UCSF
UC San Francisco Electronic Theses and Dissertations

Title
The interaction between alpha 2 and Tup1

Permalink
https://escholarship.org/uc/item/8tc430ct

Author
Komachi, Kelly,

Publication Date
1996

Peer reviewed|Thesis/dissertation

eScholarship.org
Powered by the California Digital Library
University of California

The Interaction between alpha 2 and Tup1

by

Kelly Komachi

DISSERTATION

Submitted in partial satisfaction of the requirements for the degree of

DOCTOR OF PHILOSOPHY

in

Biochemistry

in the

GRADUATE DIVISION

of the

UNIVERSITY OF CALIFORNIA

San Francisco

Acknowledgments

When you're not particularly proud of your thesis work, Mike says that it makes more sense to blame people than to thank them.

In addition to their original sin, Mom and Dad are to blame for providing a happy childhood that lasted a good thirty years and for sending me to college instead of sticking me in the army. Then there are the sisters for being fun and interesting and other things I'm not, thus forcing me to seek refuge in the sartorially and socially challenged environment of academic science. Without the family, I would have lost what little sanity I had coming in, and since I am beginning to suspect that mental imbalance is crucial to success in this (and perhaps every) field, it's obvious where the blame for my failures lies.

Any flaws in the data can be blamed on Arkady, who taught me all of the techniques necessary for survival in the early years of the Johnson lab: namely, how to gel shift, footprint, sequence, purify $\alpha 2$, and go standing room to the opera. She tried to teach me how to be a good friend and a nice person, but clearly that is a useless skill. Mike is at fault for instigating almost all of the work in this thesis, for writing the better half of Chapter One, for convincing me to graduate when the rest of the lab wished I would evaporate, and for being one of the few people who (a) makes me laugh and (b) procrastinates as badly as I do. If he had gotten his degree like a normal person, I probably would have graduated sooner out of sheer boredom.

I've often wished the lab would self-destruct, but it hasn't, and the postdocs are largely to blame, especially Nancy, who captained the ship during the Dark Years, and Danesh who continues to bail water out of the leaky hold. Martha isn't a postdoc, but her scientific

prowess, uncanny ability to cut through bullshit, and extraordinary cooking skills have kept lab afloat. Damn her.

Evi, Brenda, and Owen abandoned me and disappeared into the virtual void.

If Sudarsi, Anne-Marie, and Karina hadn't poured all those plates, I wouldn't have done so many stupid screens.

The official censors of this thesis, Andrew and Cynthia, were shamelessly indulgent of my decidedly unprofessional writing style; so if anything in this thesis offends, it's their fault. I can't actually blame them for torturing me during thesis committee meetings, as I didn't die, faint, or vomit during either. As a matter of fact, they have been suspiciously nice to me. On the other hand, Cynthia is so cheerfully optimistic that she must be guilty of something. Andrew invaded our lab, stole our technician, and never did give Johnson the thrashing he so thoroughly deserves but can be forgiven almost anything for being the only faculty member to suggest that I sue the Department to get my stipend back.

Ira and Bruce were clever enough not to take me into their labs. In addition, Bruce pretty much crushed any promise Johnson had as a P.I. by granting him MBoC knighthood. But I think I've punished Ira enough by making him chair my orals, read my lousy manuscripts and listen to my incessant whining and Bruce by making him give my rotation group meeting and coach my first student-faculty journal club during one of UCSF's infamous power shortages.

Those people who gave me reagents and other scientific paraphenalia are cited in the text. I could go through and finger all the other unfortunate souls who helped make this thesis the mess that it is, but I'm in a benevolent mood, and I only have an hour to turn the whole

tamale in to the graduate division without whom this silly acknowledgement section would not exist, since they were kind enough to give me three deadline extensions.

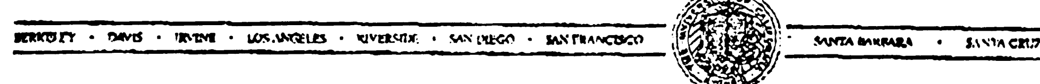
I don't think I'm violating any copyright laws, since it's sort of obvious that the "art" on the frontispieces is a parody of other people's work. However, to be explicit, the people who will sue me if they ever see this are as follows: whoever owns the rights to van Gogh's "Starry Night" (Chapter One), Dennis Worden (Appendices to Chapter One), the estate of Rene Magritte (Chapter Two), and Allan D'Arcangelo (Miscellaneous Appendices).

That leaves Johnson. But I think I've finally learned better than to try to pin anything on him.

MAR-13-1997 11:07 FROM 2 TO 14154760939 P.02

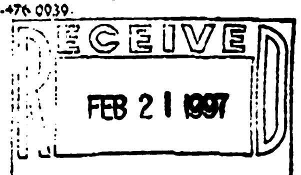
FEB-20-97 THU 19:50 UCSF MICROBIOL/IMMUNOL FAX NO. 4154768201 P.01

UNIVERSITY OF CALIFORNIA, SAN FRANCISCO



SCHOOL OF MEDICINE
Department of Microbiology and Immunology

SAN FRANCISCO, CALIFORNIA 94143-0502
FAX: 415-476-0939



FAX TRANSMISSION
TO: Val Pakaluk, Journal Secretary
Genes and Development
Editorial Office
FAX #: 516. 367. 8492

Dear Val Pakaluk,

I would like permission to include in my thesis a copy of a paper published in *Genes and Development* (cited below). The thesis will be microfilmed by University Microfilms Incorporated, and they request permission to supply single copies on demand.

Komachi, K, M.J. Redd, and A. D. Johnson (1994) The WD repeats of Tup1 interact with the homeo domain protein α2. Genes and Development 8: 2857-2867.

Please respond by fax if possible (FAX #: 415. 476. 0939). Thank you for your cooperation.

Permission granted by the copyright owner, contingent upon the consent of the original author, provided complete credit is given to the original source and copyright date.

By: [signature] Date: 3/13/97

Cold Spring Harbor Laboratory, Box 100, Cold Spring Harbor, NY 11724

TOTAL P.02

ABSTRACT

The $\alpha 2$ protein regulates the expression of genes involved in mating type determination in the yeast Saccharomyces cerevisiae by binding upstream of these genes and repressing their transcription. Point mutations in the amino terminus of $\alpha 2$ compromise repression but not DNA binding and create mutants which are dominant negative when overexpressed. The dominance of two such mutants can be suppressed by overexpression of Tup1, a protein that contains seven WD repeats and that is required for the repression of many sets of genes in yeast. The Tup1 WD repeats will bind to $\alpha 2$ but not to a repression-defective $\alpha 2$ mutant, suggesting that $\alpha 2$ represses by recruiting Tup1 via a direct interaction.

The interaction between $\alpha 2$ and Tup1 was further characterized by isolating mutations in the Tup1 WD repeats that debilitate Tup1's ability to bind $\alpha 2$ but do not affect interaction with the presumptive downstream repression machinery. The positions of these mutations together with the structure of the WD repeat protein G_β suggest that Tup1 folds into a G_β-like propeller whose flat top surface is bound by $\alpha 2$.

Table of Malcontents

List of Tables x

List of Figures xiii

Foreward 1

Chapter One: The WD repeats of Tup1 interact with the homeodomain protein α2 5

Appendices to Chapter One

Appendix A. Overexpression of α2 suppresses multiple point mutations in the Mcm1-binding portion of the α2/Mcm1 operator 20

Appendix B. DNA-Binding Activity of α2 is not affected by amino-terminal point mutations 27

Appendix C. Dominance of the α2-thr4 mutant requires DNA-binding activity 33

Appendix D. An α2 homolog from Kluyveromyces lactis 39

Appendix E. Overexpression of Tup1 suppresses a deletion of *SSN6* 45

Chapter Two: Residues in the WD repeats of Tup1 required for interaction with α2 50

Appendix F. Tup1-lexA represses transcription from a lexA operator 74

Appendix G. Dominance of the *TUP1* mutants is suppressed by overexpression of Ssn6 84

Appendix H. Fragments of Tup1 smaller than Tup1(254-713) bind to both α2 and $\alpha 2^{\Delta 2\text{-}10}$ 89

Appendix I. Tup1(363-713)-N673S, F632S causes derepression of *mfa2:lacZ* when overexpressed 97

Appendix J. Ste4 can interact with α2 in vitro but does not appear to do so in vivo 101

Post-mortem 113

Miscellaneous Appendices 117

Appendix K. *TUP1* and *SSN6* are not required for silencing of the mating-type loci 118

Appendix L. Inhibition of α2 synthesis leads to rapid derepression of the **a**-specific gene *MFA2* 121

Appendix M. Multimerization of the α2 operator increases repression 127

Appendix N. Dominant negative *SSN6* mutants 129

Appendix O. Effect of histone mutations on α2-mediated repression 138

References 165

Signs of Frivolousness

The Bomber of Civilians, or "The Useless Propeller" 177

List of Tables

Table A-1. Overexpression of α2 suppresses the repression defect of the center-substituted operator 23

Table C-1. The α2 mutants are defective for **a**1/a2-mediated repression 35

Table C-2. The α2 mutants are dominant negative for **a**1/α2-mediated repression 36

Table C-3. DNA-Binding activity is required for the dominance of the α2-thr4 mutant 37

Table C-4. The α2-thr4-H3-3 mutant is dominant negative for **a**1/α2-mediated repression 38

Table E-1. Overexpression of Tup1 allows repression of an **a**-specific gene reporter but not a hypoxic gene reporter in an *ssn6Δ* strain 47

Table E-2. Overexpression of Ssn6 does not suppress a *tup1Δ*. 48

Table E-3. Overexpression of Tup1(254-713) represses an *mfa2:lacZ* reporter in the absence of wild-type *SSN6* or *TUP1* 49

Table 2-1. Tup1 mutants are defective in repression of the α2-regulated reporter *mfa2:lacZ* 62

Table 2-2. Point mutations do not affect repression by Tup1-lexA 63

Table 2-3. Effect of Tup1 point mutations on repression of other Tup1/Ssn6-regulated reporters 64

Table F-1. Tup1-lexA complements a *tup1Δ* strain for repression of *mfa2:lacZ* 77

Table F-2. Tup1-lexA represses from a lexA operator 78

Table F-3. Repression by Tup1-lexA does not require wild-type *TUP1* but may be partially dependent on *SSN6* 79

Table F-4. Repression by Tup1-lexA does not require *SRB8* or *SRB10* 80

Table F-5. Tup1(1-713), Tup1(1-253)-lexA and Tup1(254-713)-lexA repress transcription in the absence of *SSN6* 83

Table G-1. Deletion of the amino terminus of two of the Tup1 mutants destroys their ability to derepress *mfa2:lacZ* 85

Table G-2. Dominance of the *TUP1* mutants is suppressed by overexpression of Tup1 or Ssn6 86

Table G-3. The *TUP1* mutants are not suppressed by overexpression of Ssn6 in the absence of wild-type *TUP1* 88

Table I-1. Tup1(254-713)-F632S, N673S derepresses *mfa2:lacZ* 99

Table I-2. Dominance of *TUP1-(363-713)-F632S, N673S* is not suppressed by overexpression of Ssn6 100

Table J-1. Ste4 overexpression is not suppressed by α2 mutants unable to bind DNA 106

Table J-2. The α2-lacZ fusion is toxic to both *ste4* and *STE4* haploids 107

Table M-1. Greater repression is observed when the α2 operator is multimerized 128

Table N-1. Overexpression of SSN6 mutants derepresses *mfa2:lacZ* 133

Table N-2. Derepression of *mfa2:lacZ* caused by the dominant *SSN6* mutatns is suppressed by overexpression of Ssn6 or Tup1 134

Table O-1. Repression of *cyc1:lacZ* reporters by α2 in wild-type and *hhf2* strains 146

Table O-2. Repression of an *mfa2:lacZ* reporter in wild-type and *hhf2* strains 147

Table O-3. Repression of *cyc1:lacZ* reporters by α2 in wild-type and *hht2* strains 148

Table O-4. Repression of an *mfa2:lacZ* reporter in wild-type and *hht2* strains 149

Table O-5. Repression of an **a**1/α2-repressible reporter in wild-type and *hhf2* strains 150

Table O-6. Repression of an **a**1/α2-repressible reporter in wild-type and *hht2Δ1-30* strains 151

Table O-7. Repression of *cyc1:lacZ* reporters by α2 in wild-type strains and in strains having mutations in *HHF2* and/or *HHT2* 152

Table O-8. Effect of histone mutations on expression of *MATα2:lacZ*.................... 153

Table O-9. High copy plasmids carrying *TUP1*, *SSN6*, *TUP1+SSN6*, or *MATα2* do not suppress the repression defect of a *hht2Δ1-30* strain.................................. 154

Table O-9. Yeast strains used in histone experiments....................................... 156

Table O-10. Plasmids used in histone experiments 162

List of Figures

Figure A-1. α2 can bring Mcm1 to an operator in which the Mcm1-binding sequences have been replaced but not deleted 24

