the same phenotype. In fact a strain containing disruptions in *SRB8, SRB10* and *SRB11* has been created and displays the same phenotype as any of the single mutants suggesting that these genes all act in the same pathway (3). Although, Srb8, 9, 10, and 11 were shown to be part of the SRB complex that interacts with the heptad tail of polymerase, these proteins do not appear to be a part of the mediator complex identified in another laboratory, which shares many similarities with the SRB complex.

SIN4 was identified initially in a screen for mutations that derepress the *HO* promoter and has subsequently been re-isolated by a number of labs working on Ssn6/Tup1 dependent repression pathways (12, 73, 101, 102, 122). Sin4 interacts with the Rgr1 protein, as well as p50 an unidentified protein, which are components of the mediator complex required for activation of transcription in vitro (61). Mutations in *RGR1* have been isolated that affect glucose repression (93). Whether *RGR1* affects other Ssn6/Tup1 dependent repression pathways has not been determined.

ROX3 was initially isolated in a screen for genes involved in repression of the hypoxic gene *ANB1* but is also known to be involved in glucose repression (87, 101). *ROX3* is an essential gene. Recently *ROX3* has been implicated in the stress response in yeast (27). The stress response involves the activation of a group of genes in response to a variety of stresses including heat shock, starvation and high osmolarity (for review see (66). Strains bearing a mutant allele of *ROX3* are incapable of inducing *CYC7* in response to stress and are unable to grow on media of high osmolarity or under conditions of glucose starvation strongly suggesting that *ROX3* is essential for the global stress response in yeast. *ROX3* transcription is induced by stress (27). Interestingly, *SRB10* has also been shown to be induced by starvation, suggesting that the SRB proteins may also play a role in the stress response (106).

LexA fusions with Sin4, Srb11, Rox3, or Srb9 or activate transcription of test promoters containing lexA binding sites suggesting that these proteins are primarily involved in activation (45, 55, 101). However, conclusions about activation by lexA fusions are complicated by the fact that these proteins are known to associate with RNA pol II. Activation may simply be a result of recruitment of pol II and may not reflect the actual regulatory role of these proteins (for example see 4).

The ultimate downstream target of Ssn6/Tup1 repression is RNA pol II. Two general models have been proposed for the mechanism of Ssn6/Tup1 repression. One model involves the Ssn6/Tup1 dependent organization of chromatin over the target genes preventing accessibility to RNA pol II (88). The second model is that Ssn6/Tup1 directly interacts with the RNA pol II holoenzymes preventing assembly or release from the promoter (39). Evidence in favor of the chromatin model comes mostly from molecular biological approaches that led to the finding that many Ssn6/Tup1 repressed promoters are found to be covered by positioned nucleosomes (97). Furthermore, genes repressed by Ssn6/Tup1 are less accessible to bacterial methylases which could explain the ability of Ssn6/Tup1 to repress the recombination enhancer described above (52, 99). Deletions of the amino terminal portions of the histone proteins H3 and H4 have a weak affect on Ssn6/Tup1 repression in vivo, which may explain why H3 and H4 have never been isolated in the many screens for genes involved in this pathway (89). Finally, Tup1 is capable of binding histones H3 and H4 in a southwestern blot experiment (20). Attempts to identify which portion of Tup1 binds to the histone proteins were hampered by the fact that every portion of Tup1 was able to bind to the histone proteins.

Evidence for the second model comes from a variety of approaches. Mutations in genes encoding proteins known to be part of or able to associate with RNA pol II affect Ssn6 and Tup1 dependent repression (see above). In vivo footprinting experiments in combination with chromatin mapping indicate that α2 directed repression of a test promoter containing a Gal4 binding site can be repressed efficiently with no evidence for positioned

nucleosomes (85). In fact Gal4 remains bound to its site even in the repressed state suggesting that the promoter is accessible to at least Gal4. Biochemical evidence also suggests that Ssn6/Tup1 can mediate repression in vitro under conditions where nucleosomes are unlikely to form (38, 84). Since mutations in *SIN4, ROX3, SRB8, 9, 10,* and *11* only partly affect Ssn6/Tup1 repression it seems unlikely that these proteins are the target of Ssn6/Tup1. The essential components of the SRB or mediator complex may actually be the targets. The interaction of Ssn6/Tup1 with target proteins may be modulated by the Sin4, Rox3, Srb8, 9, 10 , and 11. Finally, the two models for Ssn6/Tup1 repression are not mutually exclusive; both mechanisms may play a role in repression.

Conclusion

Ssn6 and Tup1 are required for the repression of at least eight sets of genes in yeast. Most of the pleiotropic phenotypes of the original *ssn6* and *tup1* mutants can be explained by the constitutive expression of these genes. However a few of the phenotypes, such as the affect of *ssn6* and *tup1* on plasmid stability have yet to be explained, indicating that there are probably additional gene sets regulated by Ssn6/Tup1. The recurrent theme of this review is that the general Ssn6/Tup1 repression machine is targeted to gene sets by specific DNA binding regulatory proteins that are in turn regulated in response to environmental or internal cues.

One might expect that the DNA binding proteins α2, Mig1, Mig2, Rgt1, Rox1 contain homology within the domains that are involved in interaction with Tup1 and/or Ssn6. However, these proteins share no obvious regions of homology.

The role of Tup1 in repression of transcription appears to have been conserved, since direct homologs of Tup1 have been isolated in both *Candida albicans* and *Neurospora crassa* (6, 131). Moreover, a growing number of WD repeat containing proteins have been isolated and found to be involved in repression, including Hir1 and Met30 in S. cerevisiae;

SCON2 in N. crassa; extra sex combs and groucho in Drosophila; the Cop1 protein in Arabidopsis thaliana; HIRA and a family of groucho homologs called TLE proteins in humans (16, 34, 35, 56, 58, 96, 103, 111). These proteins have been implicated in repression of a wide variety of genes. For example, groucho controls genes involved in segmentation, sex determination and neurogenesis, while COP1 represses genes involved in photomorphogenesis (16, 83). In humans the HIRA protein has been implicated in the developmental disease DiGeorge syndrome (58). Thus understanding the mechanism of Ssn6/Tup1 repression in yeast should provide a paradigm for the function of the WD repeat repressor proteins.

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Table I. The a-specific genes repressed by α2/Mcm1/Ssn6/Tup1

Gene Name	Protein Function	Reference
MFa1	mating pheromone a-factor	(47)
MFa2	" " "	(47)
STE6	a-factor transporter	(47)
STE2	cell surface receptor for α-factor	(47)
BAR1	α-factor protease	(47)
AGA2	a-agglutinin binding subunit	(9)

Table II. The haploid-specific genes repressed by a1/α2/Ssn6/Tup1

Gene Name	Protein Function	Reference
GPA1	α-subunit of trimeric G protein	(70)
STE18	γ-subunit of trimeric G protein	(124)
STE4	β-subunit of trimeric G protein	(124)
STE12	Activator of mating type genes	(28)
RME1	Repressor of Ime1, meiosis	(13)
STE5	signal transduction in response to pheromone	(68)
MATα1	Activator of α-specific genes	(98)
HO	Endonuclease involved in MAT locus switching	(68)
AXL1	haploid bud site selection	(32)
SST2	Adaptation to pheromone	(17)
AGA1	a-agglutinin core subunit	(91)
Ty1	Transposable element	(25)
STA1	Glucoamylase	(18)

Table III. Genes repressed by Mig1/Ssn6/Tup1

Gene Name	Protein function	Reference
GAL4	DNA binding activator of GAL genes	(76)
GAL1	Galactokinase	(76)
SUC2	Invertase	(77)
MAL63	DNA binding activator of the MAL genes	(44)
MAL61	Maltose permease	(44)
MAL62	Maltase	(44)
CYC1	Cytochrome	(90)
CAT8	DNA binding activator of PCK1 FPB1 ICL1 CAT5	(36)
HXT2	hexose transporter	(81)
HXT4	hexose transporter	(81)

Table IV. Glucose induced genes repressed by Rgt1/Ssn6/Tup1

Gene Name	Protein	Reference
HXT1	Hexose transporter	(82)
HXT2	" "	(81)
HXT3	" "	(80)
HXT4	" "	(81)

Table V. Sporulation genes regulated by Ssn6/Tup1

Gene Name	Protein	Reference
DIT1	Required for synthesis of dityrosine, involved in spore wall maturation.	(7)
DIT2		(30)

Table VI. Two groups of Hypoxic genes repressed by Rox1/Ssn6/Tup1

Gene Name	Protein function	Reference
Rox1	Repressor of hypoxic genes	(14)
HEM13	Heme synthesis	(1)
ERG11	Sterol synthesis	(118)
CPR1	" "	(118)
SUT1	Sterol uptake	(5)
OLE1	Fatty acid synthesis	(105)

Gene Name	Aerobic homolog	Protein function	Reference
HMG2	HMG1	Sterol synthesis	(113)
COX5b	COX5a	Electron transport	(43)
CYC7	CYC1	" "	(128)
AAC3	AAC1/AAC2	" "	(92)
ANB1	TIF51a	eIF5a	(63)

Table VII. DNA-damage-inducible genes repressed by Ssn6/Tup1

Gene Name	Protein function	Reference
RNR2	Ribonucleotide reductase (RNR), small subunit	(22)
RNR3	RNR large subunit	(23)

Table VIII. The Flocculin genes

Gene Name	Ssn6/Tup1 regulated	Reference
FLO1	yes	(107)
FLO5	?	(109)
FLO9	?	(108)
FLO10	?	(108)

Fig. 1 The repression of the **a**-specific genes by α2, Ssn6, and Tup1 in **a** and **a**/α-cells. Pictured are schematic representations of the regulatory proteins involved in repression. Mcm1, Ssn6, and Tup1 are expressed in all three cell types whereas α2 is expression is limited to **a** and **a**/α–cells. Mcm1 is involved in activation of the asg in **a**-cells as well as repression of these genes in **a** and **a**/α cells.

Fig. 2 The repression of Recombination between *HMLα* and *MATα* by α2, Ssn6/Tup1. *HO* cleavage of the *MATα* locus of chromosome III stimulates mating type switching. The MAT loci replacement occurs via gene conversion with information at the silent cassettes *HMLα* or *HMRa*..

Matα is replaced by information from *HMRa*. The directionality of switching is guaranteed by the α2/Ssn6/Tup1 dependent repression of an enhancer of recombination located near the other silent cassette *HMLα*.

Fig. 3 The cell type regulation of the haploid specific genes. The three cell types haploid **a**, α-cells as well as the **a**/α diploid are represented as well as figurative renderings of the proteins involved in repression of the hsg. Ssn6 and Tup1 are expressed in all of the cell types but only pictured in the **a**/**a** diploid for simplicity. a1 and α2 are expressed in **a** or α-cells respectively, as well as in **a**/α diploid cells. In combination of a1 and α2 bind to DNA elements upstream of each hsg and recruit Ssn6/Tup1 in order to bring about repression.

Fig. 4 The regulation of the glucose repressed genes by Mig1, Ssn6/Tup1. A cartoon rendering of the glucose repressed genes in either the off state--glucose--or the on state--no glucose. A signal transduction pathway that culminates in the Snf1/Snf4 kinase regulates Mig1 activity. In glucose, the activity of the Snf1/Snf4 kinase is counteracted or repressed by the action of the Glc7/Reg1 type I protein phosphatase, and Mig1 binds DNA elements

upstream of the glucose repressed genes and recruits the Ssn6/Tup1 repression machine. In the absence of glucose, Mig1 activity is repressed by the action of Snf1/Snf4 and is found in a hyperphosphorylated state. Whether Snf1/Snf4 phosphorylates Mig1 directly is not known. It has been proposed that the inactivation of Mig1 could result from cytoplasmic localization. Grr1 and Hxk2 function upstream of the dueling Glc7/Reg1 phosphatase and Snf1/Snf4 kinase, but their function in this pathway is not understood.

Fig. 5 Regulation of the glucose induced genes by Rgt1 and Ssn6/Tup1. A schematic representation of the Rgt1 protein directing Ssn6/Tup1 dependent repression in the absence of glucose, and activating transcription of the *HXT1* gene in the presence of high glucose levels. In the presence of low glucose levels, Rgt1 is inactivated by a signal transduction pathway that begins at the cell surface with the high affinity glucose sensor, Snf3, and involves the Grr1 protein. In high glucose, the low affinity glucose sensor, Rgt2, generates a signal involving the Grr1 protein that transforms Rgt1 into an activator.

Fig. 6 The regulation of the hypoxic genes by Rox1 and Ssn6/Tup1. A cartoon representation of the regulation of the *ROX1* gene as well as the Hypoxic genes by either high levels of oxygen or in low oxygen. High levels of oxygen stimulate the synthesis of heme, which in turn stimulates the Hap1 activation of the *ROX1* gene. Rox1 protein binds to DNA sequences located upstream of the hypoxic genes and recruits the Ssn6/Tup1 repression machine to bring about repression. Under conditions of low oxygen or in the absence of oxygen, heme levels drop and consequently Hap1 is unable to promote *ROX1* transcription. Rox1 protein is rapidly protealysed, and the hypoxic genes are derepressed.

Fig. 7 The regulation of the DNA damage inducible genes *RNR2* and *RNR3*. A schematic representation of the regulation of the DNA damage inducible genes (DIN genes) by a signal transduction pathway responsive both to blocks in replication or DNA damage.

DNA pol II is involved in the generation of the signal which is then transduced by a kinase cascade beginning with Mec1 which activates Rad53 by phosphorylation. Activated Rad53 in turn phosphorylates and activates Dun1 resulting in the derepression of the DIN genes. The CRT1 gene was identified as a repressor of RNR expression. Crt1 is modeled as the DNA binding protein that directs the repression of the RNR genes by recruiting the Ssn6/Tup1 repression machine.

Fig. 8 The regulation of flocculin gene(s). The inducible flocculation genes are repressed during exponential cell growth and induced upon growth arrest due to nutrient limitation. The Sfl protein is required for repression of the FLO genes and is modeled here as the factor that directs the repression of the FLO genes by tethering Ssn6/Tup1 to the promoters of these genes. Flo8 is required for the induction of the FLO genes upon growth arrest.

Fig. 1 a-specific gene expression

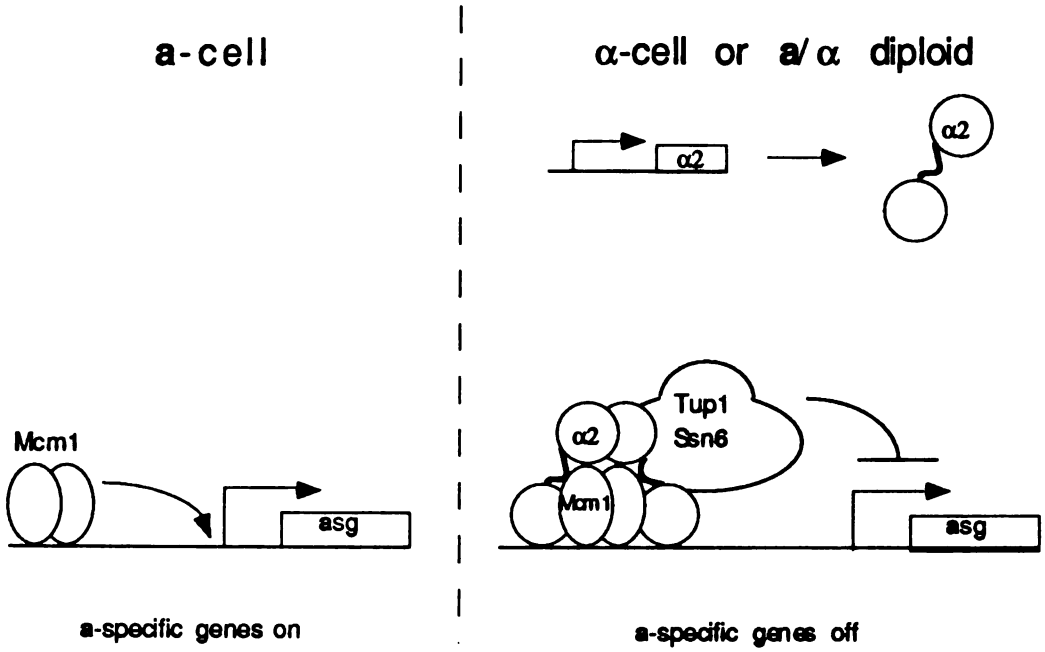


Fig. 2 α2/Ssn6/Tup1 repression of recombination

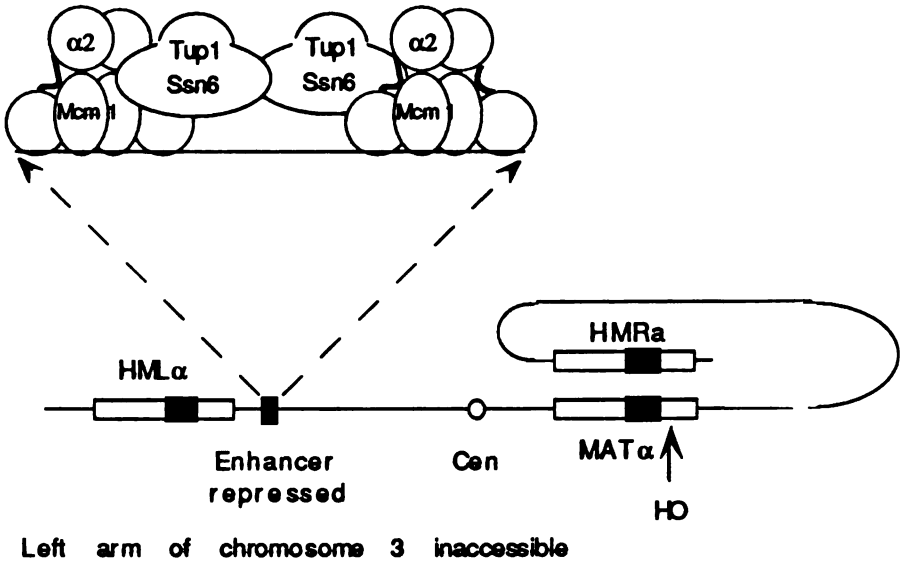


Fig. 3 Haploid-specific gene expression

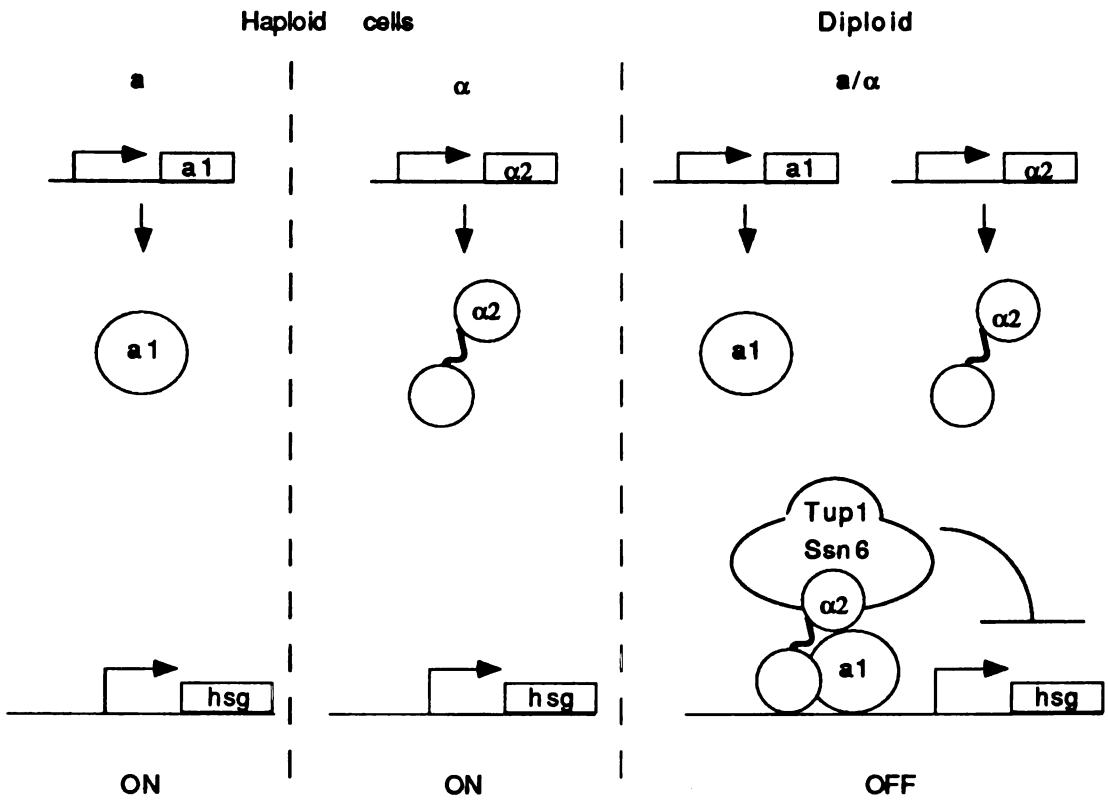


Fig. 4 Mig1 directed Glucose repression

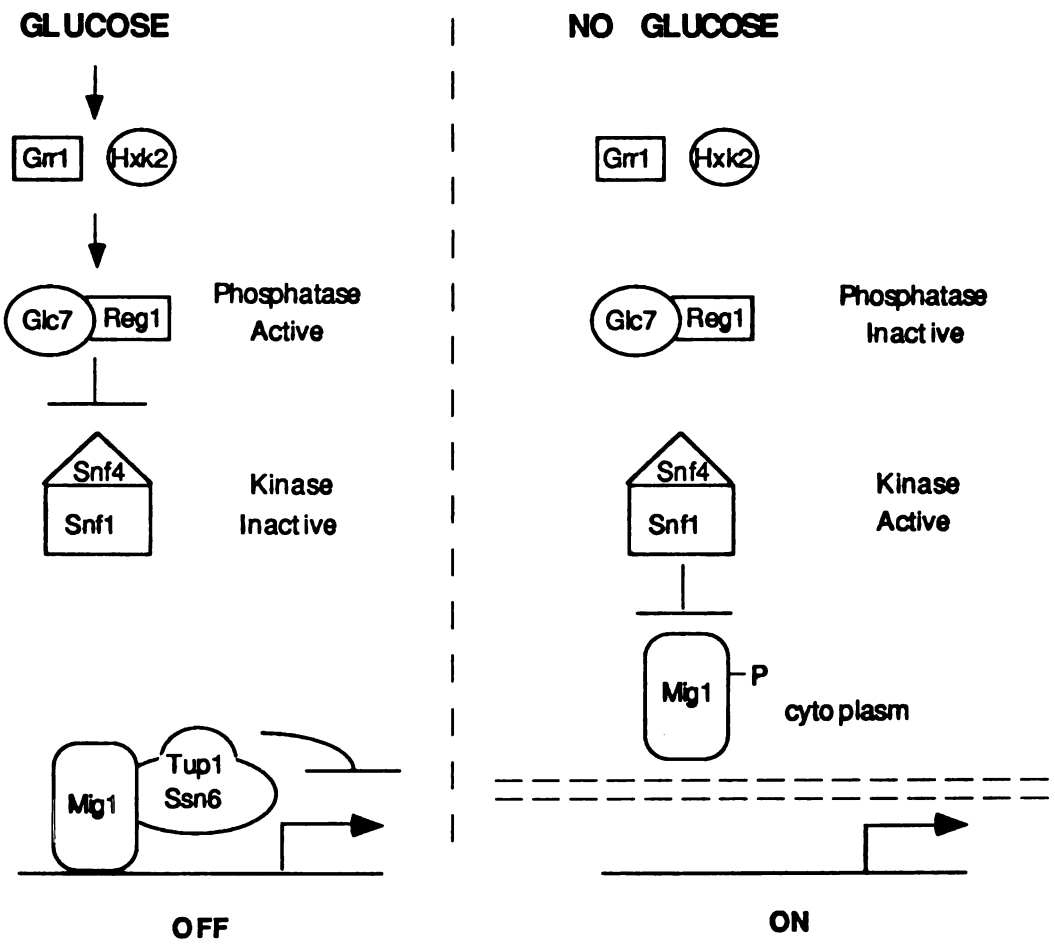


Fig. 5 Rgt1 repression of glucose induced genes

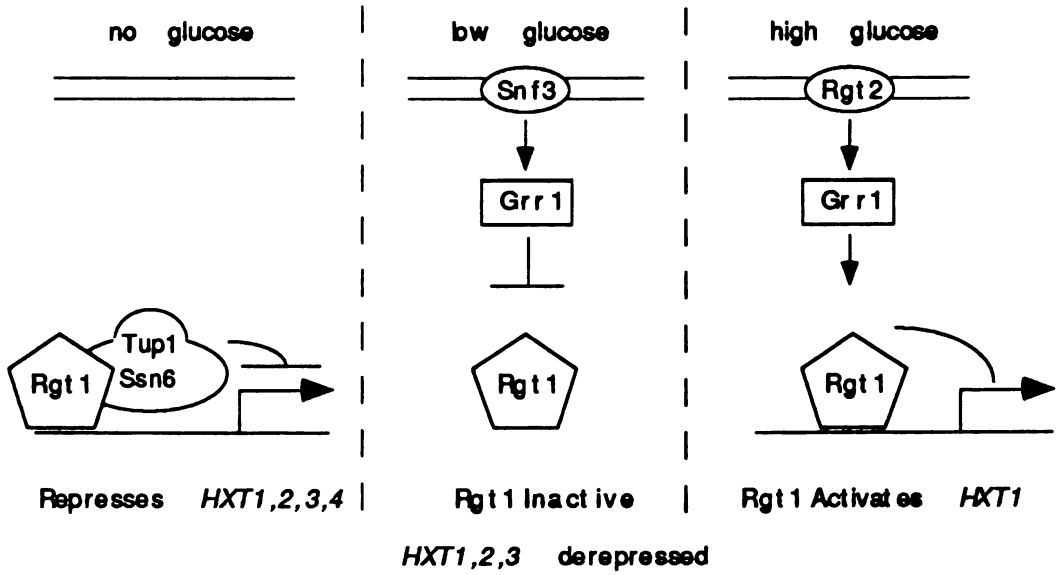


Fig. 6 Hypoxic gene regulation

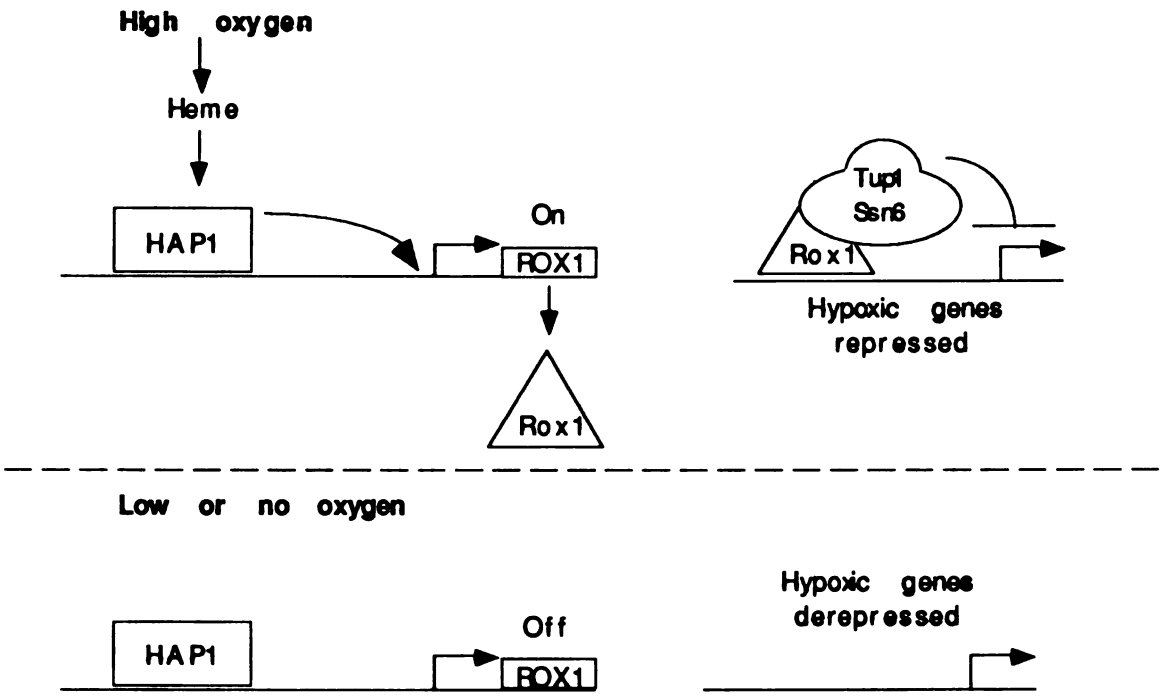


Fig. 7 Regulation of the DNA damage inducible genes

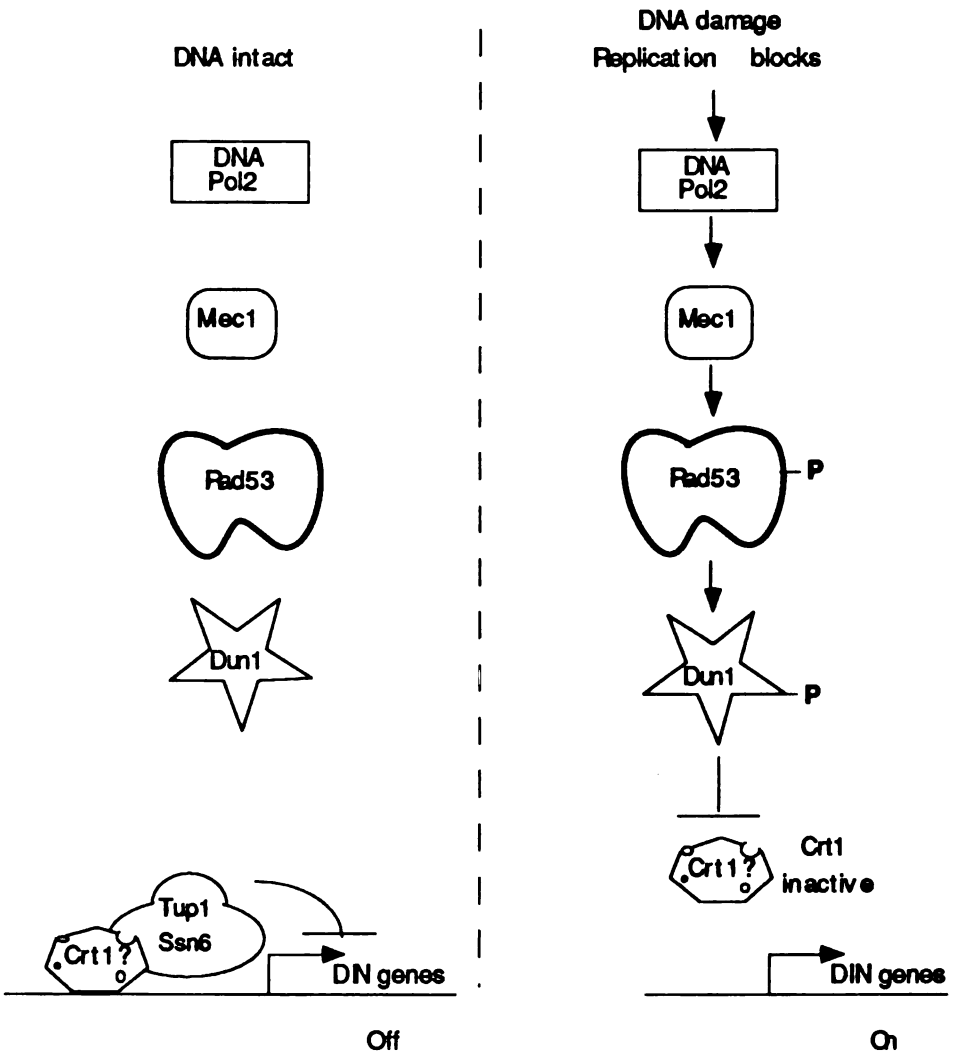
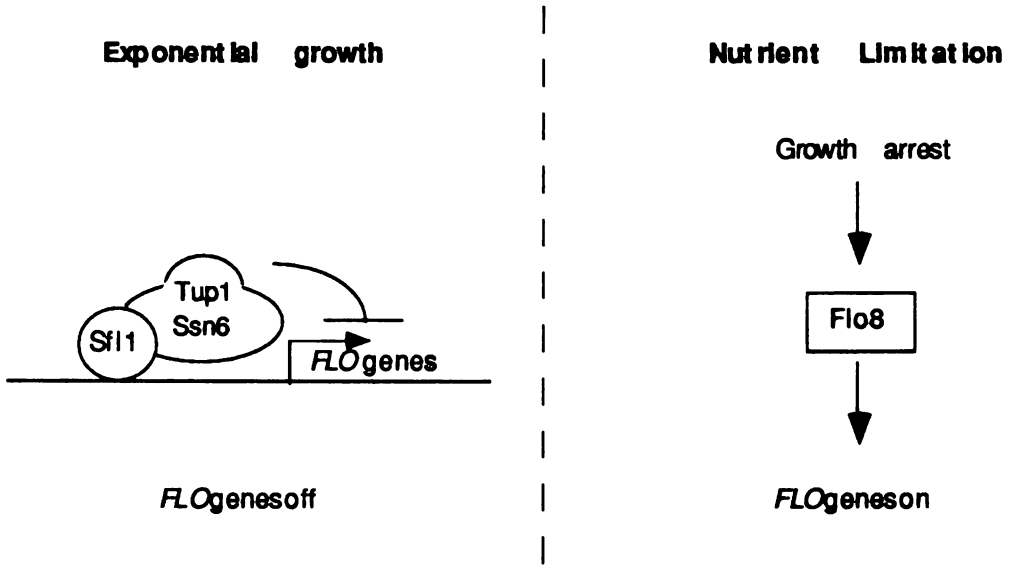


Fig. 8 Flocculation gene expression



Chapter 2

The WD repeats of Tup1 interact with the homeo domain protein α2

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The WD repeats of Tup1 interact with the homeo domain protein α2

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Tup1 and Ssn6 transcriptionally repress a wide variety of genes in yeast but do not appear to bind DNA. We provide genetic and biochemical evidence that the DNA-binding protein α2, a regulator of cell-type-specific genes, recruits the Tup1/Ssn6 repressor by directly interacting with Tup1. This interaction is mediated by a region of Tup1 containing seven copies of the WD repeat, a 40 amino acid motif of unknown function found in many other proteins. We have found that a single WD repeat will interact with α2, indicating that the WD repeat is a protein–protein interaction domain. Furthermore, a fragment of Tup1 containing primarily WD repeats provides at least partial repression in the absence of Ssn6, suggesting that the repeats also mediate interaction between Tup1 and other components of the repression machinery.

[*Key Words*: Homeo domain; WD repeat; transcriptional repression]

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Cells have evolved a variety of mechanisms for turning genes off when they are not needed. In the budding yeast *Saccharomyces cerevisiae* one repression system is remarkable for its involvement in regulating a wide variety of genes. Two proteins, Ssn6 and Tup1, are required for the repression of at least five independently regulated sets of genes: the **a** cell-specific genes and the haploid-specific genes, the glucose-repressed genes, the hypoxic genes, and the DNA damage-inducible genes (Mukai et al. 1991; Keleher et al. 1992; Trumbly 1992; Zitomer and Lowry 1992; Elledge et al.1993).

Both Ssn6 and Tup1 are members of extended protein families. Ssn6 contains 10 copies of the tetratricopeptide repeat or TPR (Schultz and Carlson 1987). Tup1 also contains a repeated sequence that was first identified in β-transducin, the WD repeat (Fong et al. 1986). Seven WD repeats have been identified within Tup1 (Williams and Trumbly 1990; see also results of this work). This motif is ~40 amino acids in length and contains a highly conserved tryptophan–aspartate or WD sequence. Proteins with WD repeats are involved in a wide variety of processes, including gene repression, signal transduction, secretion, RNA splicing, and progression through the cell cycle (for reviews, see Duronio et al. 1992; van der Voorn and Ploegh 1992).

The function of WD repeats is not known, although it has been suggested that they mediate protein–protein interactions. The function of the WD repeats of Tup1 remains obscure. Deletion of a single repeat results in the same phenotype as a complete deletion of the gene (Williams and Trumbly 1990). However, expression of the amino-terminal 200 amino acids of Tup1 lacking all of the WD repeats is able to function for glucose repression as well as function partially for hypoxic gene repression (Tzamarias and Struhl 1994).

How do Tup1 and Ssn6 regulate diverse sets of genes? It has been proposed that Ssn6/Tup1 is a general repressor in yeast, recruited to genes by specific DNA-binding proteins (see Fig. 1). These DNA-binding proteins would then be subject to regulation in response to the appropriate signals (Keleher et al. 1992). Several lines of evidence support this model. First, Ssn6 and Tup1 are found associated in a protein complex (Williams et al. 1991). Second, both LexA–Ssn6 and LexA–Tup1 fusion proteins can repress transcription of a test promoter possessing a LexA binding site (Keleher et al. 1992; Tzamarias and Struhl 1994). Third, DNA-binding proteins that are required for repression and that bind to sequences upstream of the regulated genes have been identified for all but one of the sets of genes known to be regulated by Ssn6/Tup1: α2 for **a**-specific genes and haploid-specific genes, Mig1 for glucose-repressed genes, and Rox1 for hypoxic genes (Johnson and Herskowitz 1985; Nehlin and Ronne 1990; Balasubramanian et al. 1993).