Figure B-1. Mutant α2 proteins bind to the α2/Mcm1 operator in vitro 29

Figure B-2. Mutant α2 proteins bind to the **a**1/α2 operator in vitro 31

Figure D-1. DNA and protein sequence of the α2 homolog from K. lactis 41

Figure D-2. Homology between the amino acid sequences of S. cerevisiae α2 and the K. lactis α2 43

Figure 2-1. Screen for mutations in Tup1-lexA that affect interaction with α2 65

Figure 2-2. Binding of wild-type and mutant GST-Tup1 fusions to α2 67

Figure 3-3. Similarity between the region of Tup1 that binds α2 and the region of Gβ that binds Gα 69

Figure F-1. Summary of Tup1-lexA and LexA-Tup1 fusions tested for their ability to repress *cyc1:lacZ* reporters containing lexA operators 81

Figure H-1. GST-Tup1(340-713) binds to both α2 and $\alpha2^{\Delta 2\text{-}10}$ in vitro 91

Figure H-2. Deletions of GST-WD2 disrupt binding of both α2 and $\alpha2^{\Delta 2\text{-}10}$ 93

Figure H-3. Summary of GST-Tup1 fusions tested for their ability to bind to α2 by the column assay used in Figures 1 and 2 95

Figure J-1. GST-Ste4 binds to α2 and $\alpha2^{\Delta 2\text{-}10}$ in vitro 104

Figure J-2. Tup1 does not interact with Gpa1, nor does α2 interact with Ste4 in two-hybrid type assays 108

Figure J-3. WD repeats of Tup1 and Ste4 are not interchangeable in vivo 111

Figure K-1. Northern blot of *tup1Δ* and *ssn6Δ* strains probed for *MATα2* or *MATa1* RNA 119

Figure L-1. Inhibition of α2 synthesis leads to derepression of the **a**-specific gene *MFA2* 123

Figure L-2. Establishment of α2-mediated repression occurs in α factor-arrested cells 125

Figure N-1. Ssn6 and Ssn6-d both bind to α2 in vitro 136

Foreward

Sex is one of the three best things in life (7); so it is only fair that yeast, a fountainhead of pleasure, should enjoy the thrill of mating. Many years ago, human voyeurs recognized that there are two yeast mating types, **a** and α, which meet, woo, and make exchange of vow. Yet despite all the protuberating and fusing that accompanies fungal mating rituals, the yeast cell has a sex life that would make the pope think that God is in His Heaven and all is right in the world: yeast chooses a single partner; it mates but once; it veers from heterosexuality only under bizarre circumstances (reviewed in (32, 34, 90)).

This straight and narrow lifestyle comes about in large part from the yeast cell's strong sense of gender which is genetically determined by the *MAT* locus (57, 91). In α cells, the *MATα* locus encodes α1, an activator of genes required for α-mating behavior (α-specific genes), and α2, a repressor of genes required for **a**-mating behavior (**a**-specific genes) (1, 3, 41, 92, 108). In **a** cells, ***MATa*** encodes **a**1, a protein which has no apparent function on its own (1, 92). Sex is said to be ruined by marriage, and in yeast, the rumors are true: once **a** and α have consummated their passion, the **a**1 and α2 proteins conspire to shut down the haploid-specific genes, including those required for mating (24, 38, 48, 62).

Yeast erotica spiraled further downward with the realization that the proteins encoded by the *MAT* loci are regulators of transcription (reviewed in (33, 39, 68)). In particular, the carboxy terminus of the α2 protein contains a known DNA-binding motif, the homeodomain, which was shown to bind sequences found upstream of **a**-specific genes and to be required for repression in vivo (28, 80). Because small deletions in the amino terminus of α2 that do not affect DNA binding destroy α2's ability to repress (28), α2 was deemed an active repressor as opposed to one which represses simply by occupying DNA sequences required for activator binding.

By the early 1990's, several examples of active repression had already been found in higher eukaryotes. The Drosophila proteins Krüppel, engrailed, and even-skipped and

the mammalian thyroid receptor had all been shown to possess DNA-binding activity that was necessary but not sufficient for repression (4, 13, 37, 54), and in the case of Krüppel, a repression domain which could function when transferred to a bacterial DNA-binding protein had been identified (54). However, the mechanism of active repression was still a mystery, and α2 seemed like a quick route to the solution since the vaunted tools of yeast molecular genetics could presumably be used to hasten the journey. And so it was that a hunt for α2 mutants that bound DNA but did not repress was begun with the hope that such mutants would serve as a foundation for suppressor hunts leading to proteins that interact with α2.

What with one thing and another, four years passed.

In that time, three results were reported. First, mutations in *SSN6* and *TUP1* were demonstrated to obliterate repression of many sets of genes (71, 94, 96, 113, 114), including those regulated by α2 (31, 46, 60, 67). Second, a lexA-Ssn6 fusion was shown to repress transcription from a lexA operator in a Tup1-dependent fashion (46). Third, a LexA-Tup1 fusion was shown to repress transcription from a lexA operator in an Ssn6-independent fashion (97). These results suggested that α2's role in repression was to recruit Tup1 via Ssn6 and that Tup1 was the protein which actually interfered with transcription.

The additional finding that Tup1 bound to the supposed repression domain of α2 helped to confirm the suspicion that α2 had simply been taking credit for Tup1's handiwork, and attention drifted away from α2 and toward this odd gene that had been lurking around in obscure journals (20, 56, 93, 112) ever since its initial identification in a screen for mutants defective in thymidine uptake (75). By the time the α2 connection had been firmly established, *TUP1* had been cloned, sequenced, and found to encode a protein containing seven WD repeats (107), or stretches of amino acids with the general sequence X_{6-94}-[GH-X_{23-44}-WD] that tend to stutter their way through proteins in four- to ten-unit arrays (reviewed in (69)). WD madness began in 1986 with the discovery of repeats in the

β subunit of the heterotrimeric G protein transducin (G_β) (19) and continued into the 1990's, with repeats eventually being found in proteins involved in almost every eukaryotic process from the trendy to the passé (reviewed in (15, 69, 101)). Thus, the presence of WD repeats in Tup1 would have been cause for champagne except that no one knew what WD repeats actually did other than show up in homology searches.

Like the thunder that follows a lightning bolt, much of the speculation that came in the wake of the WD repeat was nothing more than vacuum-produced noise. As far as anyone could tell, WD repeats had no enzymatic activity, and the only property that members of the WD family seemed to share was that several were subunits of large multiprotein complexes (reviewed in (69)). Process of elimination and lack of imagination led aficionados to assume that WD proteins had structural or regulatory function and that the repeat somehow mediated protein-protein interaction.

Being one of the few examples of a WD protein in which the repeats themselves were known to mediate an interaction, Tup1 presented a unique opportunity for examining how WD repeats bind to other proteins. But there were a few problems. For instance, although an isolated WD repeat from Tup1 would bind to α2 in vitro, it was unclear if this interaction had any significance in vivo, especially since α2 mutants that did not bind to full-length Tup1 would bind to the single repeat. So biochemistry was out for the time being, and it seemed like the most logical approach to the problem was to look for mutations in Tup1 that specifically eliminated binding to α2. Such mutations would presumably be useful in at least three ways. First, the mutations would point out which repeats bound to α2. Second, the mutations would highlight which part of each repeat bound α2. And third, the mutations would give some clue as to the structure of Tup1, since the mutations would presumably lie close together on the surface of the protein.

In short then, this thesis is the simple story of two proteins, α2 and Tup1, and the mutations that keep them apart. Chapter One recounts the isolation of α2 mutants proficient for operator binding but defective for repression and reveals that these mutants

have lost their affection for Tup1. Chapter Two describes the isolation of Tup1 mutants unable to bind $\alpha 2$ but able to repress transcription when brought to the DNA by artificial means and argues that Tup1 is structurally similar to G_β.

CHAPTER ONE

The WD repeats of Tup1 interact with $\alpha 2$

This chapter is a reprint of the material as it appears in Genes and Development (1994), volume 8, pages 2857-2867. The Introduction and Sections 5 and 6 of the Results were written by Michael J. Redd who performed the experiments summarized in Figures 4 and 5. The remainder of the paper was written by Kelly Komachi, who performed the experiments summarized in Figures 2, 3, and 6. Alexander Johnson directed and supervised the research.

Alexander D. Johnson, thesis adviser

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

Kelly Komachi,[1,3] Michael J. Redd,[1,3] and Alexander D. Johnson[1,2]

[1]Department of Biochemistry and Biophysics, [2]Department of Microbiology and Immunology, University of California, San Francisco, California 94143 USA

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]

Received September 15, 1994; revised version accepted October 18, 1994.

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.

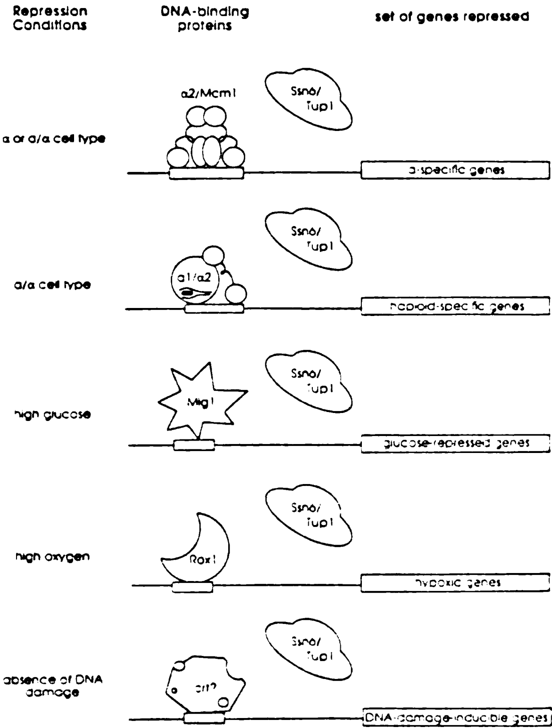


Figure 1. Ssn6 and Tup1 are required for repression of many different genes. Ssn6 and Tup1 mediate repression of the five sets of genes listed at *right*. Repression of each particular set of genes also requires specific upstream sequences and the DNA-binding protein or proteins indicated to the *left*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

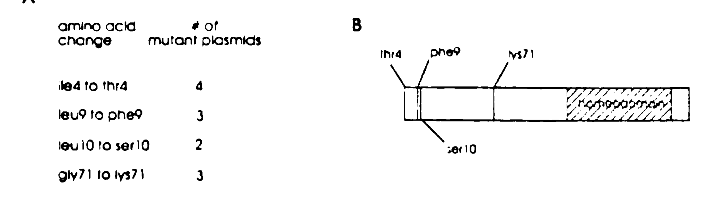
To better understand how α2 directs repression after it has bound to its operator, we isolated mutants of α2 defective in repression but competent for DNA binding. To facilitate the identification of such mutants, we exploited the fact that α2 binds cooperatively to its operator with an activator, Mcm1 (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



C

CELL TYPE	β-GALACTOSIDASE ACTIVITY
MATa	150 ± 20
MATα	0.7 ± 0.1
MATα2lys71	75 ± 7
MATα2phe9	120 ± 30
MATα2thr4	100 ± 10
MATα2ser10	150 ± 20

D

CELL TYPE	α2 PLASMID	β-GALACTOSIDASE ACTIVITY
MAT α	CV13 (2μm vector)	0.8 ± 0.3
MAT α	MATα2/CV13	≤ 0.1
MAT α	MATα2lys71/CV13	2.3 ± 0.3
MAT α	MATα2phe9/CV13	50 ± 1
MAT α	MATα2thr4/CV13	34 ± 10
MAT α	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 *mfa2:lacZ* reporter. Each reported value is the average of three β-galactosidase assays. (*D*) A *MATα mfa2: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 α	MATα2^{lys71}/CV13	YEp24 (2μm URA3 vector)	2.5 ± 0.5
MAT α	MATα2^{lys71}/CV13	SSN6/YEp24	3.3 ± 0.2
MAT α	MATα2^{lys71}/CV13	TUP1/YEp24	0.8 ± 0.1
MAT α	MATα2^{lys71}/CV13	(SSN6+TUP1)/YEp24	0.9 ± 0.3
MAT α	MATα2^{phe9}/CV13	YEp24	13.4 ± 2.0
MAT α	MATα2^{phe9}/CV13	SSN6/YEp24	22.0 ± 8.9
MAT α	MATα2^{phe9}/CV13	TUP1/YEp24	1.7 ± 0.4
MAT α	MATα2^{phe9}/CV13	(SSN6+TUP1)/YEp24	0.9 ± 0.1
MAT α	MATα2^{thr4}/CV13	YEp24	47 ± 5
MAT α	MATα2^{thr4}/CV13	SSN6/YEp24	46 ± 13
MAT α	MATα2^{thr4}/CV13	TUP1/YEp24	24 ± 3
MAT α	MATα2^{thr4}/CV13	(SSN6+TUP1)/YEp24	8.7 ± 1.5
MAT α	MATα2^{ser10}/CV13	YEp24	112 ± 10
MAT α	MATα2^{ser10}/CV13	SSN6/YEp24	90 ± 7
MAT α	MATα2^{ser10}/CV13	TUP1/YEp24	73 ± 3
MAT α	MATα2^{ser10}/CV13	(SSN6+TUP1)/YEp24	23 ± 3
MATa		YEp24	156 ± 23
MATa		SSN6/YEp24	155 ± 15
MATa		TUP1/YEp24	160 ± 10
MATa		(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).