One of the best characterized of this group of DNA-binding proteins is the α2 protein. Budding yeast exists as three different cell types, **a** cells, α cells, and **a**/α diploid cells. α2 is expressed in α cells, where it is required for the repression of **a**-specific genes, and in **a**/α diploid cells, where it is required for the repression of both **a**-specific genes and haploid-specific genes. The **a**-specific genes are constitutively expressed in **a** cells because this cell type does not contain the α2 gene. In α cells α2 binds cooperatively with the Mcm1 protein to a DNA se-

[3]These authors contributed equally to this work.

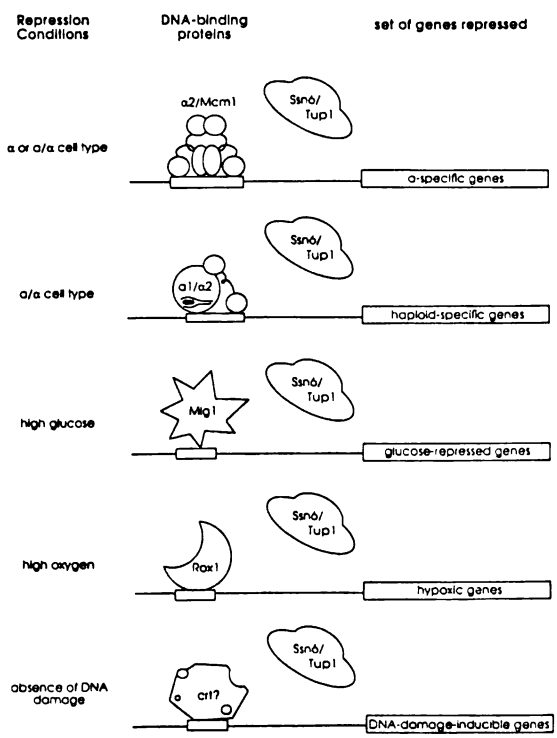


Figure 1. Ssn6 and Tup1 are required for repression of many different genes. Ssn6 and Tup1 mediate repression of the five sets of genes listed at *right*. Repression of each particular set of genes also requires specific upstream sequences and the DNA-binding protein or proteins indicated to the *left* of the each set of genes.

quence called the **a**-specific gene operator located upstream of each **a**-specific gene (for review, see Johnson 1992). Once bound to the operator α2 directs the Ssn6/Tup1-dependent repression of the gene. Placing an α2 operator upstream of other yeast genes—*CYC1*, *TRP1*, *URA3* and *GAL1*—brings them under the control of α2-directed repression, indicating that the repression is not specific for particular activators (Johnson and Herskowitz 1985; Roth et al. 1990; K. Komachi and M. Redd, unpubl.). Occupancy of the operator by α2 is not sufficient to bring about repression. In vivo dimethyl sulfate (DMS) footprinting of the **a**-specific gene *STE6* demonstrated that α2 is able to bind the *STE6* operator in the absence of Ssn6 but is unable to bring about repression (Keleher et al. 1992).

These results show that α2 carries out two functions: (1) It binds to operators upstream of specific genes; and (2) it directs the Ssn6/Tup1 dependent repression of those genes. α2 possesses a homeo domain located at the carboxyl terminus that is responsible for its DNA binding. The portion of α2 involved in directing repression has not been clearly identified, although the amino terminus has been implicated (Hall and Johnson 1987). In this paper we examine, both genetically and biochemically, the link between α2 and the Ssn6/Tup1 repressor. We show that α2 binds to Tup1 and that this interaction is mediated by the WD repeats of Tup1.

Results

Isolation of α2 mutants defective in repression but not DNA binding

To better understand how α2 directs repression after it has bound to its operator, we isolated mutants of α2 defective in repression but competent for DNA binding. To facilitate the identification of such mutants, we exploited the fact that α2 binds cooperatively to its operator with an activator, Mcm1 (Keleher et al. 1989), and designed a screen in which repression-defective mutants would activate transcription by helping Mcm1 bind DNA. Our screen was based on the following observations: (1) In the absence of α2, Mcm1 binds to the center of the wild-type operator and activates transcription (Bender and Sprague 1987; Keleher et al. 1988; Passmore et al. 1989); (2) a mutant operator in which the Mcm1-binding portion has been replaced by an unrelated sequence (the center-substituted operator) does not bind Mcm1 and does not activate transcription (Keleher et al. 1988); and (3) the cooperative interaction between α2 and Mcm1 allows formation of the α2/Mcm1 complex on the center-substituted operator in the presence of high levels of α2 (C.A. Keleher and A.D. Johnson, pers. comm.). In principle, overexpression of an α2 mutant defective only in repression should activate transcription from the center-substituted operator by recruiting Mcm1 to the DNA.

A plasmid that overexpresses α2 was mutagenized and transformed into a yeast strain carrying a *lacZ* reporter in which the upstream activating sequences have been replaced by the center-substituted operator. Transformants (36,000) were screened for β-galactosidase activity, and 20 positives were picked. Of these 20, 12 yielded plasmids that reproduced the original phenotype when reintroduced into the reporter strain. The 12 plasmids were sequenced and found to contain one of four point mutations, as summarized in Figure 2A. Two of the plasmids also contained silent mutations, and one of the plasmids bearing the thr4 mutation had an additional Arg → Gly amino acid change at position 60. Plasmids containing more than one mutation were not used in any of the subsequent work.

To further test the idea that the mutants we have isolated are defective in a repression function other than DNA-binding, we expressed each of the mutant proteins in bacteria. Using the gel-mobility shift assay, we found that the mutant proteins bind to the α2 operator both alone and cooperatively with Mcm1 in a manner indistinguishable from that of wild-type α2 (data not shown).

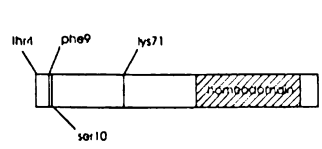
*The α2 mutants are defective in repressing authentic **a**-specific genes*

To show that the inability of the α2 mutants to repress is

A

amino acid change	# of mutant plasmids
Ile4 to thr4	4
leu9 to phe9	3
leu10 to ser10	2
gly71 to lys71	3

B



C

CELL TYPE	β-GALACTOSIDASE ACTIVITY
MATα	150 ± 20
MAT a	0.7 ± 0.1
MAT α2 lys71	75 ± 7
MAT α2 phe9	120 ± 30
MAT α2 thr4	100 ± 10
MAT α2 ser10	150 ± 20

D

CELL TYPE	α2 PLASMID	β-GALACTOSIDASE ACTIVITY
MAT a	CV13 (2μm vector)	0.8 ± 0.3
MAT a	MATα2/CV13	≤ 0.1
MAT a	MATα2lys71/CV13	2.3 ± 0.3
MAT a	MATα2phe9/CV13	5.0 ± 1
MAT a	MATα2thr4/CV13	34 ± 10
MAT a	MATα2ser10/CV13	53 ± 3

Figure 2. α2 Repression-defective mutants. (*A*) Summary of α2 mutations and the frequency with which they were isolated. (*B*) Location of mutations relative to the homeo domain. (*C*) Yeast strains differing only at the *MAT* locus (cell type) were assayed for β-galactosidase activity from an integrated *mfα2:lacZ* reporter. Each reported value is the average of three β-galactosidase assays. (*D*) A *MATα mfα2:lacZ* strain was transformed with a high-copy mutant α2 plasmid and assayed for β-galactosidase activity. Each reported value is the average of three β-galactosidase assays performed on three individual transformants.

not peculiar to transcription of the reporter used in our screen, we replaced the wild-type copy of α2 at the *MAT* locus with each of the mutant copies in a strain carrying an *mfa2:lacZ* reporter and examined the ability of the mutants to direct repression of this **a**-specific gene fusion. *MFA2* encodes the mating pheromone, **a**-factor and is normally repressed in α cells. As shown in Figure 2C, the *mfa2:lacZ* reporter is expressed in **a** cells, repressed in α cells, and derepressed to various levels in mutant α cells. In addition, the mutant strains produce extracellular **a**-factor and barrier activity as determined by bioassay (Sprague 1991; data not shown) and hence must also express the **a**-specific genes *STE6* and *BAR1*, which encode a pheromone export protein and the barrier protease, respectively (MacKay et al. 1988; McGrath and Varshavsky 1989).

The α2 mutations are dominant negative

If the mutant proteins are defective in repression but not in binding to the operator with Mcm1, we expect these alleles to be dominant when the mutant proteins are overexpressed because they should bind to the operator and block access to wild-type α2. To test this prediction, we transformed high-copy plasmids containing the mutant α2 genes into a wild-type α strain carrying the *mfa2:lacZ* reporter and assayed the transformants for β-galactosidase activity. Results are summarized in Figure 2D. Each of the four mutant proteins caused derepression of the reporter, showing that all of the mutations are dominant negative for α2-mediated repression.

In summary, we believe we have isolated mutant versions of α2 that occupy the operator but fail to repress transcription of the **a**-specific genes. We refer to these mutant proteins as repression-defective mutants.

The dominance of some of the α2 mutants is suppressed by overexpression of Tup1

We speculated that the repression-defective mutants fail to interact with another protein of the repression complex, most likely Ssn6 or Tup1, both which are required for repression of the **a**-specific genes and have been proposed to interact with DNA-binding proteins (see Introduction). If this hypothesis is correct, we expected that increasing the concentration of Ssn6 or Tup1 might offset the decreased affinity of the repression-defective mutants for these proteins and restore repression. We first tested whether overexpression of Ssn6 and/or Tup1 suppressed the inability of the α2 mutants to repress transcription by transforming strains carrying a chromosomal *matα2* mutation with high-copy plasmids bearing *SSN6*, *TUP1*, or *SSN6* and *TUP1* and monitoring the expression of an *mfa2:lacZ* reporter. None of the plasmids restored repression in any of the strains (data not shown).

We next tested whether overexpression of Ssn6 and/or Tup1 would suppress the *dominance* of the repression-defective mutants. Because α2 binds its site as a dimer, the mutants can presumably exclude the wild-type protein from the operator by binding the site as either homodimers or heterodimers with wild-type α2. We reasoned that interaction of a heterodimer with the downstream protein might be restored at a concentration lower than that required for interaction with a mutant homodimer. Overexpression of Ssn6 and Tup1 from a high-copy plasmid, though unable to suppress the defect of cells expressing only the mutant forms of α2, does restore repression to an *mfa2:lacZ* reporter in cells expressing both wild-type α2 and the dominant-negative forms of α2 (Fig. 3). Moreover, overexpression of Tup1 alone suppresses the dominance of the weaker mutants, suggesting that Tup1 might interact directly with α2 and that the mutants that we have isolated might be defective in binding to Tup1. We therefore set out to look for an interaction between α2 and Tup1 in vitro.

α2 binds to Tup1 in vitro

To test the hypothesis that Tup1 and α2 interact, we first fused the *TUP1* gene to the glutathione *S*-transferase

CELL TYPE	α2 PLASMID	SUPPRESSION PLASMID	β-GALACTOSIDASE ACTIVITY
MAT a	MATα2^{lys71}/CV13	YEp24 (2μm URA3 vector)	2.5 ± 0.5
MAT a	MATα2^{lys71}/CV13	SSN6/YEp24	3.3 ± 0.2
MAT a	MATα2^{lys71}/CV13	TUP1/YEp24	0.8 ± 0.1
MAT a	MATα2^{lys71}/CV13	(SSN6+TUP1)/YEp24	0.9 ± 0.3
MAT a	MATα2^{phe9}/CV13	YEp24	13.4 ± 2.0
MAT a	MATα2^{phe9}/CV13	SSN6/YEp24	22.0 ± 8.9
MAT a	MATα2^{phe9}/CV13	TUP1/YEp24	1.7 ± 0.4
MAT a	MATα2^{phe9}/CV13	(SSN6+TUP1)/YEp24	0.9 ± 0.1
MAT a	MATα2^{thr4}/CV13	YEp24	47 ± 5
MAT a	MATα2^{thr4}/CV13	SSN6/YEp24	46 ± 13
MAT a	MATα2^{thr4}/CV13	TUP1/YEp24	24 ± 3
MAT a	MATα2^{thr4}/CV13	(SSN6+TUP1)/YEp24	8.7 ± 1.5
MAT a	MATα2^{ser10}/CV13	YEp24	112 ± 10
MAT a	MATα2^{ser10}/CV13	SSN6/YEp24	90 ± 7
MAT a	MATα2^{ser10}/CV13	TUP1/YEp24	73 ± 3
MAT a	MATα2^{ser10}/CV13	(SSN6+TUP1)/YEp24	23 ± 3
MATα		YEp24	156 ± 23
MATα		SSN6/YEp24	155 ± 15
MATα		TUP1/YEp24	160 ± 10
MATα		(SSN6+TUP1)/YEp24	146 ± 19

Figure 3. The dominance of the α2 mutants is suppressed by overexpression of Tup1 and Ssn6. A *MATα mfα2:lacZ* strain was transformed with a high copy mutant α2 plasmid and a suppression plasmid and then assayed for β-galactosidase activity. The last set of assays shows that the suppression plasmids do not affect β-galactosidase expression in the absence of α2. Each reported value is the average of three β-galactosidase assays performed on three individual transformants.

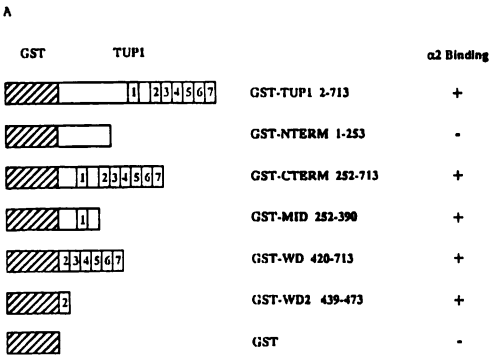
(GST) gene and purified the fusion protein from *Escherichia coli* (GST–TUP1, Fig. 4A). The purified GST–TUP1 (Fig. 5A, lane 7) was then coupled to a column matrix through which bacterial extracts containing the α2 protein (lane 1) were passed. The flowthrough fractions contained most of the bacterial proteins but lacked α2, indicating that α2 was selectively retained on the column (lanes 18–22). The bound α2 protein was then eluted from the column by high salt (lane 4).

To determine which portion of Tup1 is required for binding α2, we constructed two additional GST fusion proteins: GST–NTERM consisting of amino acids 1–253 of Tup1, and GST–CTERM, consisting of the remaining carboxy-terminal portion of Tup1 fused to GST (Fig. 4A). α2 (again present in a bacterial extract) was specifically retained on the GST–CTERM column (Fig. 5A, lanes 13–17) and was eluted from the column by high salt (lane 3). In contrast, α2 flowed through the column bearing the GST–NTERM protein (lanes 8–12), and salt elution yielded only a small fraction of the α2 loaded (lane 2). These results indicate that α2 binds specifically to Tup1 and that the carboxy-terminal portion of Tup1 mediates this interaction.

The carboxyl terminus of Tup1 contains the six WD repeats identified by Williams and Trumbly (Fig. 4A, labeled 2–7). To determine whether these repeats mediate the interaction with α2, we further subdivided Tup1 into two more GST fusion proteins: the middle region consisting of amino acids 252–390 (GST–MID), and the WD region consisting of amino acids 420–713 (Fig. 4A, GST–WD). Surprisingly, α2 bound to both GST–MID and GST–WD (data not shown; summarized in Fig. 4A). This result indicates that Tup1 contains at least two separable α2-binding domains, one that is essentially a series of WD repeats, suggesting that a function of these repeats is binding α2.

α2 binds to a single WD repeat

Comparison of the portion of Tup1 within the GST–MID fusion protein with the Tup1 WD repeats revealed a region of similarity indicating that Tup1 may have a seventh WD repeat. Amino acids 341–383 of the middle portion of Tup1 do not contain the highly conserved tryptophan–aspartate motif characteristic of the repeat, but do share significant sequence homology with the



B

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341-383   LDETSVVCCVK------FSNDGEYLATGC-NKTTQVYRVSDGSLVARLSD

410-481   PSSDLYIRSVC------FSPDGKFLATGAEDRLIRIWDIENRKIVMIL
482-525   QGHEQDIYSLDY-----F-PSGDKLVSGSGDRTVRIWDLRTGQCSLTLSI
527-567   DGVTTVAVSPG---------DGKYIAAGSLDRAVRVWDSETGFLVERLDS
573-616   TGHKDSVYSVV------FTRDGQSVVSGSLDRSVKLWNLQNANNKSDSKT
627-668   IGHKDFVLSVA------TTQNDEYILSGSKDRGVLFWDKKSGNPLLML
669-713   QGHRNSVISVAVANGSSLGPEYNVFATGSGDCKARIWKYKKIAPN

Consensus .GH...V.SV.------FS.DG..aATGS.DR.VRaWD...G.....L..
                            T     S
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Figure 4. GST–TUP1 fusion proteins with a summary of α2 binding results and alignment of Tup1 WD repeats. (*A*) The GST portion is represented by the hatched portion. The numbered boxes represent the WD repeats of Tup1. The amino acids of Tup1 included in each fusion protein are indicated. (*B*) Amino acids 341–383 aligned with the six WD repeats of Tup1. The alignment and consensus were made by hand. The dashes represent gaps, as the repeat lengths differ. In the consensus, ø represents hydrophobic residues.

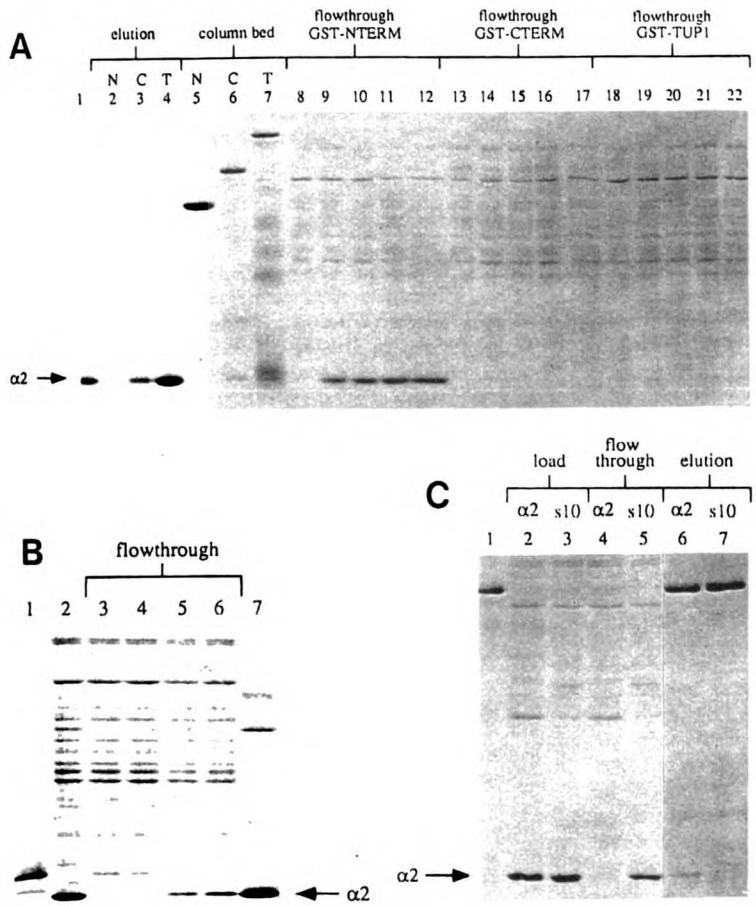

Figure 5. α2 binds to a single WD repeat of Tup1. (*A*) Coomassie blue-stained SDS gel showing the results of passing a bacterial extract containing α2 (lane *1*) over columns bearing purified GST–NTERM (lane *5*), GST–CTERM (lane *6*), and GST–TUP1 (lane *7*). Flowthrough fractions from each column are as indicated. The columns were eluted with 1 M salt. The peak fractions were pooled and are shown in lanes *2–4* (N, C, and T, indicate GST–NTERM, GST–CTERM, and GST–TUP1, respectively). α2 is indicated (→). (*B*) Coomassie blue-stained SDS gel containing the results from passing a bacterial extract containing α2 over a column bearing GST–WD2. (Lane *1*) A sample of the column bed; (lane *2*) a portion of the α2 extract; (lanes *3–6*) fractions of the column flowthrough; (lane *7*) a sample of the high salt eluate from the column. (*C*) Bacterial extracts containing either wild-type α2 (lane *2*) or mutant $\alpha2^{Ser10}$ (lane *3*) were passed over columns bearing GST–CTERM Tup1 (lane *1*). Mutant α2 flowed through the column (lane *5*), whereas wild-type α2 was retained. The columns were eluted with glutathione, displacing GST–CTERM and α2 from the column loaded with wild-type α2, but only GST–CTERM from the column loaded with mutant α2 (lanes 6 and 7, respectively).