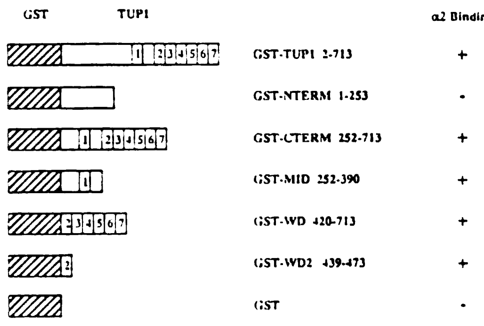
To determine which portion of Tup1 is required for binding α2, we constructed two additional GST fusion proteins: GST–NTERM consisting of amino acids 1–253 of Tup1, and GST–CTERM, consisting of the remaining carboxy-terminal portion of Tup1 fused to GST (Fig. 4A). α2 (again present in a bacterial extract) 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

A



B

```
341-383   LDBTSVVCCVK------FSNDGEYLATGC-NKTTQVYRVSDGSLVARLSD

440-481   PSSDLYIRSVC------FSPDGKFLATGAEDRLIRIWDIENRKIVMIL
482-525   QGHEQDIYSLDY-----F-PSGDKLVSGSGDRTVRIWDLRTGQCSLTLSI
527-567   DGVTTVAVSPG---------DGKYIAAGSLDRAVRVWDSETGFLVERLDS
573-616   TGHKDSVYSVV------FTPDGQSVVSGSLDRSVKLWNLQNANNKSDSKT
627-668   IGHKDFVLSVA------TTQNDEYILSGSKDRGVLFWDKKSGNPLLML
669-713   QGHRNSVISVAVANGSSLGPEYNVFATGSGDCKARIWKYKKIAPN

Consensus .GH...V.SV.------FS.DG..øATGS.DR.VRøWD...G.....L..
                         T        S
```

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.

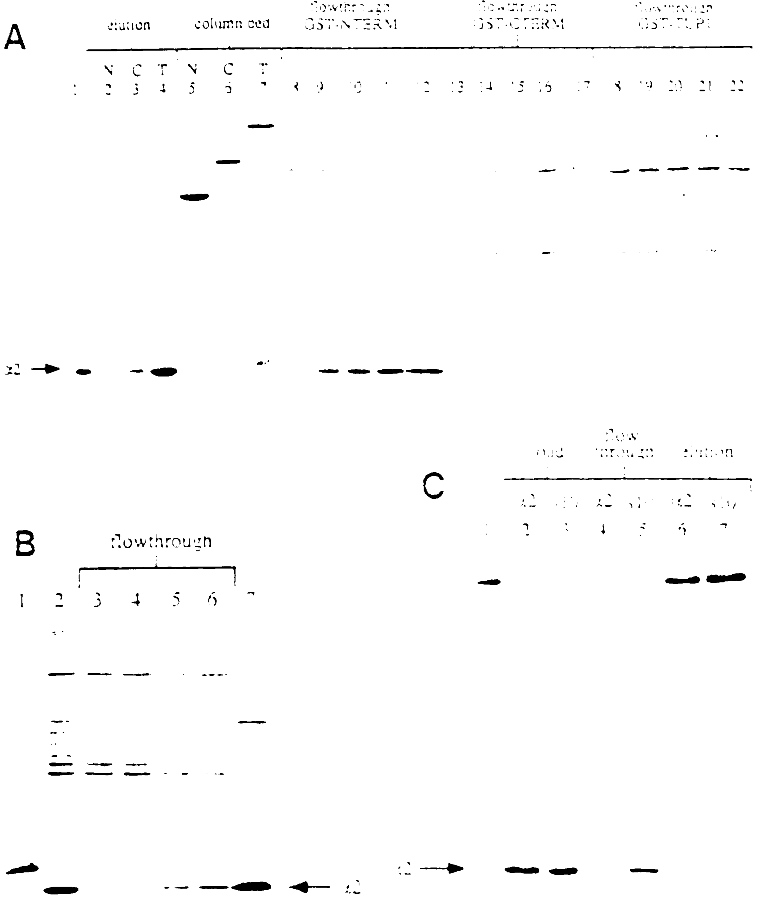


Figure 5. α2 binds to a single WD repeat of Tup1. (A) Coomassie blue-stained SDS gel showing the results of passing a bacterial extract containing α2 (lane 1) over columns bearing purified GST–NTERM (lane 5), GST–CTERM (lane 6), and GST–TUP1 (lane 7). Flowthrough fractions from each column are as indicated. 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 430–473 (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 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.

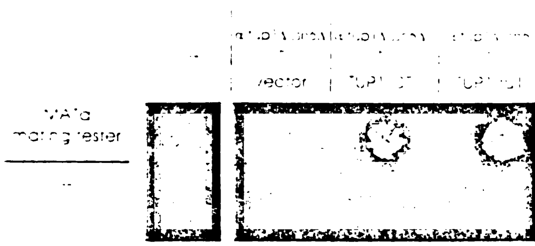


Figure 6. Overexpression of Tup1 suppresses the mating defect of a *MATα ssn6Δtup1Δ* strain. A *MATα ssn6Δ tup1Δ* strain was transformed with plasmids expressing no Tup1, the Tup1 carboxyl terminus (CT), or full-length Tup1 (full) from the *GAL10* promoter. Transformants were mixed with a *MATa* tester 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.

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-

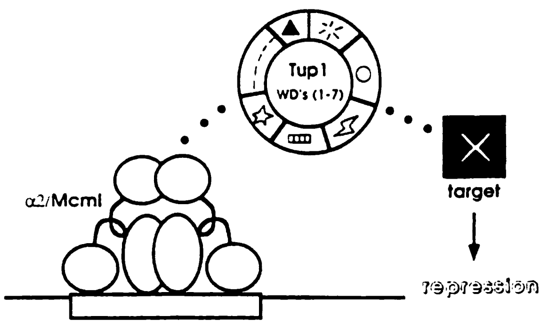


Figure 7. Model for α2 repression. Tup1 is recruited to the **a**-specific genes by binding to α2 and represses transcription by interacting with a downstream target. The WD repeats (represented by the different symbols) mediate both of these interactions, which can occur in the absence of Ssn6.

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 *MATα* 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 (*MATα mfa2::lacZ trp1 leu2 ura3 his4*) (Hall and Johnson 1987). KKY122 was constructed by replacing *MATα2* of SM1196 with *URA3*. All mutant *α2 mfa2::lacZ* strains were constructed by cotransforming KKY122 with YEp13 and a *Hin*dIII–*Nde*I fragment containing the mutant *MATα2*, 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 $\alpha 2^{Ser10}$, 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 $\alpha 2^{Ser10}$ 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 (*MATα 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 100,000*g* 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 equilibrated 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 100,000*g* 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 *MAT*a *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.

Acknowledgments

We thank Burkhard Braun, Cynthia Keleher, Robert Trumbly, and Madhu Wahi for communication of results prior to publication. We are particularly grateful to B. Braun and M. Wahi for sharing the observation that Tup1 overexpression suppresses the mating defect caused by an *SSN6* deletion and point mutation, respectively. We thank Doug Bob Kellogg for valuable advice and technical assistance with the GST protein purification; Anita Sil, Robert Trumbly, and Andrew Vershon for plasmids; B. Braun for yeast strains; Andrew W. Murray for prompt and perspicacious comments on the manuscript; Henry Bourne and members of his laboratory for useful discussions; Rebecca Smith for help with Figure 4; members of the Johnson laboratory, past and present, for advice, tolerance and constructive confabulation. This work was supported by a grant from the National Institutes of Health (NIH) to A.D.J. K.K. was supported in part by a National Science Foundation predoctoral fellowship and by a training grant from the NIH to the Department of Biochemistry and Biophysics at the University of California, San Francisco.

The publication costs of this article were defrayed in part by payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 USC section 1734 solely to indicate this fact.

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.

References

Alani, E., L. Cao, and N. Kleckner. 1987. A method for gene disruption that allows repeated use of *URA3* selection in the construction of multiply disrupted yeast strains. *Genetics* **116:** 541–545.

Astell, C.R., L. Ahlstrom-Jonasson, M. Smith, K. Tatchell, K.A. Nasmyth, and B.D. Hall. 1981. The sequence of the DNAs coding for the mating-type loci of *Saccharomyces cerevisiae*. *Cell* **27:** 15–23.

Balasubramanian, B., C.V. Lowry, and R.S. Zitomer. 1993. The ROX1 repressor of *Saccharomyces cerevisiae* hypoxic genes is a specific DNA-binding protein with a high-mobility-group motif. *Mol. Cell. Biol.* **13:** 6071–6078.

Bender, A. and G.F. Sprague. 1987. MATα1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific genes. *Cell* **50:** 681–691.

Bradford, M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.* **72:** 248–254.

Broach, J.R., J.N. Strathern, and J.B. Hicks. 1979. Transformation in yeast: Development of a hybrid cloning vector and isolation of the *CAN1* gene. *Gene* **8:** 121–133.

Clapham, D.E. and E.J. Neer. 1993. New roles for G protein β-γ dimers in transmembrane signalling. *Nature* **365:** 403–406.

Clark, K.L., D. Dignard, D.Y. Thomas, and M. Whiteway. 1993. Interactions among the subunits of the G protein involved in *Saccharomyces cerevisiae* mating. *Mol. Cell. Biol.* **13:** 1–8.

Conklin, B.R. and H.R. Bourne. 1993. Structural elements of Gα subunits that interact with Gβγ, receptors and effectors. *Cell* **73:** 631–641.

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

Duronio, R.J., J.I. Gordon, and M.S. Boguski. 1992. Comparative analysis of the β transducin family with identification of several new members including *PWP1*, a nonessential gene of *Saccharomyces cerevisiae* that is divergently transcribed from *NMT1*. *Proteins* **13:** 41–56.

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

Fong, H.K.W., J.B. Hurley, R.S. Hopkins, R. Miake-Lye, M.S. Johnson, R.F. Doolittle, and M. Simon. 1986. Repetitive segmental structure of the transducin β-subunit: Homology with the CDC4 gene and identification of related mRNAs. *Proc. Natl. Acad. Sci.* **83:** 2162–2166.

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

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

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

Johnson, A.D. 1992. A combinatorial regulatory circuit in budding yeast. In *Transcriptional regulation* (ed. S.L. McKnight and K.R. Yamamoto), pp. 975–1006. Cold Spring Harbor Laboratry Press, Cold Spring Harbor, New York.

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

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

Keleher, C.A., S. Passmore, and A.D. Johnson. 1989. Repressor α2 binds to its operator cooperatively with yeast protein Mcm1. *Mol. Cell. Biol.* **9:** 5228–5230.

Keleher, C.A., M.J. Redd, J. Schultz, M. Carlson, and A.D. Johnson. 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. *Cell* **68:** 708–719.

Liu, F. and M.R. Green. 1994. Promoter targeting by adenovirus E1a through interaction with different cellular DNA-binding domains. *Nature* **368:** 520–525.

MacKay, V.L., S.K. Welch, M.Y. Insley, T.R. Manney, J. Holly, G.C. Saari, and M.L. Parker. 1988. The *Saccharomyces cerevisiae BAR1* gene encodes an exported protein with homology to pepsin. *Proc. Natl. Acad. Sci.* **85:** 55–59.

Mak, A. and A.D. Johnson. 1993. The carboxy-terminal tail of the homeo domain protein α2 is required for function with a second homeo domain protein. *Genes & Dev.* **7:** 1862–1870.

McGrath, J.P. and A.E. Varshavsky. 1989. The yeast *STE6* gene encodes a homolog of the mammalian multi-drug resistance P-glycoprotein. *Nature* **340:** 400–404.

Miller, J.H. 1972. *Experiments in molecular genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, New York.

Mukai, Y., S. Harashima, and Y. Oshima. 1991. AAR1/TUP1

protein, with a structure similar to that of the β subunit of G proteins, is required for a1-α2 and α2 repression in cell type control of *Saccharomyces cerevisiae*. *Mol. Cell. Biol.* **11:** 3773–3779.

Murakami, T., W. Simmons, and A. Spiegel. 1992. Site-specific antibodies directed against G protein β and γ subunits: Effects on α and βγ subunit interaction. *Biochemistry* **31:** 2905–2911.

Nehlin, J.O. and H. Ronne. 1990. Yeast MIG1 repressor is related to the mammalian early growth response and Wilms' tumour finger proteins. *EMBO J.* **9:** 2891–2898.

Nelson, H.C.M., M.H. Hecht, and R.T. Sauer. 1983. Mutations defining the operator-binding sites of bacteriophage lambda repressor. *Cold Spring Harbor Symp. Quant. Biol.* **47:** 441–449.

Passmore, S., R. Elble, and B.K. Tye. 1989. A protein involved in minichromosome maintenance in yeast binds a transcriptional enhancer conserved in eukaryotes. *Genes & Dev.* **3:** 921–935.

Roth, S.Y., A. Dean, and R.T. Simpson. 1990. Yeast α2 repressor positions nucleosomes in *TRP1*/ARS1 chromatin. *Mol. Cell. Biol.* **10:** 2247–2260.

Roth, S.Y., M. Shimizu, L. Johnson, M. Grunstein, and R.T. Simpson. 1992. Stable nucleosome positioning and complete repression by the yeast α2 repressor are disrupted by amino-terminal mutations in histone H4. *Genes & Dev.* **6:** 411–425.

Salama, N.R., T. Yeung, and R.W. Schekman. 1993. The Sec13p complex and reconstitution of vesicle budding from the E.R. with purified cytosolic proteins. *EMBO J.* **12:** 4073–4082.

Sauer, R.T., D.L. Smith, and A.D. Johnson. 1988. Flexibility of the yeast α2 repressor enables it to occupy the ends of its operator leaving the center free. *Genes & Dev.* **2:** 807–816.

Schena, M., L.P. Freedman, and K.R. Yamamoto. 1989. Mutations in the glucocorticoid receptor that distinguish interdigitated DNA binding and transcriptional enhancer activities. *Genes & Dev.* **3:** 1590–1601.

Scheuermann, R., S. Tam, P.M. Burgers, C. Lu, and H. Echols. 1983. Identification of the epsilon-subunit of *Escherichia coli* DNA polymerase III holoenzyme as the *dnaQ* gene product: A fidelity subunit for DNA replication. *Proc. Natl. Acad. Sci.* **80:** 7085–7089.

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

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

Smith, D.B. and K.S. Johnson. 1988. Single-step purification of polypeptides expressed in *Escherichia coli* as fusions with glutathione S-transferase. *Gene* **67:** 31–40.

Sprague, G. 1991. Assay of yeast mating reaction. In *Guide to yeast genetics and molecular biology* (ed. C. Guthrie and G.R. Fink), pp. 77–93. Academic Press, San Diego, CA.

Tatchell, K., K.A. Nasmyth, B.D. Hall, C. Astell, and M. Smith. 1981. In vitro mutation analysis of the mating type locus in yeast. *Cell* **42:** 237–247.

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

Tzamarias, D. and K. Struhl. 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. *Nature* **369:** 758–761.

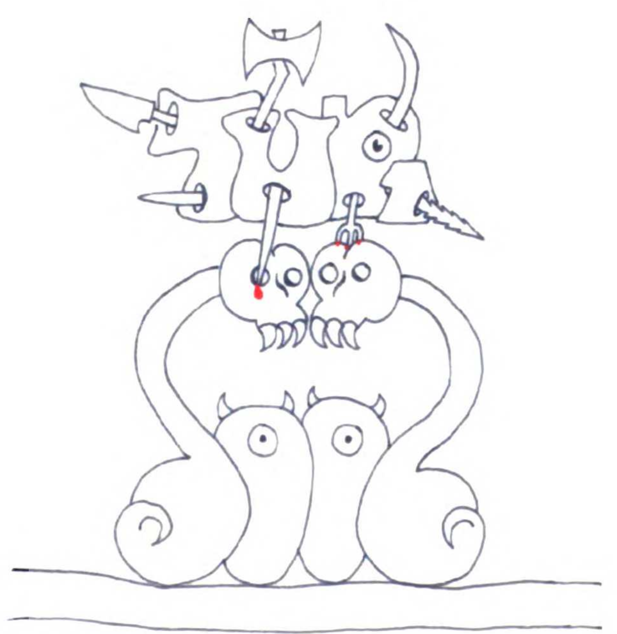
van der Voorn, L. and H.L. Ploegh. 1992. The WD-40 repeat. *FEBS Lett.* **307:** 131–134.

Whiteway, M., K.L. Clark, E. Leberer, D. Dignard, and D.Y. Thomas. 1994. Genetic identification of residues involved in association of α and β G-protein subunits. *Mol. Cell. Biol.* **14:** 3223–3229.

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

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

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



APPENDICES TO CHAPTER ONE

Appendix A. Overexpression of α2 suppresses multiple point mutations in the Mcm1-binding portion of the α2/Mcm1 operator

The two proteins α2 and Mcm1 bind cooperatively to the **a**-specific gene operator in vitro (45). Mutant operators in which the Mcm1-binding sequences have been either deleted (center-deleted operator) or replaced with random sequences (center-substituted operator) are unable to bind Mcm1 in vitro and fail to repress transcription in vivo (44). Presumably these mutant operators do not repress transcription because they fail to bind an α2/Mcm1 complex. Here we show that the center-substituted operator but not the center-deleted operator will repress transcription in vivo when α2 is overexpressed. In addition, at high concentrations of α2, an α2/Mcm1 complex will form on the center-substituted operator but not the center-deleted operator in vitro. These results suggest that the interaction between α2 and Mcm1 is strong enough to promote formation of an α2/Mcm1/operator complex in the absence of specific Mcm1-DNA contacts and provided the impetus for the α2 mutant screen described in Chapter One.

To test whether α2 overexpression suppresses the inability of the mutant operators to repress transcription, we transformed a *cyc1:lacZ* reporter containing either the center-substituted or center-deleted operator in between the UAS and TATA into *matΔ* and *MATα* yeast strains. These strains were then transformed with a high-copy plasmid bearing either *MATα2* or no insert and assayed for β-galactosidase activity. Both the center-substituted and center-deleted operators caused a decrease in transcription of the *cyc1:lacZ* reporter in an α2-independent fashion for unknown reasons. More importantly, overexpression of α2 led to a significant decrease in transcription of the reporter containing the center-substituted but not the center-deleted operator (Table 1). These results indicate that α2 is either binding only to the center-substituted operator in vivo or is binding to both operators but repressing only from the center-substituted operator. If the former is the case,

it should be possible to form an α2/Mcm1 complex on the center-substituted operator but not the center-deleted operator in vitro. Because preliminary experiments had indicated that an α2/Mcm1 complex will form on the center-substituted operator at high concentrations of α2 (C. Keleher, unpublished observations), we decided to test whether an α2/Mcm1 complex will also form on the center-deleted operator under similar conditions.

In order to compare α2/Mcm1 complex formation on the center-substituted operator versus the center-deleted operator in vitro, we performed gel-mobility shift assays with purified α2, Mcm1 from a crude yeast extract, and a radiolabeled DNA probe containing the center-substituted, center-deleted, or wild-type operator. A shifted species the size of an α2/Mcm1 complex appeared in the presence of the center-substituted or wild-type but not center-deleted operator (Figure 1). Furthermore, an α2/Mcm1 complex formed on the center-substituted operator at concentrations of α2 that were too low to support an α2 shift in the absence of Mcm1. Hence, it would appear that interactions between α2 and Mcm1 allow an α2/Mcm1 complex to form on the center-substituted operator despite the lack of specific Mcm1-binding sequences. Presumably the center-deleted operator does not bind an α2/Mcm1 complex because the space between the two α2 half-sites is too small to accommodate Mcm1.

While these results are consistent with α2 and Mcm1 binding cooperatively in vivo to the wild-type and center-substituted but not center-deleted operator in vivo, an alternative explanation for the lack of repression from the center-deleted operator is that α2 binds to both the center-substituted and center-deleted operators when overexpressed but cannot repress without Mcm1. Another possibility is that the shorter space between the α2 half-sites in the center-deleted operator may change the shape of the α2 dimer and prevent it from interacting with the repression machinery. The slight amount of repression observed in the presence of the center-deleted site suggests that α2 may in fact be binding the center-deleted operator to some degree. However, the ability of α2 to repress as an **a**1/α2 heterodimer in the presumed absence of Mcm1 disfavors these alternative explanations.

Methods

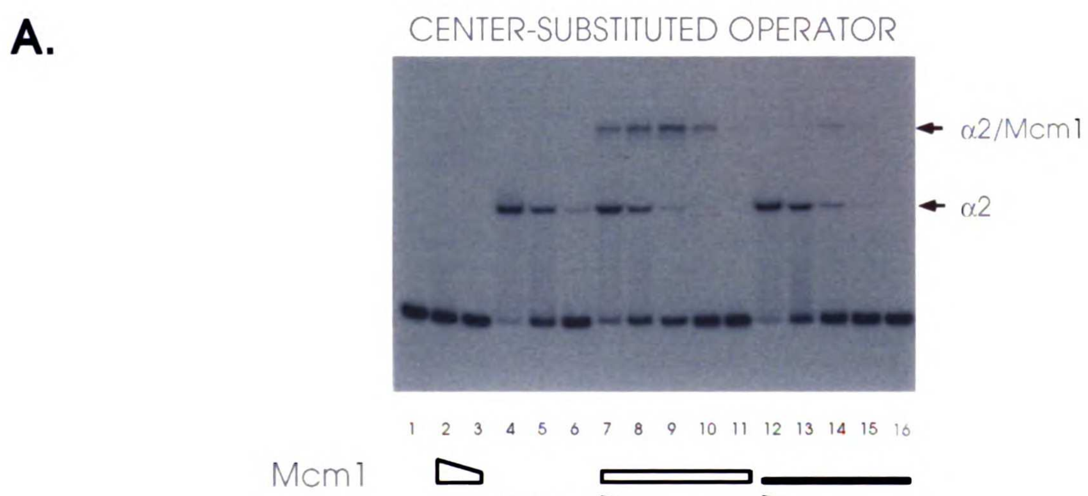
Yeast extracts were prepared as described (24). Gelshifts were performed in 10 mg/ml BSA, 10 ng/μl HaeIII-cut E. coli DNA, 5 mM $MgCl_2$, 10 mM NaCl. The probes have been previously described (44). Purified $\alpha 2$ was provided by Arkady Mak.

Table A-1. Overexpression of $\alpha 2$ suppresses the repression defect of the center-substituted operator. Yeast strains 246-1-1 (*MATα*) and AJY85 (*matΔ*) were transformed with *cyc1:lacZ* reporters pCGb and pCG19 which contain the center-substituted and center-deleted operator, respectively, in between the UAS and TATA (44). The resulting strains were transformed with a 2 μm plasmid vector containing no insert or *MATα2* and assayed for β-galactosidase activity.

MAT allele	operator	2 μm plasmid	β-galactosidase activity	fold repression
matΔ	center-substituted	vector	6.9 ± 1	
MATα	center-substituted	vector	3.4 ± 2	2
matΔ	center-substituted	*MATα*	0.4 ± 0.3	17
matΔ	center-deleted	vector	35.6 ± 8	
MATα	center-deleted	vector	35.7 ± 3	1
matΔ	center-deleted	*MATα*	14.5 ± 4	2.5

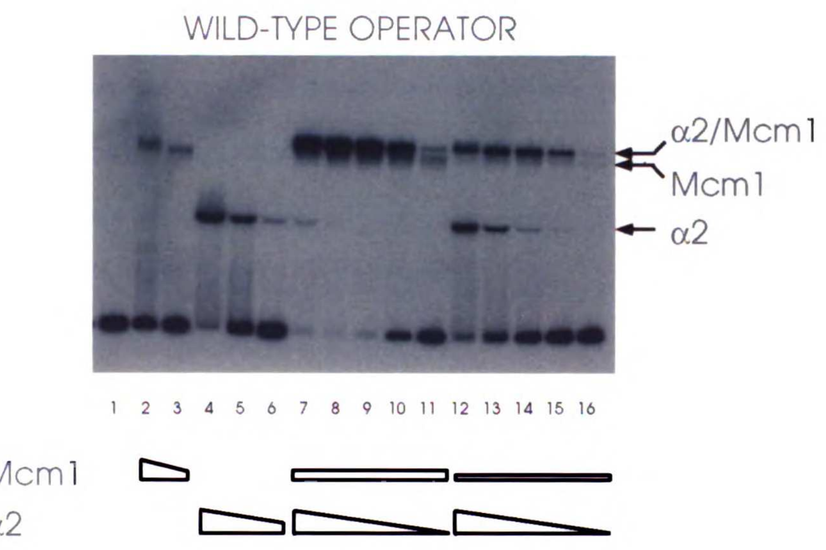
Figure A-1. $\alpha 2$ can bring Mcm1 to an operator in which the Mcm1-binding sequences have been replaced but not deleted. (**A**) Mcm1 can bind to the center-substituted operator in the presence of high concentrations of $\alpha 2$. Purified $\alpha 2$ was added to a yeast extract containing Mcm1 and a radioactively labeled DNA fragment containing the center-substituted operator. Lane 1 contains no protein. Lanes 2 and 3 contain 2 μl and 0.4 μl yeast extract, respectively. Lanes 4-16 contain five-fold serial dilutions of $\alpha 2$ and either 0 μl (lanes 4-6), 2 μl (lanes 7-11) or 0.4 μl (lanes 12-16) yeast extract. The left-most lane in each $\alpha 2$ dilution series contains 10^{-8} M $\alpha 2$. The positions of the $\alpha 2$/operator and $\alpha 2$/Mcm1 operator complexes are indicated to the right. (**B**) Mcm1 cannot bind to the center-deleted operator, even in the presence of elevated concentrations of $\alpha 2$. Purified $\alpha 2$ was added to a yeast extract and the radioactively labeled center-deleted operator. Lane 1 contains no protein. Lanes 2 and 3 contain 2 μl and 0.4 μl yeast extract, respectively. Lanes 4-14 contain five-fold serial dilutions of $\alpha 2$ and either 0 μl (lanes 4-6), 2 μl (lanes 7-10), or 0.4 μl (lanes 11-14) yeast extract. The left-most lane in each $\alpha 2$ dilution series contains 10^{-8} M $\alpha 2$. The position of the $\alpha 2$/operator complex is indicated to the right. (**C**) Positive control showing that Mcm1 binds to the wild-type operator in the presence and absence of $\alpha 2$. Lanes 1-16 are as in (A) except that the radioactively labeled DNA fragment contains the wild-type operator instead of the center deleted operator. The positions of the $\alpha 2$/operator, Mcm1/ operator, and $\alpha 2$/Mcm1/operator complexes are indicated to the right.