Tup1 WD repeats (Fig. 4B). If this WD repeat-like portion of middle region of Tup1 is responsible for α2 binding, then a single WD repeat should be sufficient to bind α2. To test this idea, amino acids 439–473, comprising the second Tup1 WD repeat, were fused to the GST domain creating GST–WD2 (see Fig. 4A). A column bearing GST–WD2 (Fig. 5B, lane 1) specifically retained α2, which was eluted from the column with high salt (lane 7). This result indicates that a single WD repeat of Tup1 is sufficient for binding α2.

Amino-terminal α2 mutant fails to bind to Tup1

The behavior of α2 repression-defective mutants discussed above suggested that the amino-terminal region of α2 may be required for binding Tup1. To test this model we made extracts from *E. coli* expressing the $\alpha2^{Ser10}$ mutant. Extracts containing $\alpha2^{Ser10}$ or wild-type α2 were passed over columns bearing the GST–CTERM Tup1 fusion protein. The results of this experiment are shown in Figure 5C. Lane 1 represents the GST–CTERM column bed. Lanes 2 and 3 are samples of the extracts containing the wild-type α2 protein and the $\alpha2^{Ser10}$ mutant, respectively. The wild-type α2 protein was retained on the column as indicated by the absence of the α2 in the column flowthrough (lane 4). In contrast, the $\alpha2^{Ser10}$ mutant protein appeared in the flowthrough (lane 5). The columns were washed and subsequently eluted with free glutathione, which displaces GST–CTERM protein and any protein bound to it from the glutathione–agarose bed. Elution of the column loaded with wild-type α2 displaced both α2 and GST–CTERM (lane 6), whereas elution of the column loaded with $\alpha2^{Ser10}$ yielded only the GST–CTERM protein (lane 7). These results indicate that the wild-type α2 amino terminus is required for Tup1 binding. Consistent with this idea, a deletion mutant of α2 lacking amino acids 2–10 ($\alpha2^{\Delta2-10}$) also failed to bind to a GST–CTERM Tup1 column (data not shown). Curiously, when the same experiment is repeated with a column bearing the single WD repeat, $\alpha2^{\Delta2-10}$ bound as well as wild-type α2 (data not shown). Thus, the amino terminus of α2 is required for binding a

portion of Tup1 containing all seven WD repeats; it is not required to bind to WD2 in isolation. A possible explanation is that the WD repeat(s) of Tup1 required for binding α2 are masked in some way, and the amino terminus of α2 is required to unmask these WD repeats allowing Tup1 and α2 to associate.

The carboxyl terminus of Tup1 is sufficient for α2-mediated repression in vivo

Repression of the **a**-specific genes requires that Tup1 both bind to α2 and interfere with transcription. The affinity column experiments demonstrated that the carboxyl terminus of Tup1 interacts with α2 in the absence of Ssn6. Next, we wished to determine whether a carboxy-fragment of Tup1 is sufficient for in vivo repression as well. α cells lacking either Tup1 or Ssn6 are sterile, because of the derepression of the **a**-specific genes. If the carboxyl terminus of Tup1 is capable of both binding α2 and repressing transcription in the absence of Ssn6, then expressing the carboxyl terminus in an α strain lacking both Tup1 and Ssn6 should restore repression of the **a**-specific genes and correct the mating defect. As shown in Figure 6, a *MATα tup1Δ ssn6Δ* strain transformed with a vector plasmid is sterile, but the same strain transformed with a plasmid overexpressing either full-length Tup1 or Tup1(336–713) mates as an α cell, indicating that repression of the **a**-specific genes has been at least partially restored. Hence, a fragment of Tup1 consisting almost exclusively of WD repeats is capable of both interacting with α2 and bringing about repression. Furthermore, Ssn6 is not absolutely required for either function, as overexpression of Tup1 partially compensates for a lack of Ssn6. Overexpression of Ssn6, in contrast, has no effect on the mating behavior of the *MATα tup1Δ ssn6Δ* strain (data not shown).

We wish to emphasize that suppression of the phenotypes of a *tup1Δ ssn6Δ* strain by the fragment of Tup1

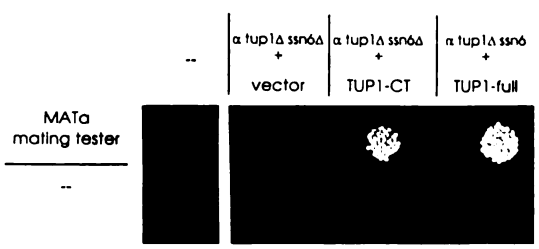

Figure 6. Overexpression of Tup1 suppresses the mating defect of a *MATα ssnΔtup1Δ* strain. A *MATα ssn6Δ tup1Δ* strain was transformed with plasmids expressing no Tup1, the Tup1 carboxyl terminus (CT), or full-length Tup1 (full) from the *GAL10* promoter. Transformants were mixed with a *MATa* tester strain (*top*) or no tester strain (*bottom*) and grown on a plate that selects for diploids resulting from conjugation. The unmated *MATa* tester strain is plated to the *far left*. Unmated transformants plated in the *bottom* row do not form a background patch because of their slow growth and clumpiness.

containing only WD repeats is not complete. In addition to exhibiting α-specific sterility, yeast strains lacking Ssn6 or Tup1 are clumpy and slow growing, presumably because of the inappropriate expression of normally repressed genes. Overexpression of Tup1 does not correct the slow growth or clumpiness of the *tup1Δ ssn6Δ* strain, indicating that the absence of Ssn6 cannot be completely compensated for by increased levels of Tup1. Also, although overexpression of Tup1 (336–713) provides sufficient repression of **a**-specific genes to suppress the mating defect of an *αtup1Δ ssn6Δ* strain, the level of repression of an *mfa2:lacZ* reporter in these strains is quite weak; much stronger repression is observed when either Tup1(254–713) or full-length Tup1 is overexpressed.

Discussion

Tup1 interacts with a DNA-binding protein

Although Ssn6 and Tup1 are required for the transcriptional repression of a wide variety of genes, neither Ssn6 nor Tup1 has been reported to bind DNA, nor does there appear to be any promoter element common to all sets of Ssn6/Tup1-repressed genes. Our present studies show that Tup1 interacts directly with α2, a homeo domain protein that binds to sequences found upstream of the **a**-specific genes. The in vivo relevance of the in vitro interaction between Tup1 and α2 is supported by our isolation of α2 repression-defective mutants that occupy the operator but fail to repress and by our observation that the strongest of these mutants does not bind Tup1 in vitro. These results imply that α2 directs repression by interacting with Tup1 and that the failure of the mutants to repress transcription is attributable to their inability to recruit Tup1 to the operator.

We predict that Tup1 also interacts with the DNA-binding proteins found upstream of other Ssn6/Tup1-regulated genes, thus explaining how Ssn6 and Tup1 are able to inhibit expression of a wide variety of genes having no common upstream sequences. This arrangement of a transcriptional regulator influencing many diverse genes by interacting with a multitude of site-specific DNA-binding proteins has also been reported for the viral activator E1A, which interacts directly with various gene regulatory proteins that bind upstream of E1A-regulated genes (e.g., see Liu and Green 1994). Unlike E1A, which interacts directly with the DNA-binding domains of various transcriptional activators, Tup1 requires a region of α2 outside of the DNA-binding domain. The lesions in three of the four repression-defective mutants that we have isolated lie in the extreme amino terminus of α2, indicating that this stretch of amino acids is important for interaction with Tup1.

A single WD repeat is a protein–protein interaction domain

The carboxyl terminus of Tup1 contains seven copies of a repeating 40 amino acid motif known as the WD repeat. Originally identified in the β-subunit of the het-

erotrimeric G protein transducin, the WD repeat has since been found in a wide variety of proteins. Because members of the WD family of proteins share no obvious functional properties and are often engaged in multisubunit complexes, it has been assumed that the WD repeat is a structural element involved in protein–protein interaction. This assumption is supported by several observations. First, proteins such as β-transducin and Sec13, which are known to interact biochemically with other proteins, consist mainly of WD repeats, suggesting that binding might occur through the repeats (for review, see Conklin and Bourne 1993; Salama et al. 1993). Second, antibodies raised to peptides within the repeats of β-transducin can inhibit its ability to interact with the transducin α subunit (Murakami et al. 1992). Third, Gpa1 and Ste4, the α and β subunits of a yeast G protein, interact in vivo in the two-hybrid fusion assay; this interaction is disrupted by mutations in the second WD repeat of Ste4 (Clark et al. 1993; Whiteway et al. 1994). In this study we have shown directly that a single WD repeat of Tup1 will bind to α2 and can therefore function as a discrete unit.

The ability of an isolated WD repeat to mediate protein binding raises the question of why WD repeats tend to be found in iterated arrays. One possibility is that the repeats are functionally redundant. Tup1, for example, probably has at least two WD repeats capable of binding α2 if WD1 is responsible for the binding of the middle region to α2. The presence of more than one α2-binding WD repeat might allow full-length Tup1 to interact with more than one domain or molecule of α2 and thus strengthen overall binding. Another possibility is that interactions between the WD repeats themselves influence the binding properties of the protein as a whole. A fragment of Tup1 containing all seven WD repeats binds to wild-type α2 but not to an α2 negative control mutant; a single WD repeat binds to both wild-type and mutant α2. Apparently the presence of other WD repeats somehow confers specificity upon the binding of an individual repeat to α2. Finally, it is possible that different WD repeats bind different proteins. In addition to binding to α2, Tup1 presumably interacts with various DNA-binding proteins found upstream of other Ssn6/Tup1-repressed genes as well as with other components of the repression machinery (see below). Each of these interactions could, in principle, be carried out by a different WD repeat. The presence of multiple repeats might allow WD proteins in general to interact with several proteins at once and to direct the assembly of a variety of multiprotein complexes.

The WD repeats of Tup1 bind α2 and partially repress transcription in vivo

Because α cells lacking either Ssn6 or Tup1 aberrantly express their a-specific genes, it was thought that both proteins were necessary for α2-mediated repression. Surprisingly, we have found that overexpression of the carboxyl terminus of Tup1 allows partial repression of the a-specific genes in a strain lacking Ssn6, indicating that the WD repeats of Tup1 can mediate both binding to α2 and partial repression of transcription and that Ssn6 is not absolutely required for either of these activities. Hence, it appears that α2-directed repression involves a complex of α2, Tup1, and Ssn6, in which α2 binds DNA, Tup1 both binds α2 and interferes with transcription, and Ssn6 plays a peripheral role, perhaps serving to stabilize the Tup1/α2 complex (Fig. 7).

The inability of Tup1 overexpression to correct all of the defects of a *tup1Δ ssn6Δ* strain indicates that Ssn6 is required for repression of some sets of genes even in the presence of excess Tup1. One possible explanation for this observation is that the interaction between Tup1 and α2 is stronger than the interaction between Tup1 and other DNA-binding repressors and that we cannot achieve sufficiently high levels of Tup1 in vivo to drive formation of the other complexes in the absence of Ssn6. Alternatively, the Ssn6–Tup1 complex might interact differently with the individual DNA-binding proteins that mediate repression of the various repressed gene sets. This latter explanation is supported by the observation that a fragment of Tup1 lacking WD repeats will partially repress a hypoxic gene and a glucose-repressed gene but not an α2-regulated gene in the presence of Ssn6 (Tzamarias and Struhl 1994; K. Komachi and A.D. Johnson, unpubl.). These results are not necessarily contradictory, given that Tup1 appears to have two repression domains (Tzamarias and Struhl 1994): one in the amino terminus, which also contains an Ssn6-binding domain; and one in the carboxyl terminus, in a region overlapping with the first WD repeat. It is possible that the Ssn6–Tup1 complex interacts with Rox1 and Mig1 mainly through Ssn6 but with α2 mainly through Tup1. Thus, the amino terminus of Tup1 could repress transcription of the hypoxic and glucose-repressed genes by tethering the amino-terminal repression domain to Rox1 and Mig1 via Ssn6; likewise, the carboxyl terminus of Tup1 could repress transcription of the a-specific genes by recruitment of the carboxy-terminal repression do-

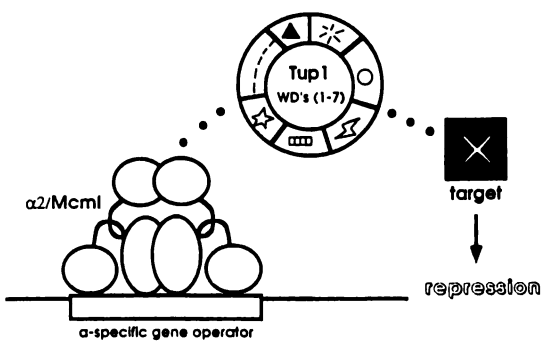

Figure 7. Model for α2 repression. Tup1 is recruited to the a-specific genes by binding to α2 and represses transcription by interacting with a downstream target. The WD repeats (represented by the different symbols) mediate both of these interactions, which can occur in the absence of Ssn6.

main via a direct interaction between Tup1 and α2. Complete repression of all sets of Ssn6–Tup1-regulated genes, however, would require both full-length Tup1 and Ssn6.

Possible targets of Tup1 repression

Although it is formally possible that Tup1 represses merely by binding to α2 and providing some sort of steric block to transcription, we believe that Tup1 interferes with transcription by interacting with a downstream target for the following reasons. First, mutations in genes other than *SSN6* and *TUP1* disrupt α2-mediated repression and cause pleiotropic phenotypes similar to those engendered by disrupting *SSN6* or *TUP1* (M. Wahi and A. Johnson, pers. comm.). The products of these *ARE* (alpha2 repression) genes represent possible downstream targets of Tup1. Second, there exist dominant alleles of *TUP1* whose mutations map to WD repeats other than those thought to bind α2 (K. Komachi and A.D. Johnson, unpubl.). Such mutants might be dominant because they fail to interact with the downstream target but are able to bind α2 and displace wild-type Tup1. Finally, deletion analysis by Tzamarias and Struhl (1994) has identified at least two regions of Tup1 that are capable of repressing transcription from a LexA operator when fused to LexA and may interact with downstream targets.

Although the ultimate target of Tup1 repression is the transcription machinery, the direct downstream target remains a mystery. One possibility is that Tup1 interacts with nucleosomes or some component of chromatin, as α2 has been shown to position nucleosomes in an Ssn6/Tup1-dependent manner, and correlations have been made between nucleosome positioning and repression (Roth et al. 1990; Cooper et al. 1994). However, it is unlikely that nucleosomes are the sole target because mutations in histone H4 that disrupt nucleosome positioning by α2 cause only slight derepression of the a-specific genes (Roth et al. 1992). Furthermore, α2 can direct Tup1-dependent repression of basal transcription in an in vitro system that presumably lacks nucleosomes, suggesting that another target of Tup1 might be RNA polymerase and its entourage of initiation factors (Herschbach et al. 1994). Given that each WD repeat theoretically allows interaction with at least one other protein, the ability to bind multiple targets may be a general characteristic of WD proteins. β-Transducin, for example, is thought to act in signal transduction by influencing a variety of downstream effectors, including β-adrenergic receptor kinase, phospholipases A_2 and C, and adenyl cyclase (for review, see Clapham and Neer 1993). Likewise, Tup1 might repress transcription by interacting with a number of different proteins, such as histones, the *ARE* gene products, or components of the general transcription machinery.

Materials and methods

Plasmids and strains

Plasmid pAV101 was constructed by Andrew Vershon (Waksman Institute, Rutgers, Piscataway, NJ) and contains the *Hin*dIII–*Hin*dIII *MAT*α fragment (Astell et al. 1981) into which a *Bgl*II site has been engineered at the codon for the eighth amino acid of α2 and in which the *Hpa*I site immediately downstream of α2 has been replaced by a *Bam*HI site. Plasmid pKK63 was constructed from pAV101 by removing the 0.7-kb *Nde*I fragment containing α1 and inserting the resulting 3.6-kb *Hin*dIII fragment into the *Hin*dIII site of YEp13 (Broach et al. 1979).

Plasmid pKK68 was constructed by inserting the center-substituted operator (Keleher et al. 1988; see Fig. 4) into the *Xho*I site of a version of pLGΔSS (Johnson and Herskowitz 1985) from which the 2μ sequences have been removed. Yeast strain KKYd25 was constructed by integrating pKK68 at the *URA3* locus of KT23αx8 (*matΔ trp1 leu2 ura3 his4*) (Tatchell et al. 1981; Siliciano and Tatchell 1984). Single-copy integration was confirmed by DNA–DNA hybridization.

Unless noted otherwise, all yeast strains used were constructed in the EG123 background (Astell et al. 1981). The α *mfa2:lacZ* fusion strain used was SM1196 (*MAT*α *mfa2:lacZ trp1 leu2 ura3 his4*) (Hall and Johnson 1987). KKY122 was constructed by replacing *MAT*α*2* of SM1196 with *URA3*. All mutant α*2 mfa2:lacZ* strains were constructed by cotransforming KKY122 with YEp13 and a *Hin*dIII–*Nde*I fragment containing the mutant *MAT*α*2*, selecting for growth on medium lacking leucine and subsequently selecting for loss of the *URA3* marker on medium containing 5-fluoro-orotic acid. Integration at *MAT* was determined by DNA–DNA hybridization.