A.



B.

C.



Appendix B. DNA-Binding Activity of α2 is not affected by amino-terminal point mutations

The α2 amino-terminal point mutants are unable to repress transcription from an α2/Mcm1 operator. This defect is thought to be due to an inability to bind to Tup1 rather than a failure to occupy the α2/Mcm1 operator. We provide evidence for this idea by showing that all of the α2 mutants bind cooperatively with Mcm1 to DNA in vitro.

Gel-mobility shift assays were performed using crude bacterial extracts containing either wild-type or mutant α2 protein and a radioactively labeled fragment of DNA containing the α2/Mcm1operator. As shown in Figure 1A, all of the mutant proteins bound to the α2/Mcm1 operator in the absence of Mcm1. In the presence of Mcm1, all of the mutants formed an α2-Mcm1 complex on the DNA (Figure 1B). Binding with Mcm1 by each of the mutants was indistinguishable from binding by wild-type α2.

We also tested the ability of the mutant α2 proteins to bind cooperatively with the a1 protein to the **a**1/α2 operator. In the absence of **a**1, wild-type α2 and all of the mutants formed dimers on the **a**1/α2 operator at high protein concentrations (Figure2A). In the presence of **a**1, wild-type α2 and all of the mutants except α2-lys71 formed an **a**1/α2 complex (Figure 2B). The α2-lys71 mutant only formed an **a**1/α2 complex at high concentrations of α2-lys71, and the complex had a higher mobility than the wild-type a1/α2 complex. Since residue 71 lies in a region of α2 that is thought to contact **a**1 (32), the lys71 mutation may affect the ability of α2 to interact with both **a**1and Tup1. Consistent with this idea is the observation that the α2-lys71 is not dominant negative for **a**1/α2 repression (see Appendix C), as one would expect if the dominant phenotype involves displacing a wild-type **a**1/α2 complex from the operator.

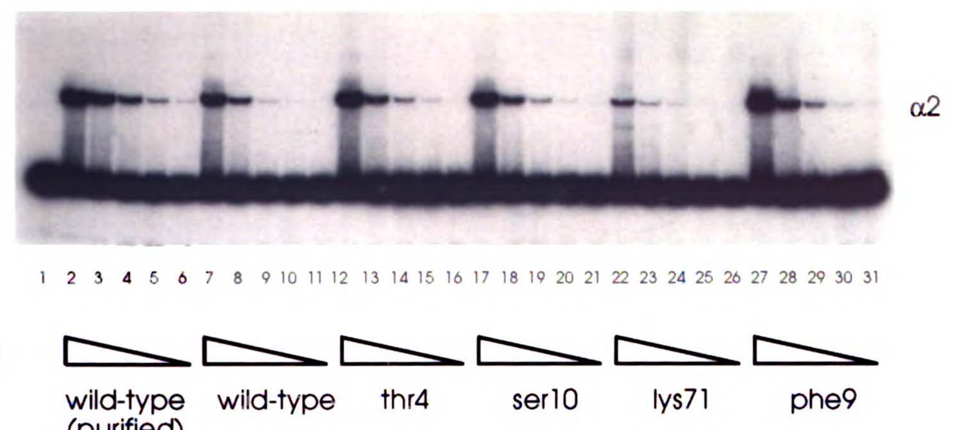
In summary, the α2 mutants are competent for binding DNA cooperatively with Mcm1 or **a**1 in vitro. Hence, it seems unlikely that their inability to repress transcription from an α2/Mcm1 operator is due to an inability to bind DNA.

Methods

Plasmids pKK211, pKK212, pKK355, and pKK496 are derivatives of pAV100 and were used to express α2-thr4, α2-ser10, α2-lys71, and α2-phe9, respectively, in E. coli strain XA-90. pAV100 was created by deleting the BamHI fragment from pAV99 (50). Bacterial and yeast extracts were prepared as described (22, 43). Mcm1/α2 gel shifts were performed in 10 mg/ml BSA, 10 ng/μl HaeIII-cut E. coli DNA, 5 mM $MgCl_2$, 10 mM NaCl, using an 86 bp ^{32}P end-labeled probe containing the wild-type STE6 operator (37). **a**1/α2 gel shifts were performed in 20 mM Tris (pH8), 0.1 mM EDTA, 10 mg/ml BSA, 10 ng/μl HaeIII-cut E. coli DNA, 5 mM $MgCl_2$, 0.1% Nonidet P40, 2.5% glycerol, using a 73 bp ^{32}P end-labelled probe containing the asymmetric consensus **a**1/α2 operator (23). Purified α2 was provide by Arkady Mak. Purified **a**1 was provided by Caroline Goutte.

Figure B-1. Mutant α2 proteins bind to the α2/Mcm1 operator in vitro. (**A**) Binding of wild-type and mutant α2 proteins to the α2/Mcm1operator in the absence of Mcm1. Three-fold serial dilutions of purified α2 (lanes 2-6) or of a bacterial extract containing wild-type α2 (lanes 7-11), α2-thr4 (lanes 12-16), α2-ser10 (lanes 17-21), α2-lys71 (lanes 22-26) or α2-phe9 (lanes 27-31) were added to a radioactively labeled DNA fragment containing the α2/Mcm1 operator and run on a nondenaturing polyacrylamide gel. Lane 1 contains no protein. The left-most lane in each α2 dilution series contains 1.7 x 10^{-8} M α2, as estimated from Coomassie-stained gels. The position of the α2-operator complex is incicated to the right. (**B**) Cooperative binding of wild-type and mutant α2 proteins with Mcm1 to the α2/Mcm1 operator. Five-fold serial dilutions of a bacterial extract containing wild-type α2 (lanes 5-7), α2-thr4 (lanes 8-10), α2-ser10 (lanes 11-13), α2-phe9 (lanes 14-16), or α2-lys71 (lanes 17-19) were added to a yeast extract containing Mcm1 and the radioactively labeled α2/Mcm1 operator. Lane 1 contains no protein; lanes 2-4 contain five-fold serial dilutions of the yeast extract; lanes 5-19 contain the same amount of yeast extract as does lane 3. The left-most lane in each α2 dilution series contains 1.7 x 10^{-8} M α2. The positions of the α2/operator, Mcm1/operator, and α2/Mcm1/operator complexes are indicated to the right.

A.



B.

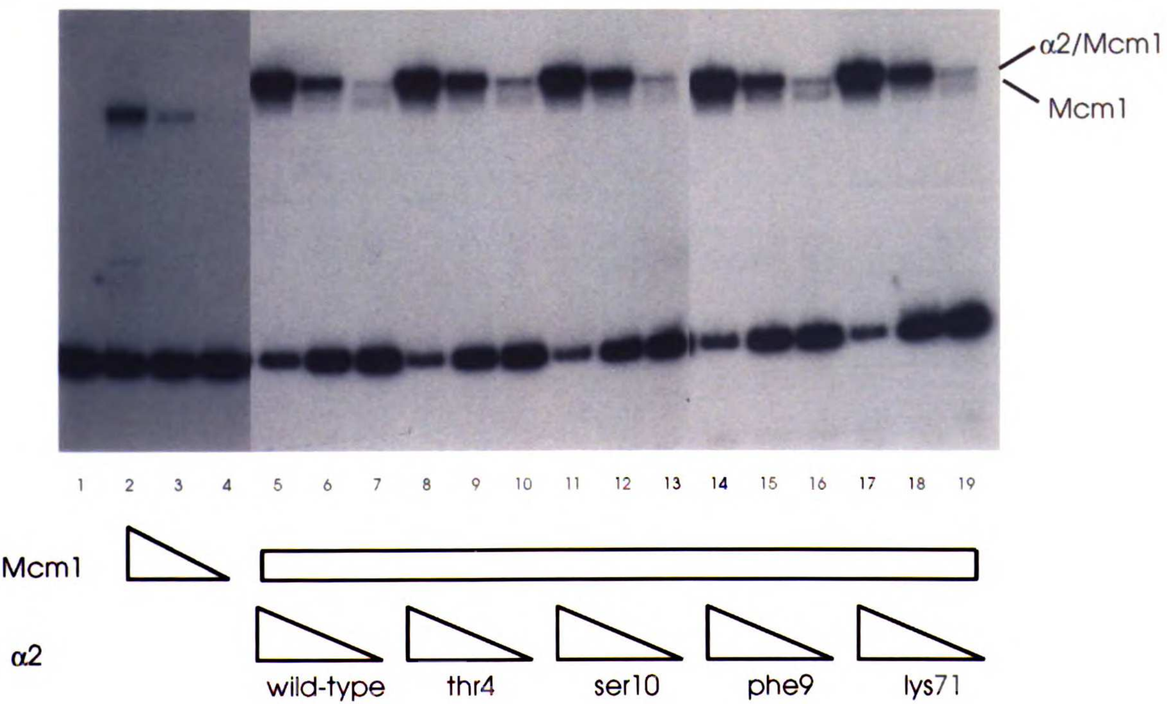
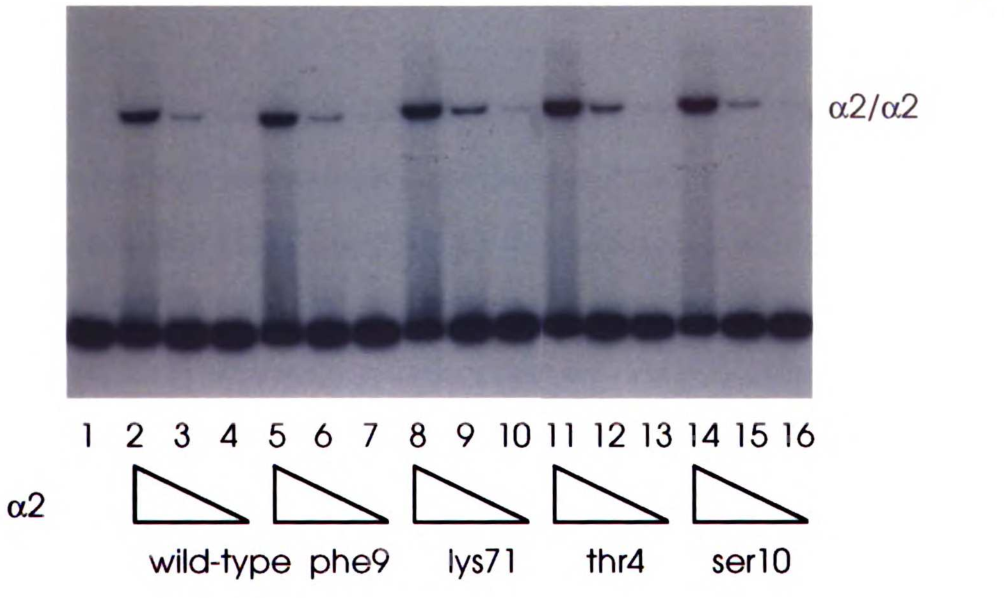
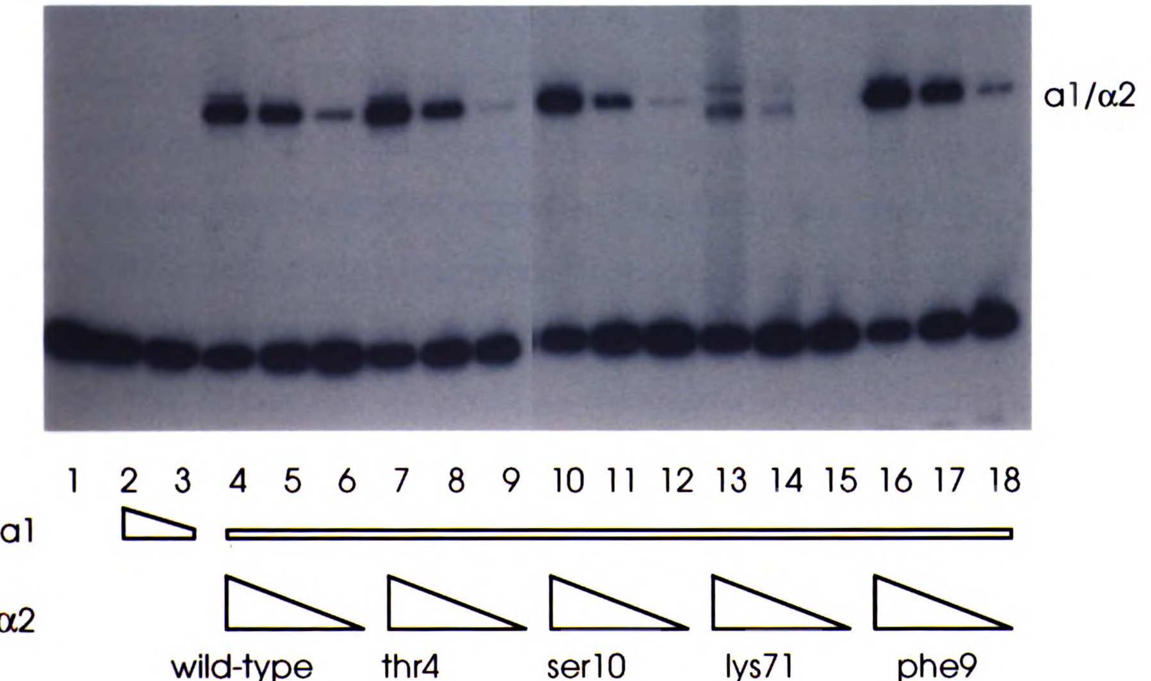