The *TUP1* and *SSN6* high-copy plasmids used were pFW28 and pLN113-3, respectively (Schultz and Carlson 1987; Williams and Trumbly 1990). Plasmid pKK371 was constructed by inserting the *Sph*I fragment containing *SSN6* from pLN113-3 into the *Sph*I site of pFW28, creating a high-copy plasmid containing both *TUP1* and *SSN6*.

The GST–CTERM expression vector was constructed by ligating the *Bam*HI fragment from plasmid pFW28 (Williams and Trumbly 1990) containing a portion of the *TUP1* seqence into pGEX-2T (Smith and Johnson 1988). The remaining GST–fusion expression vectors were constructed by amplification of the appropriate *TUP1* seqences with the polymerase chain reaction (PCR). Oligonucleotides (5′ and 3′) containing restriction sites were utilized to facilitate cloning into vectors pGEX-3X or pGEX-2T (Smith and Johnson 1988).

Plasmids pAV99 (Mak and Johnson 1993) and pKK211 were used to create *E. coli* expression vectors for α2 and $\alpha2^{Ser10}$, respectively. Plasmid pKK211 was constructed by replacing the *Bgl*II–*Bam*HI fragment of pAV99 with the *Bgl*II–*Bam*HI fragment of pKK99, the Ser-10 mutant version of pKK63. For overexpression in *E. coli*, the 1.0-kb *Bam*HI fragment was removed from downstream of the $\alpha2^{Ser10}$ coding sequence, and the resulting plasmid was transformed into an *E. coli* strain containing an $F'lacI^{Q1}$.

Plasmids for expressing full-length Tup1 or the carboxyl terminus of Tup1 in yeast were constructed using pSJ1 (Herschbach et al. 1994), which contains the *GAL10* promoter upstream of a polylinker. Plasmid pΔSJ was constructed by Andrew Vershon by deleting the *Xho*I–*Sal*I fragment containing the translational start of pSJ1. Plasmid pKK391 was constructed by replacing the *Bam*HI–*Hin*dIII fragment of pΔSJ with a PCR fragment containing the entire coding sequence of *TUP1*. Plasmid pKK462 was constructed by replacing the *Bam*HI–*Hin*dIII fragment of pSJ1 with a PCR fragment containing the coding sequence for amino acids 336–713 of *TUP1*.

Yeast strain BB-2c (*MAT*α *trp1 leu2 ura3 his4 ssn6Δ9 tup1Δ::LEU2*) was provided by Burkhard Braun (University of California, San Francisco). KKY144 was constructed by replacing the *tup1Δ::LEU2* allele of BB-2c with an unmarked *TUP1* deletion and transforming the resulting strain with pAS107, an

integrating *GAL2*-bearing plasmid provided by Anita Sil (University of California, San Francisco). The unmarked *TUP1* deletion was introduced into BB-2c using plasmid pRT164 which contains a *TUP1* deletion disrupted by *URA3* flanked by *hisG* repeats (Alani et al. 1987); pRT164 was provided by Robert Trumbly (Medical College of Ohio, Toledo).

Plasmid mutagenesis

Mutagenesis of pKK63 by passage through a mutator strain of *E. coli* was achieved by transforming the plasmid into TAM12mutD5 (Scheuermann et al. 1983). A single transformed colony was isolated, picked, and grown to saturation in 50 ml of LB medium plus 100 μg/ml of ampicillin, and plasmid DNA was isolated from these cells. Hydroxylamine mutagenesis of pKK63 was performed as described previously (Nelson et al. 1983) except that the DNA was incubated in hydroxylamine at 65°C for 90 min and the hydroxylamine was removed by passing the sample over a P10 resin spin column. Mutagenized plasmid DNA was used to transform JA194, a *leuB*$^-$ strain of *E. coli* whose inability to grow on leucine can be complemented by the *S. cerevisiae LEU2* gene. Transformed JA194 colonies able to grow on LB plus 50 mg/ml of ampicillin but unable to grow on media lacking leucine were found at an approximate frequency of 10^{-4}.

Mutant screen and yeast plasmid isolation

KKYd25 was transformed with mutagenized plasmid DNA by the lithium acetate method (Ito et al. 1983) and plated at a density of ~500 colonies per plate on plates lacking leucine and uracil (−Ura–Leu plates). Transformants were replica plated onto nitrocellulose filters on −Ura–Leu plates and grown for 12 hr at 30°C. The colonies were scored for β-galactosidase production by immersing the filter in liquid nitrogen for 20 sec, placing the filter on a disc of Whatman 3MM paper in a petri dish containing 2.2 ml of 0.3 μg/ml 5-bromo-4-chloroindolyl-β-D-galactopyranoside (Xgal) in Z buffer (Miller 1972), and incubating the filter for 10 hr at 30°C.

Potential positives were picked from the original transformation plate, streaked for single colonies, and retested for blueness by the filter assay. Mutant plasmids were isolated from positive colonies as described in Schena et al. (1989). Yeast plasmids were transformed into the *E. coli* strain HB101 by the $CaCl_2$ method.

Liquid β-galactosidase assays

β-Galactosidase assays were performed as described (Miller 1972), except that yeast cells were permeabilized with 0.0025% SDS and 5% chloroform, the assays were performed at 25°C, and the cell debris was removed by centrifugation prior to reading the OD_{420} of the sample, thus eliminating the need to correct for light scatter. Activities are reported in Miller units.

Purification of GST–fusion proteins

GST–fusion protein expression vectors were transformed into *E. coli*, and cells carrying expression vectors were grown to saturation in 300 ml of LB medium containing 100 μg/ml of ampicillin. This culture was then used to inoculate 3 liters of 2× LB containing 100 μg/ml of ampicillin. Cells were grown to an optical density of ~0.8. IPTG was added to 0.1 mM. Cells were grown for 3 hr and subsequently harvested by centrifugation. Cells were washed once in ice-cold PBS [140 mM Na_2HPO_4, 1.8 mM KH_2PO_4 (pH 7.2), 138 mM NaCl, 2.7 mM KCl] and frozen in liquid nitrogen. Cells were lysed by the addition of 5 volumes of PBS containing 1 mM EGTA, 1 mM EDTA, 1 mM PMSF, and 200 μg/ml of lysozyme. Cells were stirred for 30 min to break up the pellet. Lysis was completed with sonication or several minutes. KCl and DTT were added to 0.3 M and 15 mM, respectively. Extracts were then centrifuged at 100,000g for 1 hr prior to loading over a 6-ml glutathione–agarose column at 40 ml/hr. The columns were then washed with PBS containing 0.3 M KCl and 1 mM DTT at a flow rate of 60 ml/hr until no proteins could be detected in the flowthrough. The columns were eluted with 50 mM Tris (pH 8.0), 0.3 M KCl, and 5 mM glutathione. Protein was detected by Bradford assay (Bradford 1976). Peak fractions were pooled and dialyzed into 50 mM HEPES (pH 7.6), 0.25 M KCl, 30% glycerol, 1 mM EGTA, 1 mM $MgCl_2$, and 1 mM DTT. Protein yields varied between 5 and 50 mg, depending on the particular fusion protein. Proteins were frozen in liquid nitrogen and stored at −75°C.

α2 and α2^{Ser10} extracts

E. coli extracts containing α2 or α2^{Ser10} were prepared as described in Sauer et al. (1988), except that the extracts were prepared from cells grown at 37°C and were purified no further than the ammonium sulfate precipitation step. The ammonium sulfate pellet was resuspended in U buffer (50 mM Tris (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol, 5 M urea), dialyzed against U buffer, and centrifuged at 10,000 rpm in an SS34 rotor for 30 min. The supernatant was then dialyzed against S + 500 buffer [500 mM NaCl, 50 mM Tris-HCl (pH 8.0), 1 mM EDTA, 10 mM 2-mercaptoethanol] and centrifuged at 10,000 rpm in an SS34 rotor for 30 min. Extracts were stored at −75°C in S + 500 buffer.

Column chromatography

GST–fusion proteins were immoblized on glutathione–agarose (Sigma) by incubating overnight in binding buffer (250 mM KCl, 50 mM HEPES at pH 7.6, 10% glycerol, 2 mM DTT, 1 mM $MgCl_2$). Columns were then constructed containing 0.5 ml of glutathione–agarose bound to ~0.5 mg of GST–fusion protein. The columns were pre-eluted with 2 ml of elution buffer (1 M NaCl, 2 mM DTT, 50 mM HEPES at pH 7.6, 2 mM EDTA) and equlibrated with 4 ml of wash buffer (30–50 mM NaCl, 50 mM HEPES at pH 7.6, 10% glycerol, 2 mM DTT, 1 mM $MgCl_2$).

Bacterial extracts containing α2 or derivatives were diluted to a final salt concentration of 30–50 mM NaCl in 50 mM HEPES at pH 7.6, 2 mM DTT, 1 mM $MgCl_2$, 0.1 mM AEBSF (Calbiochem). Diluted extracts were centrifuged at 100,000g for 1 hr prior to loading over columns. Extract (4 ml) was loaded at 1.5 ml/hr on columns. Fractions (0.5 ml) were collected. The columns were washed with 2 of ml wash buffer then eluted with elution buffer. Peak fractions were identified by Bradford assays and pooled. Pooled elution fractions and flowthrough fractions were then precipitated with 10% trichloroacetic acid. Protein pellets were resuspended in SDS sample buffer and loaded onto 12% or 14% SDS–polyacrylamide gels for eletrophoresis. Gels were then stained with Coomassie blue.

Mating tests

Transformed strains and a *MATa lys1* tester strain were grown to saturation in liquid media containing 2% galactose and lacking leucine (SGAL − Leu). The transformants were mixed with the tester at a ratio of 10:1 (transformant/tester), spotted onto SGAL − Leu plates, and incubated at 30°C for 24 hr. The grown

patches were then replica plated onto minimal plates and incubated at 30°C for 24 hr to select for diploids.

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Note added in proof

After this manuscript was accepted for publication, Elizabeth Reisinger and Cynthia Wolberger (Johns Hopkins University School of Medicine, Baltimore, MD) alerted us to a mutation present in our GST–WD2 expression plasmid. The mutation changes the TGG coding for amino acid 470 of Tup1 to TGC, resulting in the substitution of cysteine for tryptophan at that position. This change bears only on the experiment shown in Figure 5B and summarized in Figure 4A, line 6, and may affect its interpretation. We apologize for this mistake and are now repeating the relevant experiment using a construct with the wild-type sequence.

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Chapter 3

Accessibility of α2-Repressed Promoters to the Activator Gal4

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Accessibility of α2-Repressed Promoters to the Activator Gal4

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It has been proposed that eukaryotic repressors of transcription can act by organizing chromatin, thereby preventing the accessibility of nearby DNA to activator proteins required for transcription initiation. In this study, we test this idea for the yeast α2 repressor using a simple, artificial promoter that contains a single binding site for the activator protein Gal4 and a single binding site for the repressor α2. When both the repressor and the activator are expressed in the same cell, the artificial promoter is efficiently repressed. In vivo footprinting experiments demonstrate that Gal4 can occupy its binding site even when the promoter is repressed. This result indicates that α2-directed repression must result from interference with some stage in transcription initiation other than activator binding to DNA.

Negative regulation of transcription in eukaryotes occurs by a variety of mechanisms. Some repressors act by preventing the DNA binding of activators, some bind DNA and interact with nearby activators, "quenching" their activation surface, and some communicate directly with the general transcription machinery, blocking its function or assembly (for reviews, see references 14, 16, 18, and 26). Still other repressors appear to organize repressive forms of chromatin that block the accessibility of proteins to DNA (for reviews, see references 31, 33, and 45). For some repressors, more than one of these mechanisms is thought to function simultaneously, resulting in a very low level of gene expression under repressing conditions.

One case in which two mechanisms of repression have been proposed is that of the yeast α2 protein. This protein is responsible for repressing the expression of two sets of cell-type-specific genes, a-specific genes and haploid-specific genes (for reviews, see references 7, 15, and 17). To repress a-specific genes, α2 binds cooperatively with the Mcm1 protein to a 34-bp DNA sequence called the a-specific gene operator. α2/Mcm1 binds a second protein complex composed of the Tup1 and Ssn6 proteins. Tup1 and Ssn6 are required for the repression of at least five sets of yeast genes and have been proposed to function as a general repression machine in *Saccharomyces cerevisiae*, recruited to DNA by a variety of sequence-specific DNA-binding proteins (21, 24, 41, 42).

The a-specific gene operator will bring about repression when placed in many positions upstream of a target gene, and models for repression by α2/Mcm1/Ssn6/Tup1 (referred to as the α2 repression complex) must account for this action at a distance (20, 32). One model proposes that the α2 repression complex interacts directly with the general transcription machinery at the promoter, blocking its assembly or maturation (13, 20). A second model proposes that the α2 repression complex positions nucleosomes over promoter elements, blocking the accessibility of nearby DNA to proteins (23, 34, 35, 37). In this work, we wished to determine whether an α2-repressed promoter is accessible to Gal4, a yeast activator protein that binds DNA.

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MATERIALS AND METHODS

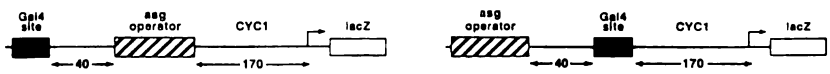
Plasmids. The a-specific gene operator used in this study is derived from *STE6* (20). The Gal4-binding site is the consensus site (CGGAGGACTGTCCTCCGT GCA) (44). The Gal4-binding site and the *STE6* operator were ligated into the *Pst*I site and the *Sal*I site, respectively, of the Bluescript polylinker and were subsequently subcloned into the blunted *Sal*I site of pΔSS (19) in either orientation to produce $pASG_{2\mu}$ and $pGAL_{2\mu}$. Promoter regions were then sequenced. Integrating plasmids were constructed by removing the 2μm sequences, resulting in $pASG_{int}$ and $pGAL_{int}$.

Yeast strains and β-galactosidase assays. All four yeast strains used in this study are derivatives of EG123 (*MATa trp1 leu2 ura3 his4*). *matΔ* is KT23αx8, created by deletion of *MAT*α from 246-1-1 (*MAT*α *trp1 leu2 ura3 his4*) (36, 39). Plasmid pSJ4*LEU* was used to make a deletion insertion of *LEU2* at the *GAL4* gene (10). Plasmids $pASG_{int}$ and $pGAL_{int}$ were integrated into the *ura3*-52 allele. Integrations were confirmed by Southern analysis (38). β-Galactosidase assays were performed as described by Goutte and Johnson (12). Cells were grown initially on synthetic medium minus uracil plus 2% glucose and then transferred to synthetic medium minus uracil plus 2% galactose, 2% ethanol, and 3% glycerol for several cell doublings.

Competitive PCR for quantitation of mRNA. The levels of repression of an a-specific gene, STE2, were compared at the RNA level between *MAT*α and *matΔ* cells. Quantitative PCR (9) was used to detect the very low levels of a-specific gene mRNA present in α cells. Briefly, RNA was isolated from cells, reverse transcribed (Superscript II; BRL) by using a *STE2*-specific primer, and added to PCR mixtures containing known amounts of a competitor DNA that was amplified with the same *STE2* primers as the cDNA but that resulted in a smaller PCR product due to an internal deletion in the *STE2* gene. The relative amounts of target cDNA versus competitor can be measured by direct scanning of ethidium-stained gels (1-D Multi-Lane Scan, IS-1000 Digital Imaging System), and these amounts can be compared between *MAT*α and *matΔ* cells to determine the level of repression of an a-specific gene.

Genomic footprinting. In vivo footprinting was performed as previously described, with modifications (1). Yeast strains were grown in 100 ml of synthetic medium minus uracil plus 2% galactose, 3% glycerol, and 2% ethanol to a density of 10^7 cells per ml. The cells were pelleted and resuspended in ice-cold medium to a final volume of 1 ml. A 5-μl volume of dimethyl sulfate was added with vigorous mixing. The cells were incubated at 20°C for 5 min, after which the reaction was quenched with 50 ml of ice-cold 10 mM Tris (pH 7.5)–1 mM EDTA. The cells were pelleted and resuspended in 900 μl of lysis buffer (50 mM morpholinepropanesulfonic acid [pH 7.0], 200 mM NaCl, 5 mM EDTA, 0.5% Triton X-100). The cells were lysed with glass beads (0.5-mm diameter) for 45 s in a bead beater (Biospec Products). The lysate was removed from the glass beads and diluted in 3.5 ml of additional lysis buffer. The lysate was treated with RNase A (250 μg/ml) and proteinase K (100 μg/ml) for 1 h at 37°C. The cellular debris was pelleted (12,000 × *g* for 20 min), and genomic DNA was prepared by loading the supernatant onto a Qiagen column (Qiagen Inc., Studio City, Calif.). DNA was then digested with *Hae*III, phenol chloroform extracted, ethanol precipitated, and resuspended in 100 μl of Tris-EDTA. Finally, the DNA was dialyzed against water (12,000- to 14,000-Da exclusion limit) for 2 h.

Methylated bases were detected by multiple rounds of primer extension with *Taq* polymerase. A 0.5-μg amount of DNA from cells with 2μm plasmids or 10 μg from cells with single-copy reporters, 1 pmol of end-labeled primer, 1 U of *Taq* polymerase, 200 μM each deoxynucleoside triphosphate, and 1× *Taq* buffer (40 mM NaCl, 10 mM Tris [pH 8.9], 5 mM $MgCl_2$, 0.01% gelatin [30]) were combined in a total volume of 50 μl. Mineral oil was layered over the samples,



	$pASG_{2\mu}$	$pASG_{int}$		$pGAL_{2\mu}$	$pGAL_{int}$
MATα	3	0.15	MATα	88	11
matΔ	2500	125	matΔ	3900	217
MATα gal4::LEU2	2	0.17	MATα gal4::LEU2	2	0.14
matΔ gal4::LEU2	220	8	matΔ gal4::LEU2	30	2

FIG. 1. α2 represses test constructs activated by Gal4. The test constructs are diagrammed at the top of the figure. Each construct consists of a single Gal4-binding site and a single a-specific gene (asg) operator upstream of a *CYC1 lacZ* promoter fusion. The distances in base pairs between the promoter elements are indicated. At the bottom of the figure are the results of β-galactosidase activity assays performed with four different strains. Values are the averages of assays performed in duplicate on three independent transformants.

which were then subjected to 10 to 20 rounds of thermal cycles (1 min at 94°C, 2 min at 55 to 63°C, and 1 min at 72°C). The mineral oil was extracted with chloroform, and the samples were ethanol precipitated. The pellets were washed with 70% ethanol, dried briefly, and resuspended in 4 μl of formamide loading buffer. The primer extension products were then electrophoresed through a 6% polyacrylamide sequencing gel. The gels were dried and exposed to Kodak XAR-5 film for 12 to 24 h. Note that many methylated guanines appear as doublets by *Taq* polymerase primer extension because of the variable addition of an extra nucleotide. This does not affect the interpretation of these results.