Figure B-2. Mutant α2 proteins bind to the **a**1/α2 operator in vitro. (**A**) Binding of wild-type and mutant α2 proteins to the **a**1/α2 operator in the absence of **a**1. Five-fold serial dilutions of bacterial extracts containing wild-type α2 (lanes 2-4), α2-phe9 (lanes 5-7), α2-lys71 (lanes 8-10), α2-thr4 (lanes 11-13), or α2-ser10 (lanes 14-16) were added to a radioactively labeled DNA fragment containing the **a**1/α2 operator and run on a nondenaturing polyacrylamide gel. Lane 1 contains no protein. The left-most lane in each α2 dilution series contains 1.7 x 10^{-8} M α2. The position of the α2/operator complex is indicated to the right. (**B**) Cooperative binding of wild-type and mutant α2 proteins with **a**1. Five-fold serial dilutions of bacterial extracts containing wild-type α2 (lanes 4-6), α2-phe9 (lanes 7-9), α2-lys71 (lanes 10-12), α2-thr4 (lanes 13-15), or α2-ser10 (lanes 16-18) were added to purified **a**1 protein and the radioactively labeled **a**1/α2 operator. Lane 1 contains no protein. Lane 2 contains 10^{-5} M **a**1. Lanes 3-18 contain 2 x 10^{-6} M **a**1. The position of the **a**1/α2 complex is indicated to the right.

A.



B.



Appendix C. Dominance of the α2-thr4 mutant requires DNA-binding activity

The α2 amino-terminal point mutants are unable to repress from an α2/Mcm1 operator. Because these mutants were identified on the basis of their ability to bind cooperatively with Mcm1 to DNA and because the mutants are dominant negative for α2/Mcm1-mediated repression, the mutants were thought to be defective in binding to some element of the downstream repression machinery. We provide further evidence for this idea by showing that the mutants are also defective for **a**1/α2-mediated repression and will derepress an **a**1/α2- repressed reporter when overexpressed in wild-type **a**/α cells. We then show that the dominance of the α2-thr4 mutant requires that the mutant protein be able to bind DNA, suggesting that the α2 amino-terminal mutants derepress transcription by displacing wild-type α2 from the operator and not by titrating some component of the repression machinery away from the DNA.

In order to show that the α2 mutants are unable to repress from an **a**1/α2 site, we transformed a *MATa* strain with an **a**1/α2-repressible reporter and a high copy plasmid bearing wild-type α2, mutant α2, or no insert and assayed the transformants for β-galactosidase activity. Whereas wild-type α2 caused a 12-fold decrease in transcription of the reporter, the mutants caused only a 4- to 8-fold decrease in transcription, indicating that the mutants are defective for **a**1/α2 repression (Table 1). We next transformed a *MATa/MATα* strain with the **a**1/α2-repressible reporter and a high copy plasmid bearing wild-type α2, mutant α2, or no insert and assayed the transformants for β-galactosidase activity. With the exception of α2-lys71, all of the mutant α2 plasmids caused an increase in expression of the reporter, indicating that three of the mutants are dominant negative for **a**1/α2 repression (Table 2).

In order to ascertain whether or not DNA-binding activity is required for the dominance of the α2-thr4 mutant, we examined the behavior of three α2 mutants: one

containing only the thr4 mutation (α2-thr4), one containing three mutations in helix 3 of the homeodomain that decrease the ability of α2 to bind DNA with Mcm1 but do not affect the ability of α2 to bind with **a**1 (α2-H3-3) (99), and one containing both the thr4 and helix three mutations (α2-thr4-H3-3). When a *MATα mfa2:lacZ* strain was transformed with α2-thr4 or α2-H3-3, the *mfa2:lacZ* reporter was derepressed, indicating that both α2-thr4 and α2-H3-3 are dominant negative mutants (Table 3). In contrast, α2-thr4-H3-3 did not derepress the *mfa2:lacZ* reporter, indicating that the α2-thr4 mutant cannot derepress transcription when its ability to bind DNA is destroyed.

In order to show that the presence of both the thr4 and helix three mutations in the same polypeptide does not simply unfold or destabilize α2, we also tested the ability of the α2-thr4-H3-3 mutant to derepress an **a**1/α2-repressed reporter. Because the helix three mutation does not affect the ability of α2 to bind to an **a**1/α2 operator, the α2-thr4-H3-3 mutant is expected to maintain its ability to derepress an **a**1/α2-repressed reporter. The α2-thr4-H3-3 mutant was dominant negative for **a**1/α2 repression, demonstrating that the mutant is able to interfere with repression from an operator to which it can bind (Table 4).

It is unclear why the α2-H3-3 mutant is dominant negative for α2/Mcm1-mediated repression. The most likely explanations are (1) α2-H3-3 and wild-type α2 form heterodimers that are unable bind to the α2/Mcm1 operator and (2) α2-H3-3 titrates some other proteins such as Tup1 or Ssn6 away from the operator-bound α2/Mcm1 complex. Both explanations are consistent with the observation that the α2-thr4-H3-3 mutant is no longer dominant negative for α2/Mcm1-mediated repression. Hence we believe that these results provide additional evidence that the α2 amino-terminal mutants can bind DNA in vivo and further demonstrate the separability of α2's DNA-binding activity from its ability to repress.

Table C-1. The α2 mutants are defective for **a**1/α2-mediated repression. EG123 (*MAT**a***) strain was transformed with the reporter pAJ79 and a plasmid bearing wild-type or mutant α2. Reporter pAJ79 is *cyc1:lacZ* with an **a**1/α2 operator in between the UAS and TATA.

MATα2 plasmid	β-galactosidase activity
vector	25.9 ± 2
α2 (wild-type)	2.1 ± 0.7
α2-thr4	6.5 ± 0.9
α2-ser10	7.0 ± 1
α2-phe9	3.0 ± 0.2
α2-lys71	4.3 ± 1

Table C-2. The $\alpha2$ mutants are dominant negative for **a**1/$\alpha2$-mediated repression. AJY87 (*MATa/MATα*) was transformed with the reporter pAJ79 and a plasmid bearing wild-type or mutant $\alpha2$ on a high copy plasmid and asssayed for β-galactosidase activity.

MATα2 plasmid	β-galactosidase activity
vector	2.5 ± 0.2
$\alpha2$ (wild-type)	1.1 ± 0.1
$\alpha2$-thr4	7.7 ± 1
$\alpha2$-ser10	7.0 ± 1
$\alpha2$-phe9	4.6 ± 0.4
$\alpha2$-lys71	1.8 ± 0.4

Table C-3. DNA-Binding activity is required for the dominance of the α2-thr4 mutant. Yeast strain SM1196 (*MATα mfa2:lacZ*) was transformed with a plasmid bearing wild-type or mutant *MATα2* and assayed for β-galactosidase activity.

MATα2 plasmid	β-galactosidase activity
vector	0.8 ± 0.4
α2-thr4	28.2 ± 2
α2-H3-3	19.4 ± 7
α2-thr4-H3-3	0.9 ± 0.4

Table C-4. The α2-thr4-H3-3 mutant is dominant negative for **a**1/α2-mediated repression. Yeast strain AJY87 (*MATa/ MATα*)was transformed with the reporter pAJ79 and a wild-type or mutant α2 plasmid and assayed for β-galactosidase activity. As a control, 246-1-1 (*MATα*) was transformed with the reporter pAJ3 and a wild-type or mutant α2 plasmid and assayed for β-galactosidase activity. Reporter pAJ3 is *cyc1:lacZ* with one α2/Mcm1 operator in between the UAS and TATA.

MAT allele	reporter	α2 plasmid	β-galactosidase activity
MATa/MATα	pAJ79	vector	3.6 ± 0.4
MATa/MATα	pAJ79	α2-H3-3	5.1 ± 0.1
MATa/MATα	pAJ79	α2-thr4-H3-3	26 ± 10
MATα	pAJ3	vector	0.6 ± 0.1
MATα	pAJ3	α2-H3-3	0.4 ± 0.1
MATα	pAJ3	α2-thr4-H3-3	0.7 ± 0.1

Appendix D. An α2 homolog from *Kluyveromyces lactis*

The *MATα* locus of *Saccharomyces cerevisiae* contains two divergently transcribed genes, *MATα1* and *MATα2*, which are both required to produce the α mating type. A *MATα1* homolog from the related yeast *Kluyveromyces lactis* (*KlMATα1*) was cloned by transforming a *K. lactis* genomic library into a *matα1* strain of *S. cerevisiae* and screening for restoration of α mating (111). In sequencing *KlMATα1*, Yuan et. al noticed part of an upstream open reading frame (ORF) encoding a potential *MATα2* homolog. We obtained a clone containing *KlMATα1* and a large fragment of upstream DNA and sequenced the remainder of the putative α2 ORF (Figure 1). The protein encoded by the *K. lactis* α2 gene (*KlMATα2*) contains homology to *S. cerevisiae* *MATα2* at the extreme N-terminus and in the region of the homeodomain (Figure 2).

In an attempt to test whether the *K.lactis* α2 homolog behaves like *S. cerevisiae* α2, we transformed the *KlMATα2* plasmid into various *S. cerevisiae* strains lacking *MATα2*. The *KlMATα2* plasmid was unable to complement the mating defect of a *matα2* strain, the sporulation defect of a *matα2/MATa* strain or the inability of a *matΔ* strain to repress an *mfa2:lacZ* reporter (data not shown). The K. lactis plasmid also did not cause derepression of an *mfa2:lacZ* reporter in a *MATα* strain, indicating that KlMatα2 does not behave in a dominant negative fashion, as one would expect if KlMatα2 could bind the operator but not the repression machinery or vice versa. Extracts of a *matΔ* strain bearing the *KlMATα2* contained no α2 protein, as assayed by Western blot using antibodies directed against *S. cerevisiae* α2, indicating that the lactis protein either is not expressed well from the plasmid or differs enough from *S. cerevisiae* α2 to prevent reaction with the antibodies (data not shown).

Despite the lack of functional data regarding the *K. lactis* α2 homolog, the sequence similarity between the extreme amino termini of the *K. lactis* and *S. cerevisiae* proteins is intriguing because *K. lactis* also has a *TUP1* homolog. This *TUP1* homolog is able to

complement the mating and growth defects of a *tup1Δ* strain of S. cerevisiae, suggesting that the *K. lactis* Tup1 protein can bind to *S. cerevisiae* α2 (B. Braun, unpublished observations). The similarity between the amino termini of the two α2 homologs is consistent with both of these proteins using this region to contact Tup1.

Figure 1. DNA and protein sequence of the $\alpha 2$ homolog from *K. lactis*. Amino acid positions are indicated to the left; nucleotide positions to the right. The K. lactis *MAT*α clone was provided by Olive Yuan.

```
    TTTTTGGCGT TACTTTTTTG TATTTCTTTG ATAGGATCTA TGGAAATGGT CGAGTTGTGA CCTTTCACTT

    CTGATTTAAT CATGGTATTT ATATGAAACT GACACTGTCG CCTCAAGAAA GTTTCATGTC TAAAAAAAAA  140

    TAGTATAAAG ACATTTGAGC TGAAGAATTA TACCAAATTC TTAAAATAAA TTCCAGTGAA GGACAACCCC

    AACAAGGCAA AAATGAGTAG AATACCCATA CACTCATTGC TAAACCCATC AGAAAGTTGT AAAAGCATCA  280
1                  M S R   I P I   H S L   L N P S   E S C   K S I

    GTAATGTACC CAGCAATTAC AGAGACTTAA GCACCTTCAA CAAGGAGAGA GCAAAAGTAA TTACTACATT
20  S N V P   S N Y   R D L   S T F N   K E R   A K V   I T T F

    TCAAGAGATG TTTTATTCAA TGCTAGAAAA TAATGACGAT TACAATAAAA TTGAGTCGTT GATTAGAAAC  420
44   Q E M   F Y S   M L E N   N D D   Y N K   I E S L   I R N

    TTTCAACCAA AATTGACATG GTCACACAAG TGCGAAAGCT TGACATTTAA ACAGAAGGCC TATCTCACAG
67   F Q P   K L T W   S H K   C E S   L T F K   Q K A   Y L T

    CAATAATTCA AAAGTCCATT AAGAGTTTAC TTGTGTTACT CAAAGAGAAA GGAAAAATGA GAGAGATTGA  560
90  A I I Q   K S I   K S L   L V L L   K E K   G K M   R E I E

    ATTTAAAAGA GGTCCGTAAA ATTAATAAAT ACCGACAATC CTCTAAGAAC TTTGAGGCAG GTCCGTTAAT
114  F S R   K E V   R K I N   K Y R   Q S S   K N F E   S V N

    ATAAAAATTC TAACTCAAGA TTTAATGCAC TCCAACAATA ACGAATTTAA GAAAGGAAAA AGATTTCCTA  700
137  I K I   L T Q D   L M H   S N N   N E F K   K G K   R F P

    AATCCCATAT ACAGCTCCTG GAGAACTGGT ATAGTATGAA TAGAAGAAAC CCTTACCTCG CTGAAAATGA
160 K S H I   Q L L   E N W   Y S M N   R R N   P Y L   A E N D

    TTTGGCCTAT ATAAGTAAAA ACACCACTTT GACTAAAACC CAAATAAAAA ATTGGTTAGC TAACAGAAGA  840
184  L A Y   I S K   N T T L   T K T   Q I K   N W L A   N R R

    CGAAAAGATA AAATTACTGA AGTTTCGTCA GATATAAGAA ACATTCTTAA TTAATGTAGT TAATAGAGCA
207  R K D   K I T E   V S S   D I R   N I L N

    TAGCATTACT CACACTCAAA TTGTTA
```

Figure 2. Homology between the amino acid sequences of *S. cerevisiae* α2 and the *K. lactis* α2. The *S. cerevisiae* sequence was compared to the *K. lactis* sequence by Michael Redd, using the program ALIGN. The sequences are 27% identical, with most of the similarity lying in the extreme amino terminus and the homeodomain. The three helices of the *S. cerevisiae* homeodomain are indicated above the alignment.

```
                      10            20          30          40
cerevisiae    MNKIPIKDLLNP--------QITDEFKS-SILDINK-KLFSICCNLP-KLP
              :..::::..::::       ........ :... .. :....   ..  ..
lactis        MSRIPIHSLLNPSESCKSISNVPSNYRDLSTFNKERADVITTFQEMFYSML
                      10        20        30        40        50

                     50          60        70        80
cerevisiae    ESVTTEEEVE--LRDILVFLSRANKNRKISDEEKKLLQTTSQLTT-TITVL
              :. .. ...:  .:..   :....:  ... ..:  : .. : .. .. ::
lactis        ENNDDYNKIESLIRNFQPKLTWSHKCESLTFKQKAYLTAIIQKSIKSLLVL
                     60        70        80        90       100

              90          100           110       120       130
cerevisiae    LKE---MRSIENDRSNYQLTQK----NKSADGLVFNVVTQDMINKSTKPYR
              :::   ::.:: .:.. . ..:    .:. ... ....::....... ..
lactis        LKEKGKMREIEFSRKEVRKINKYRQSSKNFESVNIKILTQDLMHSNNNEFK
                   110       120       130       140       150

                         helix 1             helix 2        helix3
                    _____________        ____________   __________
                     140       150       160       170       180
cerevisiae    -GHRFTKENVRILESWFAKNIENPYLDTKGLENLMKNTSLSRIQIKNWVSN
               :.::.:....::.:...:  :::....:. . :::.:...:::::..:
lactis        KGKRFPKSHIQLLENWYSMNRRNPYLAENDLAYISKNTTLTKTQIKNWLAN
                  160       170       180       190       200

              ____
                   190       200       210
cerevisiae    RRRKEKTITIAPELADLLSGEPLAKKKE
              ::::.:......... ..:.
lactis        RRRKDKITAVSSDIRNILN---------
                 210       220
```

Appendix E. Overexpression of Tup1 suppresses a deletion of *SSN6*

Yeast strains lacking *SSN6* are unable to repress an *mfa2:lacZ* reporter. However, this defect in α2-mediated repression can be partially suppressed by a high-copy plasmid bearing *TUP1* (M. Wahi and B. Braun, unpublished observations). Here, we quantitate the effect of Tup1 overexpression on the repression of the α2-repressed reporter pAJ3 (*cyc1:lacZ* containing an α2/Mcm1 operator) and the hypoxic reporter *anb1:lacZ* in an *ssn6Δ* strain and show that Tup1 overexpression partially suppresses the defect in repression of the α2-repressed reporter only. We also show that the N-terminus of Tup1 is not required for the ability of Tup1 to suppress an *SSN6* deletion, suggesting that the major role of the Tup1 N-terminus in α2-mediated repression is to interact with Ssn6.

In order to quantitate the ability of *TUP1* to suppress an *SSN6* deletion, we transformed a *MATα ssn6Δ* strain with either pAJ3 or the *anb1:lacZ* reporter and a 2 μm plasmid expressing full-length Tup1 (Tup1 (1-713)), the Tup1 N-terminus (Tup1 (1-253)), or no protein. Whereas none of the plasmids had any effect on the *anb1:lacZ* reporter, the Tup1(1-713) plasmid decreased expression of pAJ3 by approximately 8-fold (Table 1). In contrast, overexpression of Ssn6 from a 2 μm plasmid had no effect on the repression of an α2/Mcm1- or a1/α2-repressed reporter in a strain lacking *TUP1*, indicating that elevated levels of Ssn6 are unable to compensate for the absence of Tup1 (Table 2).

Since Ssn6 is not absolutely required for α2-mediated repression, it seemed likely that the amino terminus of Tup1 which interacts with Ssn6 might also be dispensable for α2-mediated repression. In order to test whether the Tup1 amino terminus is required for repression, we transformed KKY143 (*MATα tup1Δ ssn6Δ mfa2:lacZ*) with plasmids expressing either Tup1(1-713), Tup1(254-713), or Tup1(363-713) and assayed the transformants for β-galactosidase activity. As previously discussed, all of the Tup1 derivatives restored mating; however, only Tup1(1-713) and Tup1(254-713) restored

repression of the *mfa2:lacZ* reporter (Table 3). The ability of the Tup1(363-713) fragment to restore mating but not repression of *mfa2:lacZ* is probably an indication that even weak repression of the **a**-specific genes is sufficient to produce detectable levels of α mating.

Table E-1. Overexpression of Tup1 allows repression of an **a**-specific gene reporter but not a hypoxic gene reporter in an *ssn6Δ* strain. AJY159 (*MATα ssn6Δ9*) was transformed with either the *anb1:lacZ* reporter plasmid pKK482 or the α2-repressible reporter plasmid pAJ3. The resulting strains were then transformed with 2 μm plasmids expressing either Tup1(1-253) or Tup1(1-713) and assayed for β-galactosidase activity.

Reporter	2 μm plasmid	β-galactosidase activity
anb1:lacZ	vector (pKK412)	886 ± 200
anb1:lacZ	Tup1(1-253) (pKK369)	633 ± 70
anb1:lacZ	Tup1(1-713) (pKK396)	1074 ± 200
pAJ3	vector (pKK412)	82 ± 20
pAJ3	Tup1(1-253) (pKK369)	93 ± 30
pAJ3	Tup1(1-713) (pKK396)	10.5 ± 1

Table E-2. Overexpression of Ssn6 does not suppress a *tup1Δ*. KKY110 (*MATα tup1Δ mfa2:lacZ*) was transformed with a 2 μm plasmid carrying either *TUP1* or *SSN6*. KKY103 (*MATa/MATα tup1Δ/tup1Δ*) was transformed with reporter pAJ1 or pAJ79 and a 2 μm plasmid carrying either *TUP1* or *SSN6*. Plasmids pAJ1 and pAJ79 are 2 μm *cyc1:lacZ* reporters with no operator or one α2/Mcm1 operator between the UAS and TATA, respectively.

Strain	Plasmid	β-galactosidase activity
KKY110	Yep24 (vector)	146 ± 8
KKY110	*TUP1*/Yep24	3.7 ± 3
KKY110	*SSN6*/Yep24	114 ± 10
KKY103 + pAJ1	Yep24 (vector)	62.6 ± 5
KKY103 + pAJ1	*TUP1*/Yep24	55.6 ± 10
KKY103 + pAJ1	*SSN6*/Yep24	49 8 ± 12
KKY103 + pAJ79	Yep24 (vector)	20.9 ± 4
KKY103 + pAJ79	*TUP1*/Yep24	0.5 ± 0.1
KKY103 + pAJ79	*SSN6*/Yep24	12.3 ± 1

Table E-3. Overexpression of Tup1(254-713) represses an *mfa2:lacZ* reporter in the absence of wild-type *SSN6* or *TUP1*. KKY143 (*MATα tup1Δ ssn6Δ9 mfa2:lacZ*) was transformed with plasmids expressing either Tup1(363-713) or Tup1(254-713) under the control of the *GAL10* promoter. Transformants were grown on galactose and assayed for β-galactosidase activity.

Plasmid	β-galactosidase activity
vector (pKK412)	70.5 ± 20
Tup1(363-713) (pKK462)	27.2 ± 3
Tup1(254-713) (pKK444)	9.4 ± 1

chapter two

Residues in the WD repeats of Tup1 required for interaction with α2

Abstract

The yeast transcriptional repressor Tup1 contains seven WD repeats which interact with the DNA-binding protein α2. We have identified mutations in Tup1 that disrupt this interaction. The position of the amino acids changed by these mutations is consistent with Tup1 being folded into a seven-bladed propeller like that formed by another WD repeat-containing protein, the β subunit of the heterotrimeric G protein used in signal transduction. Our results also indicate that the interaction between Tup1 and α2 resembles the interaction between G_β and G_α, suggesting that a similar structural interface is formed by WD repeat proteins that are used in both transcriptional regulation and signal transduction.

The WD repeat is a 40-amino acid motif found in proteins involved in a wide variety of cellular processes ranging from signal transduction to RNA processing (reviewed in (15, 101)). Proteins containing WD repeats are often physically associated with other proteins and are believed in many cases to act as scaffolds upon which multimeric complexes are built (69). The structure of the GTP-binding protein (G protein) heterotrimer has revealed that the seven WD repeats of the β subunit (G_β) fold into a circular, seven-bladed propeller with a water-solvated central channel and a relatively flat top and bottom formed by the turns connecting the β-strands that make up each propeller blade (52, 88, 104). The α subunit (G_α) sits asymmetrically on top of the propeller contacting both the flat top surface and one of the sides parallel to the central channel.

Because many of the amino acids in G_β that contribute to the integrity of the propeller are hallmarks of the WD motif, it has been proposed that all WD proteins fold into propellers in which the internal β-strands form a rigid skeleton that is fleshed out on the surface by specialized loops to which other proteins bind (70). We provide evidence for this idea by examining the interaction between the yeast repressor Tup1, a WD protein whose biological function is unrelated to that of G_β, and the cell-type regulator α2. Tup1 represses the transcription of a large number of genes in *Saccharomyces cerevisiae* by interacting with various proteins bound to DNA sequences found upstream of target genes and interfering with transcription (17, 46, 67, 95, 114). Tup1 is known to interact directly with at least one of these proteins, the homeodomain protein α2 (see Chapter One). This interaction requires both the extreme N-terminus of α2 and the WD repeats of Tup1, as a deletion of the respective region of either protein results in a loss of binding. In order to delineate which parts of the WD repeats are important for this interaction, we screened for point mutations in Tup1 that affect binding to α2 but that leave other functions of Tup1 intact.

Results

Isolation of dominant negative Tup1 mutants

Our screen for *TUP1* mutants specifically defective in interacting with α2 took advantage of two properties of Tup1. First, a fragment of Tup1 lacking the WD repeats cannot bind to α2 and will disrupt repression of α2-regulated genes in wild-type strains when overexpressed, presumably because the fragment of Tup1 binds to some downstream component of the repression machinery and titrates it away from α2 (data not shown). Second, a Tup1-lexA fusion represses transcription from a lexA operator in the absence of α2 (see Appendix F and reference (97)). We therefore assumed that the two major functions of Tup1--interaction with α2 and interaction with the repression machinery--were separate and could be disarmed independently. We reasoned that a Tup1-lexA mutant defective in binding to α2 would interfere with repression of an α2-regulated reporter by wild-type Tup1 but would maintain the ability to repress from a lexA site (see Figure 1).

Using modified PCR conditions, we introduced random mutations into a *TUP1-lexA* fusion borne on a high copy plasmid and transformed the DNA into a *MATα TUP1* strain carrying an α2-repressed *URA3* gene and a Tup1-lexA-repressible *lacZ* reporter. We selected for transformants able to grow in the absence of uracil and screened the resulting Ura^+ colonies for β-galactosidase activity by filter assay. Of the 30,000 transformants examined, 150 were Ura^+, and 75 of these 150 were white by filter assay. Plasmid DNA was isolated from 24 of the white, Ura^+ colonies and sequenced; 12 unique mutations in *TUP1* were identified. Because the other 12 plasmids that we sequenced all contained one of these 12 mutations, the remaining 51 white, Ura^+ colonies were not examined further.