Plasmid DNA was methylated in vitro as described by Maxam and Gilbert (28), and 10 ng was used for primer extension as described above. Neither the methylated plasmid DNA nor the genomic DNA was treated with piperidine, since this step is unnecessary (4).

The primers used in this study were as follows. For plasmid $pASG_{2\mu}$, the bottom-strand primer (5'-ATCCACGCTATATACACGCCTGGC-3') anneals to top-strand sequences in the *CYC1* promoter from positions −236 to −212 with respect to the first codon. The $pGAL_{2\mu}$ primer (5'-CTAAAGTTGCCTGGCCA TCCACGC-3') anneals to the top strand of the *CYC1* promoter from positions −220 to −196 with respect to the first codon. The primers used for the coding and noncoding strands of plasmid $pGAL_{2\mu}$ were 5'-AACTGTATTATAAGTAA ATGCATG-3' and 5'-TGCCATATGATCATGTGTCGTCGC-3', respectively. For the integrating constructs, primers were designed that hybridized to sequences in both the *CYC1* promoter ($pASG_{int}$) and the *URA3* gene ($pGAL_{int}$), as well as in the *STE6* operator, in order to avoid background from the native yeast genes. For $pASG_{int}$, the primer used was 5'-CGGATCTGCTCGACGA GCGTGTAA-3'. The primer used for $pASG_{2\mu}$ yielded the same results. For $pGAL_{int}$, the primer used was 5'-TCAGTTATTACCCTCGACCTCGTCG-3'.

Isolation and analysis of chromatin. Chromatin was isolated from four strains (*MATα*, *matΔ*, *MATα gal4::LEU2*, and *matΔ gal4::LEU2*) containing promoter constructs $pGAL_{2\mu}$, $pGAL_{int}$, or $pASG_{2\mu}$, according to the Nonidet P-40-permeabilized spheroplast method (22). Briefly, the cells were grown in the medium used for the β-galactosidase assays to an optical density (A_{600}) of 0.8, washed with 1 M sorbitol, and digested with 0.5 mg of Zymolyase T100 (ICN) per ml. Nuclei were washed and resuspended in buffer containing 1 M sorbitol, 50 mM NaCl, 10 mM Tris-HCl (pH 7.4), 5 mM $MgCl_2$, 1 mM $CaCl_2$, 1 mM β-mercaptoethanol, and 0.075% Nonidet P-40. The nuclei were digested for 5 min at 37°C with micrococcal nuclease (MNase) (Worthington Biochemical Corp.) concentrations ranging from 0 to 25 U/ml. DNA was purified by phenol extraction after digestion with proteinase K and RNase A. Naked DNA was prepared in this manner before MNase digestion with 7.5, 15, or 30 U/ml for 1 min at 37°C. Indirect end label analysis was used to determine the positions of nuclease-sensitive regions according to the method described by Thoma et al. (40). Chromatin and naked DNA were cut with a variety of restriction enzymes that cut either in the *lacZ* gene or in the *URA3* gene. The enzymes used that cut in *lacZ* (with the distance from the start of the a-specific gene operator in pGAL constructs or from the start of the Gal4-binding site in pASG constructs indicated in parentheses) were *Hpa*I (853 bp), *Dde*I (534 bp), and *Fsp*I (451 bp). The enzymes used that cut in *URA3* were *Sna*I (441 bp) and *Dde*I (160 bp). Probes were generated by PCR and varied in length from 50 to 238 bp.

RESULTS

α2 represses Gal4-activated promoters. The chromatin reorganization model for repression predicts that DNA near the operator should be less accessible to proteins than is naked DNA. To determine whether an α2-repressed promoter is accessible to Gal4, hybrid promoters containing a single Gal4-binding site and a single **a**-specific gene operator upstream of a *CYC1* β-galactosidase promoter fusion were constructed (Fig. 1). The Gal4-binding site was placed either upstream (pASG) or downstream (pGAL) of the **a**-specific gene operator with respect to the *CYC1* promoter. The plasmid names reflect the DNA element, either the Gal4-binding site or the **a**-specific gene operator, that lies adjacent to the *CYC1* promoter. Promoter constructs either were placed on multicopy 2μm yeast plasmids ($pASG_{2\mu}$ and $pGAL_{2\mu}$) or were integrated into the chromosome at the *URA3* locus ($pASG_{int}$ and $pGAL_{int}$). To assess whether these test promoters were activated by Gal4 and whether activated transcription could be repressed by α2, the constructs were transformed into the following four different cell types: cells containing both α2 and Gal4 (*MATα GAL4*), cells containing only Gal4 (*matΔ GAL4*) or only α2 (*MATα gal4::LEU2*), and cells lacking both proteins (*matΔ gal4::LEU2*). In the presence of galactose, the promoters are activated 10- to 130-fold by Gal4 (Fig. 1; compare values from *matΔ GAL4* cells with those from *matΔ gal4::LEU2* cells). Furthermore, α2 represses transcription approximately 800-fold relative to the activated level when the operator is positioned between the Gal4 site and the *CYC1* promoter ($pASG_{2\mu}$ and $pASG_{int}$) and about 30-fold when the operator is positioned upstream of the Gal4-binding site ($pGAL_{2\mu}$ and $pGAL_{int}$; compare expression from *MATα GAL4* cells with that from *matΔ GAL4* cells). These results indicate that α2 is capable of efficiently repressing activated transcription from these constructs. The fact that the repression is greater when the operator is between the Gal4-binding site and the promoter than when the operator is upstream of the Gal4-binding site is consistent with the behavior of the operator in other test constructs (19). The expression of the constructs in *matΔ gal4::LEU2* strains is presumably due to activation by the MCM1 protein bound to the **a**-specific gene operator (2, 20).

The level of repression of an a-specific gene correlates with the repression of the hybrid reporters. We wished to know whether the strong repression (20- to 800-fold) of the test promoters is comparable to that of a bona fide **a**-specific gene. To determine the magnitude of α2 repression of the **a**-specific gene *STE2*, we employed quantitative RNA PCR analysis (9).

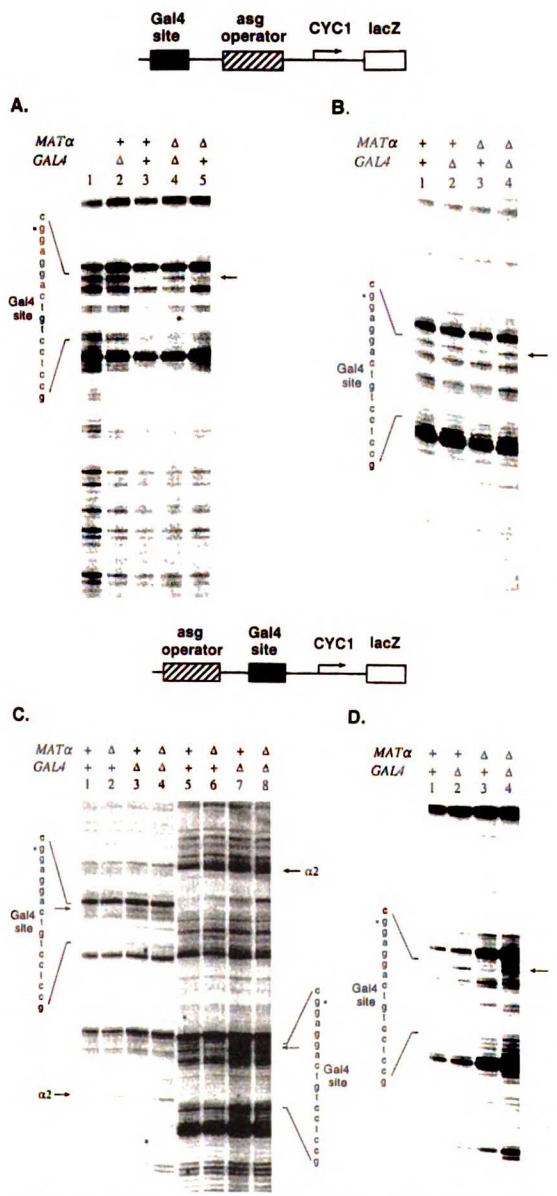


FIG. 2. Gal4 can occupy its site when the test constructs are repressed. Each panel shows the primer extensions from in vivo methylated DNA of the indicated test promoters in four different cell types: *MATα*, *matΔ*, *MATα gal4::LEU2*, and *matΔ gal4::LEU2*. The Gal4-binding site is indicated. The strong bands that bracket the Gal4-binding site are sequence-specific stops for *Taq* polymerase. The Gal4 footprint is clearly detected in *GAL4*$^+$ strains and is indicated by an arrow. The constructs are diagrammed over the appropriate panels. (A) Primer extension of the noncoding strand of promoter construct $pASG_{2\mu}$. Lane 1, extension products from in vitro-methylated plasmid DNA. Coding strand primer extension yields similar results (not shown). (B) Primer extension of the coding strand of promoter construct pASG integrated at *URA3*. (C) Primer extension of the coding strand (lanes 1 to 4) and the noncoding strand (lanes 5 to 8) of promoter construct $pGAL_{2\mu}$; (the α2 footprint is indicated by an arrow). (D) Primer extension of the noncoding strand of promoter construct pGAL integrated at *URA3*. asg, a-specific gene.

The results indicate that *STE2* transcription is repressed 200-fold in α cells relative to a cells (which lack α2), a result that is comparable to that observed in the test promoters, in which the α2 operator is located between the Gal4-binding site and the promoter (data not shown). This result indicates that the test promoters used in this study provide a legitimate model system in which to analyze α2 repression.

Gal4 can occupy its site when the test constructs are repressed. In principle, α2 repression of the test promoters could result either from interference with Gal4 DNA binding or from interference with a subsequent step in transcription initiation. In order to determine whether α2 interferes with Gal4 DNA binding in vivo, we performed dimethyl sulfate footprinting experiments on growing yeast cells. When bound to DNA, Gal4 protects a single guanine on each strand of its binding site from methylation by dimethyl sulfate (11). This protection can be seen in Fig. 2A by comparing the results from DNA isolated from strains that contain Gal4 (lanes 3 and 5) with those that lack it (lanes 2 and 4). In the case of constructs $pASG_{2\mu}$ and $pGAL_{int}$, a Gal4 footprint can be detected both in the activated state (*matΔ GAL4* cells) and in the repressed state (*MATα GAL4* cells) (compare lanes 3 and 5 in Fig. 2A and lanes 1 and 3 in Fig. 2D). For construct $pGAL_{2\mu}$, a clear Gal4 footprint is visible when the construct is active, and a weaker footprint is visible under repressed conditions (Fig. 2C; compare lanes 5 and 6). In the case of construct $pASG_{int}$, a Gal4 footprint is seen in *matΔ* cells but cannot be detected in α cells (Fig. 2B; compare lanes 1 and 3). In three of four of the test promoters (including the most strongly repressed), Gal4 occupies its binding site under conditions in which transcription is tightly repressed (*MATα GAL4* cells). These results indicate that α2 must repress transcription by some means other than preventing the DNA binding of activator proteins. We do not know the reason why Gal4 fails to occupy one of the repressed templates; however, the results obtained with the other three templates prove that repression can occur even though Gal4 is bound. We also note that the α2 footprint can be seen in these experiments (Fig. 2C, lanes 1, 3, 5, and 7, as indicated).

Nucleosomes are not positioned over test promoters. It has been observed that α2 bound to DNA positions nucleosomes adjacent to it, and it has been proposed that this positioning can contribute to transcriptional repression. In contrast to the behavior of α2, DNA-bound Gal4 is able to disrupt binding of the core histone particle both in vitro and in vivo (29, 46). To assess the role of nucleosome positioning in transcriptional repression of the test constructs used in this study, we mapped the distribution of nucleosomes over these constructs in both active and repressed states. Chromatin was isolated and digested with MNase, and the relevant regions of the DNA were displayed by indirect end labeling (40). Digestion patterns across the test promoter $pGAL_{2\mu}$ resembled those of the naked DNA controls (Fig. 3), indicating a lack of positioned nucleosomes even when Gal4 is absent (*MATα gal4::LEU2*). Moreover, the digestion patterns across test construct $pGAL_{2\mu}$ were not observably different in the presence or absence of α2, even though α2 had a dramatic effect on the expression of this construct. In the same chromatin preparations, positioned nucleosomes were seen across the *URA3* gene (in accordance with reference 3), which is located immediately upstream of the test promoter (Fig. 3; note the patterns of enhanced and protected bands in the chromatin preparations which are indicative of positioned nucleosomes [lanes 1 to 4] compared with naked DNA [lane 5]). This last observation indicates that the experiments shown in Fig. 3 are of sufficient resolution to detect positioned nucleosomes. Moreover, we detected positioned nucleosomes across the promoter of the a-specific gene

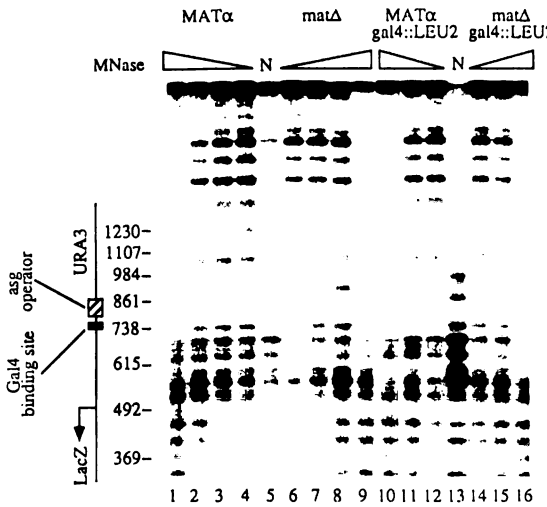


FIG. 3. MNase mapping of the $pGAL_{2\mu}$ promoter region. The indirect-end-labeling method was used to display the results of MNase digestion of chromatin isolated from four strains. Chromatin and naked DNA were cut with *Hpa*I after digestion with MNase. *Hpa*I cuts in the *lacZ* gene, 853 bp downstream of the beginning of the a-specific gene (asg) operator. The labeled primer used for indirect end labeling is 238 bp long, extending from the *Hpa*I site in *lacZ* toward the a-specific gene operator. Lanes 1 to 4, chromatin isolated from the *MATα* strain and digested with decreasing amounts of MNase (6, 3, 1.5, and 0.75 U/ml); lanes 6 to 9, chromatin isolated from the *matΔ* strain and digested with the same but increasing amounts of MNase; lanes 10 to 12, chromatin isolated from the *MATα gal4::LEU2* strain and digested with decreasing amounts of MNase (6, 1.5, and 0.75 U/ml); lanes 14 to 16, chromatin isolated from the *matΔ gal4::LEU2* strain and digested with increasing amounts of MNase (0.75, 1.5, and 3 U/ml). N, naked DNA digested with 15 U of MNase (lane 5) or 30 U of MNase (lane 13) per ml. Size markers in base pairs are indicated on the left, along with a diagram indicating the positions of the a-specific gene operator and the Gal4-binding site, as well as the *lacZ* and *URA3* genes.

STE2 (in accordance with the results described by Ganter et al. [8]), again suggesting that the failure to observe positioned nucleosomes across the artificial promoters is not due to a problem in detecting nucleosomes (data not shown). We repeated nucleosome mapping with the additional promoters ($pGAL_{int}$ and $pASG_{2\mu}$) and, in agreement with the results of Fig. 3, observed no evidence of positioned nucleosomes over any of the hybrid promoters in any of the four strains used in this work (data not shown).

DISCUSSION

This study demonstrates that α2 can efficiently repress transcription of a simple, artificial test promoter while still allowing access of the activator protein GAL4 to its binding site on the DNA. Thus, the α2 repressor must block transcription at a step subsequent to activator binding. On the surface, the presence of GAL4 on the DNA of the repressed promoters seems at odds with the proposal that α2 represses transcription by positioning nucleosomes around its binding site. On the basis of experiments performed in vivo and in vitro (29, 46), DNA-bound GAL4 appears to disrupt nucleosomes. One might have predicted that GAL4 would prevent the nucleosome positioning on the constructs described in this article. This idea was tested experimentally, and the results indicate a lack of specifically positioned nucleosomes regardless of whether GAL4 is present on the DNA.

The failure to detect positioned nucleosomes in the absence of Gal4 was initially surprising in light of the strong nucleosome positioning produced by α2 on native a-specific genes. However, the test promoter differs from those of a-specific genes in several ways. The TATA boxes and the transcription start site of the hybrid promoters are derived from the *CYC1* promoter. One feature of the *CYC1* promoter that might explain the absence of positioned nucleosomes is the constitutive binding of TBP to the TATA box of this promoter as proposed by Chen et al. (6). These investigators found that a derivative of the *CYC1 lacZ* promoter lacking upstream repressor or activator sites was free of positioned nucleosomes. Furthermore, in vivo footprinting indicated that TBP was bound to the TATA elements of this silent *CYC1 lacZ* promoter (also see reference 5). Our results could be explained by the model that TBP is bound to the TATA elements and prevents the *CYC1* promoter from being packaged in nucleosomes. With respect to TBP binding, the *CYC1* promoter may differ from other yeast promoters, including those of some a-specific genes. Despite this fact, the *CYC1* promoters used in this study were very strongly activated by Gal4 and were strongly repressed by α2, suggesting that the differences in initial TBP binding among promoters is relatively unimportant for regulation by these proteins. Finally, if TBP bound to the *CYC1* TATA elements does prevent nucleosomes from forming over this promoter, one might have predicted that a repressor that acts solely by nucleosome positioning would be unable to repress the *CYC1* promoter. As shown here and elsewhere (19, 21), α2 can tightly repress this promoter and the level of repression can be even higher than that of a bona fide a-specific gene.

If α2 does not repress transcription by controlling access of activator proteins to DNA, how does it work? Since α2 can repress basal transcription in vitro (13), it has been proposed that the α2 repression complex may act directly on the basal transcription machinery, interfering with a step in transcription initiation. In further support of this model is the discovery that components of the RNA polymerase II holoenzyme are required for efficient α2 repression (25, 27, 43). Direct interference with the basal transcription machinery seems an apt mechanism for a repressor such as α2 that must efficiently repress a large number of genes that utilize a variety of activator proteins.

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Chapter 4

A Complex Composed of Tup1 and Ssn6 Represses Transcription *in vitro*

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SUMMARY

The *Saccharomyces cerevisiae* Tup1 protein is a member of a family of WD repeat containing proteins involved in repression of transcription. Tup1, along with the Ssn6 protein, represses a wide variety of genes in yeast including cell type specific and glucose repressed genes. In this work, a protein complex containing Ssn6 and Tup1 was purified in order to determine its composition. The size of the complex is estimated to be 440kDa. Tup1 and Ssn6, which are both phosphoproteins, are the only proteins present in stoichiometric amounts. We also demonstrate that this purified complex represses transcription in an *in vitro* assay.

INTRODUCTION

The Tup1 protein of *Saccharomyces cerevisiae* is one of a family of repressor proteins that contain β-transducin or WD repeats. The majority of the WD repeat containing proteins are homologs of β-transducin and are known to be involved in heterotrimeric G protein mediated signal transduction. However, an increasing number of the proteins in this family are localized within the nucleus and are involved in repression of transcription. These include Tup1, Hir1 and Met30 in *S. cerevisiae*; SCON2 in *Neurospora crassa*; extra sex combs and groucho in *Drosophila* ; the COP1 protein in *Arabidopsis thaliana* ; HIRA and the family of TLE proteins in humans (1-10). These WD repeat repressor proteins turn off a wide variety of genes, including those involved in segmentation, sex determination and neurogenesis controlled by groucho and those involved in photomorphogenesis controlled by COP1 (7,11). The HIRA protein has been implicated in the human developmental disease DiGeorge syndrome (8,9).

Of these WD repeat repressor proteins, Tup1 is one of the best characterized. Tup1 along with another protein, Ssn6, is required for the repression of at least five sets of genes in yeast, including the glucose repressed genes, genes regulated by the presence of oxygen (hypoxic genes), the a-specific and haploid-specific genes, and a set of genes induced by DNA damage (12-16). A deletion of either or both of these proteins results in the constitutive expression of all of these genes. Tup1 and Ssn6 are recruited to these specific gene sets by interaction with sequence specific DNA binding proteins. In the case of the a-specific and haploid-specific genes in yeast, the homeodomain protein α2 binds to sequences (operators) located upstream of both these genes, and recruits Ssn6 and Tup1 by direct interaction with each of these proteins (for review see (17)).

Tup1 and Ssn6 interact directly *in vitro* and are found associated in a large complex in yeast extracts (referred to in this work for simplicity as the Ssn6/Tup1 complex) estimated at 1.2MDa (18). The size of this complex suggests that it consists of many

protein subunits. Genetic experiments have implicated a number of additional proteins in the Ssn6/Tup1 repression pathway including Rox3, Sin4, Srb8, Srb9, Srb10, and Srb11 (19-23). Each of these proteins is required for full repression of transcription by Tup1 and Ssn6 *in vivo*. In order to determine the subunit composition of the Ssn6/Tup1 complex, we purified the complex, determined its size, characterized its components, and demonstrated its activity in an *in vitro* repression assay.

EXPERIMENTAL PROCEDURES

Expression vectors

The Ssn6HIS construct was made by ligating a PCR fragment into pRS304 (24) comprised of the SSN6 coding seqence from the Eco NI site to the stop site where a Sal I site (approximately 1000 bp) was engineered by PCR. An oligonucleotide coding for 6 histidine residues followed by a stop codon was ligated in frame with the Ssn6 coding sequence. The 3 ' non-coding sequence of Ssn6 was amplified using PCR and ligated downstream of the 6xHIS tag with oligonucleotide introduced restriction sites. Finally the construct was sequenced (United States Biochemical). The construct was then digested with Pst I and integrated into the yeast genome at the native SSN6 locus resulting in the SSN6HIS fusion and a duplication of the last 1000 nucleotides of the SSN6 gene. Proper integration was confirmed by PCR. The GST-Ssn6 construct was made by cloning a PCR fragment including the entire Ssn6 coding sequence with engineered restriction sites into the plasmid pRS316-GAL1-GST (R. Deshaies unpublished, Cal. Tech.). Ssn6HIS function was checked by introducing the construct into a and α strains bearing the mfa2::lacZ reporter. Repression was measured by X-Gal filter β-galactosidase assay (25). Standard mating assays were also performed to assay complementation of the ssn6Δ9 α-mating defect (26). Gal1-GST-Ssn6 function was checked by introducing the plasmid into α ssn6Δ9 cells and assaying mating in media containing 2% galactose.

Yeast strains and growth conditions

The protease deficient strains BJ5459, MATa ura3-52 trp1 lys2-801 leu2-Δ1 his3-Δ200 pep4::HIS3 prb1-Δ1.6R can1 (27), and FM135 MATa leu2-3, 112 ura3-52 prb1-112 pep4-3 reg1-501 gal1 (R. Deshaies, Cal Tech), were used for the purification of Ssn6HIS

and GST-Ssn6 proteins respectively. FM135 cells harboring the GST-SSN6 plasmid were grown in 3 L of SD minus Uracil, minus Leucine plus 2% galactose to an A_{600} of 0.5. The reg1-501 and gal 1 mutations allow growth and induction in glucose and galactose (28). This culture was then used to inoculate 60L of YEPD plus 2% galactose in an MPP 60L fermentor. Cells were grown until they reached an A_{600} of 2.4, then harvested by filter. Cells containing the Ssn6HIS expression vector were grown in 40L of YEPD in the fermentor until they reached an A_{600} of 2.

Yeast strains SM1196 and SM1179 containing mfa2::lacZ were used to monitor the function of the Ssn6HIS construct have been described (29). Strains EG123 MATa trp1 leu2 ura3 gal2 and 246-1-1 MATα trp1 leu2 ura3 gal2 transformed with the Ssn6HIS construct were used for mating assays. The strain yCK12 MATα ssn6Δ9 trp1 leu2 ura3 gal2 containing the GST-SSN6 plasmid was used for mating assays (15).

Antibodies

Ssn6 and Tup1 antibodies were made against GST fusion proteins. The GST-SSN6 fusion used for generating antibodies was constructed by ligating the Sca I to Bst UI fragment of pSJ208 into the pGEX1 vector (Pharmacia). The GST-CTERMTup1 fusion protein has been described in (30). Purified fusion proteins were sent to Berkeley Antibody Company. Crude rabbit antibodies were then affinity purified against maltose binding protein fused to either Tup1 or Ssn6 (31). Ssn6 and Tup1 maltose binding fusion proteins were constructed by ligating PCR-derived fragments containing the tetratricopeptide coding portion of SSN6, and the WD repeat coding sequence of TUP1 into pMAL2 (New England Biolabs).

Phosphatase assays

Approximately 100ng of the GST-Ssn6/Tup1 preparation and about 10ng of the Ssn6HIS/Tup1 were added to a reaction mixture with the addition of 1μg/ml of leupeptin, pepstatin and bestatin. The inhibitor NaVanadate was added to 4 mM. 200 units of λ phosphatase was added per reaction (New England Biolabs). Reaction mixtures were incubated at 30°C for 30 minutes. The reactions were stopped by addition of Laemmli sample buffer, and heated to 65° C for 10 minutes. A sample of each reaction was then subjected to SDS-PAGE followed by Western immunoblotting.

Protein purification

A frozen 150g cell pellet containing the Ssn6HIS expression vector was lysed with glass beads (Biospec products) in an equal volume of 2x lysis buffer (1M NaCl, 100mM HEPES pH7.9, 20% glycerol, 2% triton X-100, plus the protease inhibitors 10mM benzamidine, 2mM PMSF, and 2μg/ml each of bestatin, pepstatin and leupeptin). Lysed cells were then centrifuged at 33,000rpms in a type 35 rotor for 1 hour at 4° C. The supernatant was collected and 20mls of Ni agarose (Pharmacia) was added. This mixture was rocked for 2.5 hours at 4° C after which it was poured into a column and washed with 60mls of 1x lysis buffer. The column was then washed with 80mls of 1x lysis buffer containing 5mM imidazole, 60mls of 1x lysis buffer plus10mM imidazole, and finally washed with 40mls of 20mM imidazole plus 1x buffer. The column was then washed with 40ml of low salt buffer (100mM NaCl, 20mM Tris pH 8.0, 10% glycerol, 0.1% Tween 20) and eluted with the same buffer plus 150mM imidazole. The Ssn6HIS and Tup1 elution profiles were followed by a dot blot antibody assay. The peak fractions were pooled and loaded over a 5ml HiTrap Q column (Pharmacia). Protein was eluted with a gradient of NaCl from 100mM to 500mM. The peak fractions were pooled concentrated, and loaded over a Superose-6 column (Pharmacia) in 500mM NaCl, 50mM HEPES pH8.0, 10% glycerol and 0.1% Tween 20. Fractions were collected and samples from each were subjected to

SDS-PAGE. The fractions bearing Ssn6HIS/Tup1 were identified by Western immunoblotting. The total yield was approximately 50μg of Ssn6HIS/Tup1. The Stokes radius was determined by comparing the migration of Ssn6HIS/Tup1 through the Superose-6 column with that of molecular size standards (see methods for the glycerol gradient) as in (32).

150g of cells harboring the GST-Ssn6 and Tup1 expression vectors were lysed with glass beads following the addition of an equal volume of 2x lysis buffer (1M $(NH_4)_2SO_4$, 100mM HEPES pH7.9, 10mM EDTA, 10mM DTT, 20% glycerol, with protease inhibitors 10mM benzamidine, 2mM PMSF, and 2μg/ml of bestatin, pepstatin and leupeptin). The lysate was then spun at 7000rpm in a Sorvall GSA rotor for 10 minutes to remove cellular debris. The supernatant was collected. $(NH_4)_2SO_4$ was then added to 70%. The mixture was stirred on ice for 30 minutes. Protein was then pelleted by centrifugation in a SS34 rotor at 15,000rpm for 20 minutes. The protein pellet was then resuspended in an equal volume of 50mM HEPES, 5mM DTT, 5mM EDTA, 10% glycerol plus protease inhibitors. The resuspension was then dialyzed against the same buffer for 2 hours at 4°C (14,000 molecular weight cutoff). The lysate was spun at 30,000rpm in a type 35 rotor for 45 minutes. The supernatant was loaded over a 5 ml glutathione agarose (Sigma) column. The column was washed with 100mls of 1x lysis buffer plus 0.5% Triton X-100, followed by a wash with 15mls of 200mM KOAc, 50mM KHEPES pH7.6, 1mM DTT, 10% glycerol. The column was eluted in the same plus 10mM glutathione. The proteins were concentrated approximately 10 fold by Centricon (Amicon). The total yield of GST-Ssn6/Tup1 was approximately 200μg.

Glycerol gradient sedimentation

A 5ml gradient was made by stepwise addition of 1ml of 35%, 30%, 25%, 20% and 15% glycerol plus 20mM HEPES pH 7.6, 10mM MgOAc, 1mM EDTA and 1mM DTT. The

gradient was allowed to diffuse for 3 hours at 4°C. Protein samples in the same buffer except 10% glycerol were then layered on the top of the gradient along with protein size standards thyroglobulin, ferritin, catalase, lactate dehydrogenase and BSA (Pharmacia). The gradient was then spun at 45,000rpm in a SW.1 rotor for 12 hours at 4°C. 250µl fractions were taken from the top of the gradient. Samples of each fraction were subjected to SDS-PAGE, and visualized by silver staining. The S value of the protein complexes was determined by comparison of the position in the gradient with that of the size standards of known S value.

Stoichiometry

Purified Ssn6HIS/Tup1 separated by SDS PAGE and stained with 0.25% Coomassie Brilliant Blue R-250 in 45% methanol, 10% acetic acid. The gel was destained in 25% methanol, 7% acetic acid followed by drying within cellophane membrane. The dry gel was scanned with a La Cie Silver Scan III Scanner and Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). The amount of Ssn6 relative to Tup1 was determined by densitometry of the stained bands. Since dye binding per microgram of protein varies with each protein the accuracy of this method is +/- 30%.

Immunoblotting

Protein samples were run on 8.5% SDS polyacrylamide gels followed by electrotransfer to PVDF membrane. The membrane was blocked in Tris buffered saline plus 5% milk and 0.2% Tween (TBST) for one hour, then incubated with Ssn6 or Tup1 antibodies at 1:1000 dilution for one hour. Membranes were washed 3x in TBST for 5 minutes. Anti-rabbit antibodies conjugated to horse radish peroxidase (Amersham) were then incubated with the filters in TBST at a dilution of 1:10,000 for one hour. Blots were washed 3x in TBST for

10 minutes. The blots were developed with ECL development reagents (Amersham) and exposed to Kodak XAR film. Dot blot assays were performed by dotting 1µl of a column fraction on nitrocellulose followed by the above immunoblotting protocol.

In vitro transcription

In vitro transcription experiments were conducted as in (33), with some exceptions. The yeast strain from which the transcription extracts were made does not overexpress Tup1 or Ssn6. The transcription reaction contains 3.7mM EGTA, 180mM potassium glutamate, 27 mM potassium acetate, and 6.7 µg/mL acteylated BSA. 86nM α2 was added to reactions where noted.

RESULTS

Purification of Ssn6/Tup1- The large size of the complex in which Ssn6 and Tup1 are found suggests that it has many subunits. In order to determine whether it contains components other than Ssn6 and Tup1 we purified the complex. To facilitate purification the SSN6 gene was affinity tagged at its C-terminus with a Nickel binding six Histidine sequence. The resulting Ssn6HIS fusion gene was integrated at the SSN6 locus, under the control of its own promoter. A Glutathione-S-Transferase (GST) Ssn6 fusion was also constructed with the GST protein fused to the amino terminus of Ssn6. The GST-SSN6 fusion gene was carried on a plasmid and its transcription was under the control of the GAL 1 promoter. Both tagged versions of Ssn6 were expressed in yeast and complemented a deletion of the endogenous SSN6 gene (data not shown). Expression of each of these fusion proteins was confirmed by Western immunoblotting (Fig. 1).

The Ssn6HIS fusion is expressed at approximately the same level of the as is the endogenous Ssn6 protein, and therefore should be present at normal levels in the Ssn6/Tup1 complex. Cells containing Ssn6HIS as the only source of Ssn6 were lysed and fractionated over a Nickel agarose column, and peak fractions were assayed by Western immunoblotting with antibodies prepared separately against Ssn6 and Tup1. Ssn6HIS and Tup1 co-fractionated, as expected for proteins that are tightly associated. The peak fractions were pooled and loaded over a HiTrap Q anion exchange column and eluted with a salt gradient (100-500mM NaCl). Ssn6HIS and Tup1 bound to the resin and co-eluted at approximately 170mM NaCl. The peak fractions were pooled and further fractionated by gel filtration. As determined by Western immunoblotting, both proteins eluted just after the void volume. Upon visualization of the column fractions by silver staining of an SDS-PAGE gel, it was apparent that the Ssn6HIS and Tup1 proteins were among the first proteins to flow through the filtration column, consistent with the results of Williams *et al.* (18) that these proteins are present together in a large complex (Fig. 2, fraction 9 and 10).

The purified Ssn6HIS and Tup1 migrated as doublets on polyacrylamide gels as was previously noted in yeast extracts (18). No other protein fractionated stoichiometrically with Ssn6HIS and Tup1 in this or in any other preparation, suggesting that this large complex of proteins is primarily if not exclusively composed of Ssn6 and Tup1.

Migration through gel filtration is a function the Stokes radius of a protein complex. In order to determine the Stokes radius of the Ssn6HIS/Tup1 complex, size standards of known Stokes radii were also fractionated over the same gel filtration column. Using the methods of Siegal and Monty (32) the Stokes radius of the Ssn6HIS/Tup1 complex was determined to be approximately 14.7nm.

The total yield of the Ssn6HIS/Tup1 complex was approximately 50μg from 150g of yeast. In order to obtain a greater yield of this complex, the GST-Ssn6 fusion protein was purified from a strain that overexpressed both GST-Ssn6 and Tup1. Purification of the overexpressed complex was carried out in a single step using a glutathione agarose column. Again, Tup1 co-purified with GST-Ssn6. Fig. 2C shows the purified proteins on a silver stained SDS polyacrylamide gel. The GST-Ssn6 and Tup1 proteins are indicated. The higher mobility doublet at approximately 75kDa associates with GST alone (data not shown). The overall yield of the GST-Ssn6/Tup1 complex was approximately 200μg from 150g of yeast. Only a small percentage (approximately 1%) of the GST-Ssn6 within the extract bound to the glutathione agarose column.

Determination of the size of the Ssn6/Tup1 complex- It has been suggested that the previous estimate of the molecular weight of the Ssn6/Tup1 complex of 1.2MDa may be an overestimate if the complex is elongated or extended (18). Glycerol gradient sedimentation in conjunction with gel filtration can be used to estimate the molecular weight of a molecule with greater accuracy than either method alone (32). To this end the purified Ssn6HIS/Tup1 complex was subjected to glycerol gradient sedimentation in the presence of molecular standards of known S value (Fig. 2B). The peak of the Ssn6HIS/Tup1 complex

(fraction 7) was in close proximity to that of the lactate dehydrogenase standard and corresponded to an S value of 7.3 (Fig. 2B, fraction 7). Taking into account both the Stokes radius and the S value, the molecular weight of the Ssn6/Tup1 complex was estimated at 440,000Da. The behavior of Ssn6HIS/Tup1 on gel filtration and gradient sedimentation suggests that this complex is indeed asymmetrically shaped. Since the molecular weight of Ssn6 is approximately 107,000 and Tup1 78,000, the complex must be composed of multiple Ssn6 and Tup1 molecules.

Densitometry of a Coomassie Blue-stained gel can be used to estimate stoichiometry of subunits in a complex. Coomassie Blue binding is approximately proportional to the number of positively charged groups in a protein (34). Since the Ssn6HIS and Tup1 proteins contain similar densities of Arginine, Lysine and Histidine (9.2% and 10.6% respectively), densitometry of a Coomassie-stained SDS gel was performed in order to obtain a crude estimate the stoichiometry of these protein subunits in the complex. This analysis yielded a stoichiometry of one Ssn6 to three Tup1 molecules. A complex composed of one Ssn6 and three Tup1 proteins would have a molecular mass of 342kDa, lower than the estimated molecular weight estimate of 440kDa.

Tup1 is a phosphoprotein- While Ssn6 is a known phosphoprotein (35), it has never been established whether Tup1 is modified in this way. The appearance of both Ssn6 and Tup1 as multiple bands after SDS-PAGE (see above) could be the result of phosphorylation. If phosphorylation is responsible for the observed multiple-banding pattern, then removal of the phosphates should result in each protein migrating as a single band on an SDS polyacrylamide gel. This is indeed the case as is shown in Fig. 3. Purified GST-Ssn6/Tup1 complex was subjected to λ phosphatase treatment followed by SDS-PAGE and Western blotting with antibodies against both Tup1 and Ssn6. As seen in Fig. 3, phosphatase treatment of GST-Ssn6/Tup1 results in the loss of the lower-mobility form(s) of Tup1. Since Ssn6 is known to be phosphorylated, the phosphatase-dependent

disappearance of the lower-mobility Ssn6 species serves as a control for λ phosphatase activity.

Purified GST-Ssn6/Tup1 complex has repression activity in vitro- As discussed in the introduction, the DNA binding protein α2 recruits Tup1 and Ssn6 to DNA and thereby directs repression of many target genes. Transcriptional repression directed by the α2 protein *in vivo* is thus completely dependent on both Ssn6 and Tup1. α2-directed repression has also been observed in an *in vitro* transcription system utilizing whole-cell yeast extracts (33). *In vitro*, α2 repression is dependent on the overexpression of Ssn6 and Tup1 in the yeast from which the transcription extracts are prepared, suggesting that the amount of Ssn6 and Tup1 is a limiting factor for repression in this *in vitro* system. In order to test this idea and to determine whether the purified Ssn6/Tup1 complex analyzed above can supply repressor activity, the following experiments were carried out. Transcription from a reporter containing two a-specific gene operators (α2/Mcm1 sites) upstream of a UAS-less *CYC1* promoter is measured in parallel reactions, one lacking α2 and one containing 86nM α2. Each reaction also contains a control reporter that lacks a-specific gene operators. Transcription extracts were prepared from yeast that do not overexpress Ssn6 and Tup1 and thus show no significant repression upon addition of purified α2 (Fig. 4, lanes 1 and 2). The addition of purified GST-Ssn6/Tup1 gives approximately 5-fold repression which is also dependent on added α2 (Fig. 4 lanes 3 and 4: 1.2 ug added, lanes 5 and 6: 0.24 ug added). The extent of repression is calculated as the relative amount of ^{32}P-labeled transcript from the reporter in the absence and presence of the α2 protein, normalized to the amount of transcript from the control reporter in the presence and absence of α2.

DISCUSSION

In this report we describe the purification of the Ssn6/Tup1 complex from yeast. We determined that the complex contains only Ssn6 and Tup1 proteins in stoichiometric quantities, and its size (approximately 440,000Da) is smaller than the original estimate by Willams *et al.* (18) probably due to the asymmetric shape of the complex. The molecular weight estimate of 440kDa, suggests that the complex is composed of multiple Ssn6 (107kDa) and Tup1 (78kDa) proteins, estimated at one Ssn6HIS to three Tup1 molecules. The purified Ssn6/Tup1 complex is active in an *in vitro* repression assay. Finally, we show that like Ssn6, Tup1 is a phosphoprotein.

Ssn6 contains a repeated motif termed the tetratricopeptide repeats (TPR) (35). This 34 amino acid repeat is found in proteins involved in a variety of cellular functions from cell cycle progression to mitochondrial protein import (for review see (36)). The only known function of TPRs is to mediate protein/protein interactions. The TPRs of Ssn6 are essential for repression *in vivo* and mediate *in vitro* binding to both Tup1 and α2 (35,37,38). A complex composed of several Tup1 proteins and Ssn6 would therefore contain numerous protein/protein interaction domains. In addition to interacting with one another, Tup1 and Ssn6 are known to interact the α2 protein *in vitro* (30,38). There are a number of additional proteins that have been predicted to interact with Ssn6 and Tup1: Mig1 and Rox1, the DNA binding proteins required for glucose repression and hypoxic gene regulation as well as the putative DNA binding protein required for repression of DNA damage inducible genes (15). After recruitment to a particular gene set by interaction with a DNA binding protein, the Ssn6/Tup1 repression complex may interact with downstream elements possibly Rox3, histones, Sin4, or Srb8, 9, 10,11.

The phosphorylation of Tup1 may have a regulatory significance. Groucho, another member of the WD repeat repressor proteins, is also phosphorylated, and phosphorylation has been implicated in increasing groucho's affinity for the nucleus (39).

Ste4, a WD repeat protein involved in signal transduction in yeast, is also known to be phosphorylated. Ste4 is the β-subunit of a heterotrimeric G protein complex and is multiply phosphorylated in response to mating pheromones. This phosphorylation is required for the adaptation or down-regulation of Ste4 activity in the continued presence of mating factors (40). Phosphorylation of WD proteins may be a general way of regulating protein/protein interactions. In the case of Tup1 phosphorylation could regulate interaction with either one or more of the DNA binding proteins with which it interacts or with its downstream targets.

The purified GST-Ssn6/Tup1 shows repression activity *in vitro*, in extracts from cells containing only wild type levels of Ssn6 and Tup1. Our future efforts will be directed towards identifying the components required for this repression and dissecting the mechanism of repression mediated by Ssn6/Tup1 in order to promote a general understanding of the function of the of WD repressor proteins.

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FIGURE LEGENDS

FIG. 1. **Expression of Ssn6HIS and GST-Ssn6 proteins in yeast.** Western immunoblot of yeast extracts containing wild type Ssn6, Ssn6HIS or GST-Ssn6 probed with anti-Ssn6 antibodies. Ssn6HIS has a slightly lower mobility than native Ssn6. GST-Ssn6 (the top band within the lane labeled GST-Ssn6) is overexpressed relative to native Ssn6 which is present in the same lane. The lower molecular weight bands present in the GST-Ssn6 lane are presumably due to proteolysis of overexpressed GST-Ssn6. Molecular weight markers are indicated beside the figure.

FIG. 2. **Purification and characterization of the Ssn6/Tup1 complex.** (A) Silver stained SDS polyacrylamide gel showing the elution profile of Ssn6HIS/Tup1 from a Superose-6 gel filtration column. The column was loaded with the pooled Ssn6HIS/Tup1 peak fractions from a Hi-Trap Q column (Lane labeled L). Samples of the fractions from the column were loaded as indicated. Ssn6HIS can be seen eluting in fraction 9 and 10 as indicated by arrow. Tup1 co-fractionates with Ssn6HIS and is indicated by the arrow. Size standards are indicated beside the gel. (B) Silver stained SDS polyacrylamide gel showing fractions from glycerol gradient sedimentation of pooled peak fractions 9 and 10 from Superose-6 column containing Ssn6HIS/Tup1, as well as protein size standards. Samples of fractions from the top of a glycerol gradient were loaded as indicated. Ssn6HIS/Tup1 are indicated with arrows. The protein size standards from the left are: bovine serum albumen (BSA) 3.6S, lactate dehydrogenase (LDH) 7.3S, catalase (CAT)11.3S. Molecular size standards are indicated beside the gel figure. (C) Silver stained gel showing the purified GST-Ssn6/Tup1 proteins. GST-Ssn6 and Tup1 are indicated by arrows. Size standards are indicated beside the gel figure.

FIG. 3. **Tup1 is a phosphoprotein.** Western immunoblot with Ssn6 and Tup1 antibodies against purified GST-Ssn6/Tup1 treated with λ phosphatase with the inhibitor NaVanadate, with λ phosphatase or untreated as indicated. GST-Ssn6 and Tup1 are indicated with arrows.

FIG. 4. **Purified GST-Ssn6/Tup1 complex has repression activity *in vitro*.** *In vitro* transcription reactions contain two reporter plasmids: one that contains two α2/Mcm1 operators upstream of the *CYC1* TATA region and yields a long G-less transcript and a second that lacks any operators and yields a short G-less transcript that serves as an internal control for transcription in each reaction (33). Purified GST-Ssn6/Tup1 complex was added to reactions seen in lanes 3-8, in the amounts indicated. Purified recombinant α2 protein was added to a final concentration of 86nM in reactions 2,4,6, and 8. The amount of ^{32}P-labeled transcript was quantitated with a Molecular Dynamics PhosphorImager using ImageQuant software. Repression calculated is normalized to the activity of the control reporter that lacks α2/Mcm1 operators. Lanes 1 & 2, 1.1-fold repression; lanes 3 & 4, 4.8-fold repression; lanes 5 & 6, 4.4-fold repression; lanes 7 & 8, 0.9-fold repression.

Figure 1

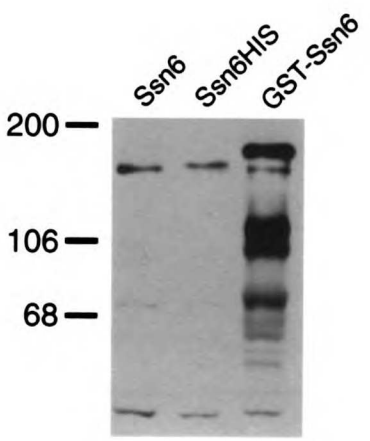
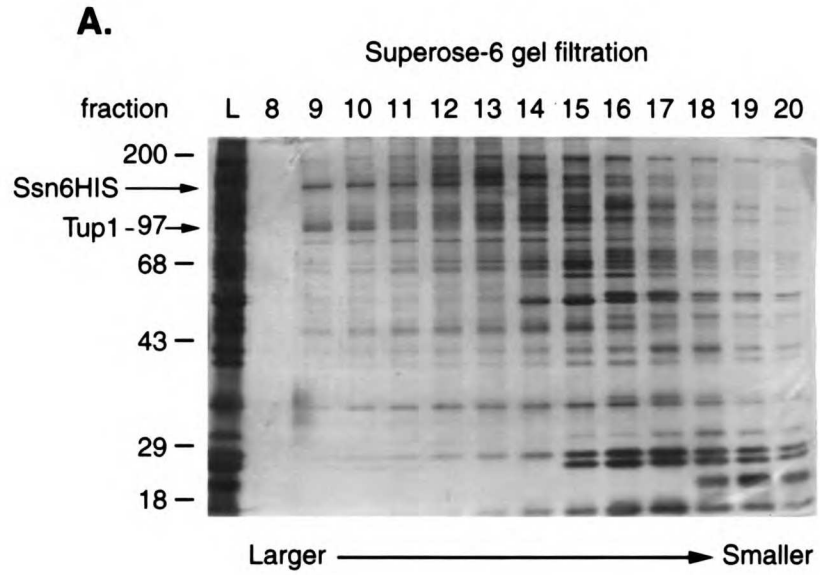


Figure 2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Superose-6 gel filtration
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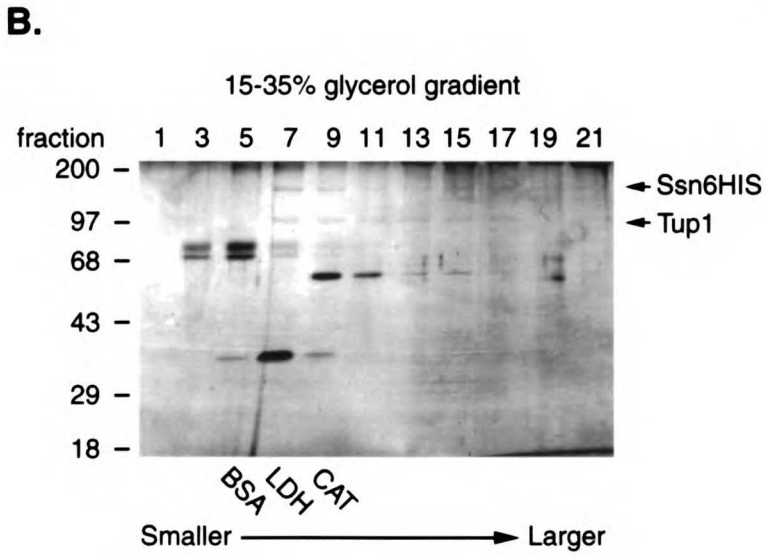
Ssn6HIS
Tup1 - 97
68
43
29
18
Larger
Smaller


B.
15-35% glycerol gradient
fraction 1 3 5 7 9 11 13 15 17 19 21
200
97
68
43
29
18
Ssn6HIS
Tup1
BSA
LDH
CAT
Smaller
Larger

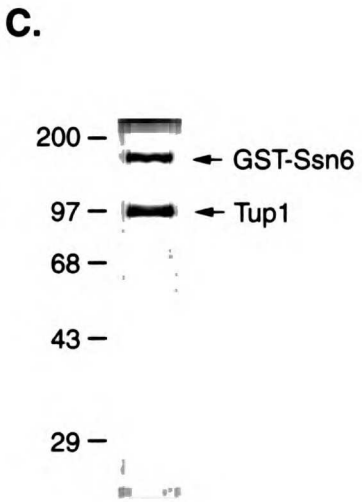

C.
200
97
68
43
29
GST-Ssn6
Tup1

Figure 3

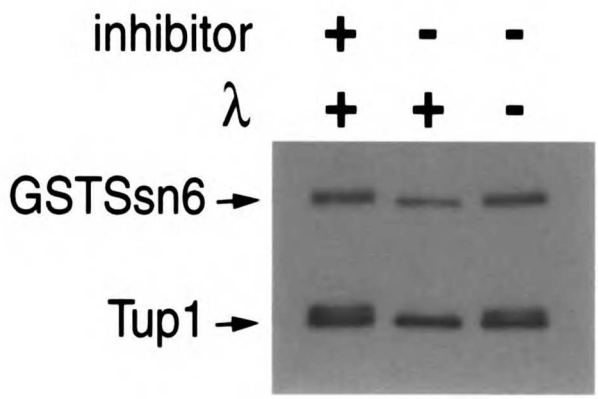
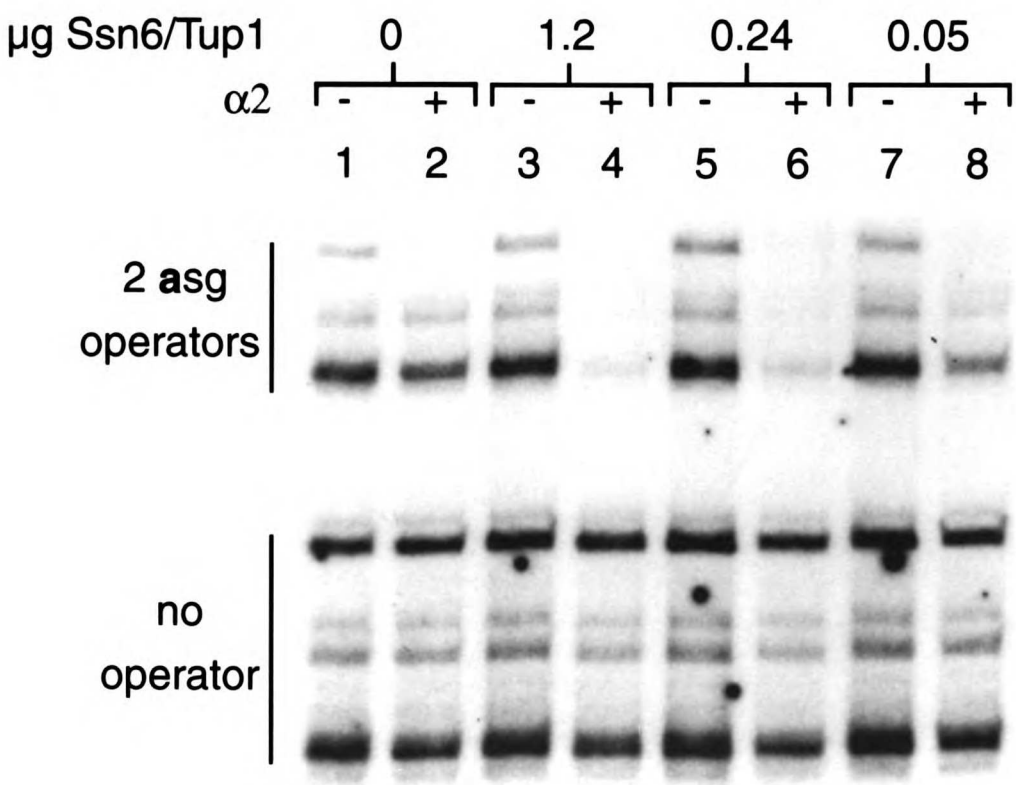
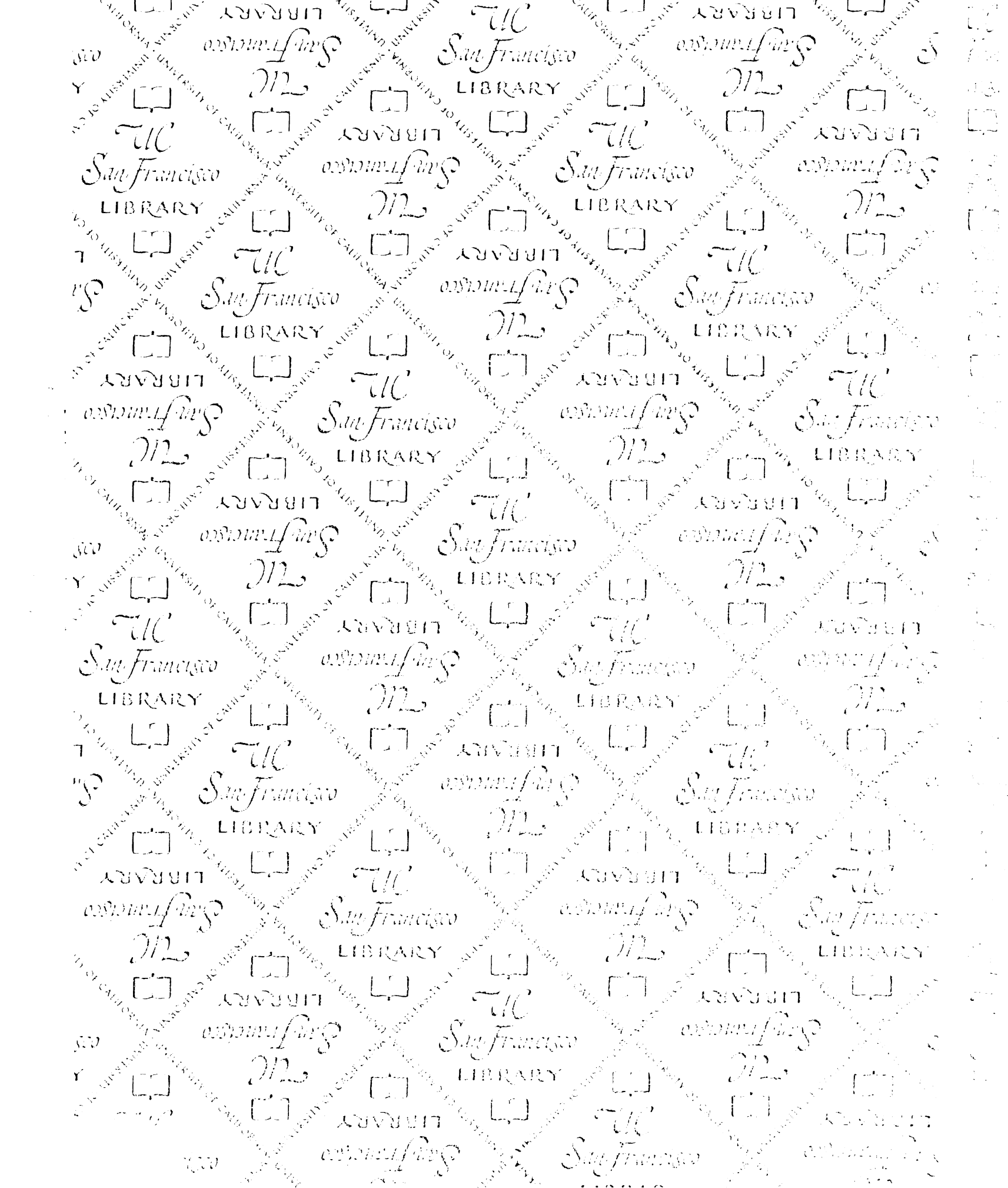


Figure 4

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