Tup1 mutants are defective for α2-mediated repression

In order to quantitate the ability of the *TUP1* mutants to complement for *TUP1* function and to confirm that any defect in repression observed is not an artifact of the lexA fusion, we introduced the mutations into a plasmid that expresses Tup1 that is not fused to lexA and transformed the resulting plasmids into a *MATα tup1Δ* strain carrying the α2-repressible reporter *mfa2:lacZ.* As expected, the mutants failed to repress the reporter gene to the same extent as does wild-type Tup1 (Table 1). In contrast, both wild-type and mutant versions of Tup1-lexA repressed from a lexA site to approximately the same degree (Table 2), suggesting that the mutations do not debilitate the interaction between Tup1 and downstream components of the repression machinery and that the defect in carrying out α2-mediated repression is due to an inability of the mutant Tup1 proteins to bind α2.

Tup1 mutants are defective in α2 binding

We next examined the ability of the mutant Tup1 proteins to bind to α2 in vitro using affinity chromatography. Each of the mutants was expressed as a GST fusion in E. coli, purified, and immobilized on glutathione agarose beads. Bacterial extracts containing α2 and $\alpha 2^{\Delta 2\text{-}12}$, a deleted version of α2 that does not bind to Tup1, were passed over the beads which were subsequently washed and eluted with high salt. As shown in Figure 2A, α2 binds to the wild-type Tup1 column and is absent from the flowthrough and wash fractions, whereas $\alpha 2^{\Delta 2\text{-}12}$ does not bind to the column and is present in the flowthrough and wash fractions. In contrast, the flowthrough and wash fractions of three of the mutant Tup1 columns contain both α2 and $\alpha 2^{\Delta 2\text{-}12}$, indicating that the mutant Tup1 columns retain α2 less efficiently than does the wild-type column (Figure 2B-D). The remaining nine Tup1 mutants also showed a decrease in α2 binding by this assay (data not shown). Each of the column experiments was repeated from two to six times with individually prepared columns, and similar results were obtained each time. Hence, we believe that the difference between the wild-type and mutant columns is unlikely to be due to slight

variations in column volume or protein concentration on the beads and reflects instead a decrease in the ability of the mutant proteins to bind α2.

Tup1 mutants fail to repress reporters repressed by DNA-binding proteins other than α2

Because Tup1 is required for the repression of many genes in addition to those regulated by α2, we examined the ability of six of the mutants to repress three other reporters that require Tup1 for repression: a glucose-repressed reporter *(suc2:lacZ)*, a hypoxic reporter *(anb1:lacZ)*, and a DNA-damage-inducible reporter *(rnr2:lacZ)*. KKY103 (*tup1Δ*) was cotransformed with a reporter and a wild-type or mutant *TUP1* plasmid and assayed for β-galactosidase activity. Most of the Tup1 mutants do not repress as strongly as does wild-type Tup1 (Table 3). In general, the mutants which are capable of only weak α2-mediated repression also show weak repression of *anb1:lacZ*, *suc2:lacZ* and *rnr2:lacZ*; however, there is no strict hierarchy for strength of repression that applies to all four reporters tested. For example, Tup1-Y580H is the weakest mutant with respect to *suc2:lacZ* repression but has an intermediate phenotype with respect to the other three reporters.

Discussion

In summary, we have genetically identified residues in the WD repeats of Tup1 that are required for the Tup1-$\alpha2$ interaction. The simplest explanation for this defect in binding is that the mutations change amino acids in Tup1 that contact $\alpha2$. Hence, the mutants provide a test for the prediction that Tup1 is folded into a β-propeller since the affected amino acids are predicted to lie close to one another on the surface of Tup1. When we used the coordinates for the G_β structure and the homology between Tup1 and G_β to construct a model for the structure of Tup1, we found that all of the amino acids that are changed in the $\alpha2$-binding mutants reside on one face of the predicted Tup1 propeller (Figure 3). This surface of Tup1 is analogous to the surface of G_β which interacts with a β-strand-β-strand-α-helix cluster in G_α.

Our results, then, provide evidence for several of the generalizations regarding WD proteins that have emerged from the structure of G_β. First, our data support the prediction that the Tup1 WD repeats form a β-propeller and are consistent with recent proteolysis experiments showing that much of Tup1 is folded into a compact, trypsin-resistant structure (21). Although the trypsin-resistant fragment of Tup1 is the size of six WD repeats rather than seven, the long linker between WD1 and WD2 contains several trypsin sites and is likely to be exposed as an extended loop on the upper surface of Tup1. Second, the mutations that we have identified affect amino acids that are completely conserved among Tup1 homologs from other yeast but not among WD proteins in general (6, 109), lending credence to the notion that surface amino acids that are evolutionarily conserved within a functional family of WD proteins are likely to be involved in interacting with specific proteins. Finally, the similarity between the Tup1-$\alpha2$ interaction and the major G_β-G_α interaction suggests that the flat surfaces of the propeller might be used by WD proteins in general as a protein-binding surface.

Although the structure of the G protein heterotrimer clearly demonstrates that the sides of the propeller are capable of making protein-protein contacts, the flat upper surface is particularly interesting because it binds not only $G\alpha$ but also some of the numerous downstream effectors that $G\beta\gamma$ consorts with after abandoning $G\alpha$ (22). Likewise, the flat upper surface of Tup1 is probably utilized to contact not only $\alpha 2$ but also the assorted DNA-binding proteins found upstream of other Tup1-regulated genes, since other genes that we have tested are partially derepressed by the *TUP1* mutations isolated in this work. Given that one of the distinguishing features of WD proteins is their ability to engage a number of different partners, it is tempting to speculate that the flat surfaces composed of flexible loops from each WD repeat are designed to provide binding sites for many proteins within a relatively small area.

Methods

Plasmids

Plasmid pKK631 is a 2μm *LEU2* plasmid containing *TUP1* fused to lexA via BamHI sites engineered at the stop codon of Tup1 and the start codon of lexA; the *TUP1* sequences in pKK631 have been modified to eliminate the naturally occurring BamHI site within the coding sequence of *TUP1* and to introduce a BamHI site at sequences coding for amino acids 333 to 335 and SalI site at sequences coding for amino acids 436 to 438. Plasmid pKK630 is identical to pKK631 except that the BamHI fragment containing sequences coding for amino acids 334 to 713 of Tup1 has been deleted. Plasmid pKK339 has three α2 operators upstream of the *URA3* gene carried on the TRP1/ARS/CEN vector pRS314 (84). Plasmid pKK602 is an *ADE2*-marked integrating version of the lexA-repressible *cyc1:lacZ* reporter pCK30 (46).

Mutant versions of the Tup1-lexA plasmid (pKK631) were recovered from yeast as described in Chapter One and sequenced. In cases where the plasmid contained more than one mutation, each of the single mutations was introduced into pKK598 by oligo-directed site-directed mutagenesis (51). All of the mutations were then subcloned into pKK448, a plasmid expressing Tup1 that is not fused to lexA, and the resulting plasmids were transformed into yeast strain SM1196 (28). Transformants were screened for β-galactosidase activity by filter assay. Table 1 summarizes the mutations which created alleles of *TUP1* that are able to derepress the *mfa2:lacZ* reporter. Plasmid pKK598 is the BamHI-HindIII fragment of *TUP1* subcloned into the f1 origin-containing plasmid pUCf1 (Promega). Plasmid pKK448 contains the *TUP1* gene with a SalI site engineered in at sequences coding for amino acids 436 to 438; the *LEU2* and 2μm sequences on pKK448 are derived from pΔSJ1 which is pSJ1 in which the SalI-XhoI fragment of the polylinker has been deleted. pSJ1 is a 2μm *LEU2* plasmid (42).

All of the GST-TUP1 expression plasmids were derived from pGST-CTERM (see Chapter One) which expresses GST fused to amino acids 254 to 713 of Tup1. The mutant versions of pGST-CTERM were generated by subcloning appropriate restriction fragments from the mutant pKK448 plasmids into pGST-CTERM. The $\alpha2^{\Delta2-12}$ expression plasmid was constructed by introducing the deletion mutation into pAV99 (58) by site-directed mutagenesis (51), using the oligonucleotide 5'-GGA TTT AAA CTC ATC TGT GAT TTG CAT ATG CTG TTT CCT GTG TGA AAT TGT TAT-3', and by subsequently removing the BamHI fragment downstream of the $\alpha2^{\Delta2-12}$ coding sequence.

The *anb1:lacZ* reporter was constructed by inserting the XhoI-BamHI fragment from pLGΔ312S (41) into the XhoI and BamHI sites of pKK480. pKK480 is the SmaI-SalI fragment of pRY52 inserted into the SmaI and XhoI sites of pRY52. pRY52 was provided by Roger Yocum and is pLG669 (26) with a BglII linker inserted into the HaeIII site. The *suc2:lacZ* reporter was constructed by inserting a BamHI-cut PCR fragment containing the upstream regulatory region of *SUC2* into the BamHI site of pLGΔSS (41). The PCR fragment was generated using the oligonucleotides 5'-GCC GGG ATC CGC TCA AAA AAG TAC GTC ATT TAG AAT TTG-3' and 5'-CTC CGG ATC CGG TCA TCA TAT ACG TTA GTG AAA AGA AAA GC-3' as primers and plasmid pRB58 (8, 81) as template. The *rnr2:lacZ* reporter is pZZ2 (113).

Yeast strains

All yeast strains are congenic to EG123 (85). KKY135'' (*MATα trp1 leu2 ura3 his4 ade2* + pKK602 + pKK339) was constructed by transforming pKK602 and pKK339 into KKY135. KKY135 is 246.1.1 (85) in which the *ADE2* gene has been partially deleted. KKY110 (*MATα trp1 leu2 ura3 his4 tup1Δ mfa2:lacZ)* and KKY103 (*MATα trp1 leu2 ura3 his4 tup1Δ*) were constructed by introducing an unmarked *TUP1* deletion into SM1196 (28) and 246.1.1, respectively, using plasmid pRT164 as described in Chapter One.

PCR mutagenesis and screen for Tup1-lexA mutants

Mutants were generated by amplifying a region of *TUP1* under mutagenic PCR conditions and cotransforming the PCR product into yeast with a gapped plasmid containing homology to both ends of the PCR product (66). The PCR product was made using the oligonucleotides 5'-CCA CTC TAA ACC TAT CCC-3' and 5'-CCT CTT CCT GCA ACA GAC GAA TCC-3' as primers and plasmid pKK631 as template DNA. Reactions were carried out in commercial 1X PCR buffer + $MgCl_2$ (Boehringer Mannheim Biochemicals) supplemented with 1 mM each dGTP, dCTP, and dTTP; 200 μM dATP; 500 μM $MnCl_2$; 3 mM $MgCl_2$; and 2.5 units Taq polymerase (Boehringer Mannheim Biochemicals). The PCR product was cotransformed with BamHI-cut pKK630 into KKY135''. The transformants were grown on synthetic -TRP-LEU plates then replica plated to -TRP-LEU-URA plates. Ura^+ colonies were patched onto -TRP-LEU-URA plates and assayed for β-galactosidase activity by filter assay as described in Chapter One.

Liquid β-galactosidase assays

Quantitative β-galactosidase assays were performed as previously described (64), except that yeast cells were permeabilized with 0.0025% SDS and 5% chloroform 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 and represent assays performed in triplicate on three independent transformants.

α2-binding assays

Bacterial extracts containing both α2 and $\alpha 2^{\Delta 2-12}$ were passed over glutathione agarose columns bearing various GST-Tup1 fusions. Purification of GST-Tup1 fusions, preparation of α2-containing bacterial extracts and affinity chromatography were performed essentially as described in Chapter One.

Acknowledgments

We thank S. Sprang for providing the coordinates of the G protein heterotrimer; B. Braun for communication of unpublished results and for assistance with the computer graphics; M. J. Redd for technical assistance; Ira Herskowitz, Cynthia Kenyon, and Andrew Murray for comments on the manuscript; members of the lab for useful discussions and advice. This work was supported by a grant from the National Institutes of Health (GM-37049) to A.D.J.

Table 2-1. Tup1 mutants are defective in repression of the α2-regulated reporter *mfa2:lacZ*. A *MATα tup1Δ mfa2:lacZ* strain was transformed with a wild-type or mutant *TUP1* plasmid and assayed for β-galactosidase activity. Allele designations in parentheses refer to the amino acid positions that are changed in the mutant Tup1 proteins. The level of repression conferred by the wild-type *TUP1* plasmid is incomplete relative to the level obtained with chromosomally expressed Tup1, possibly as a result of plasmid loss (see reference (99)).

Tup1 plasmid	β-galactosidase activity (units)
vector	170 ± 20
Tup1 (wild-type)	20 ± 6
Tup1 (C348K)	177 ± 20
Tup1 (Y445C)	57 ± 3
Tup1 (S448P)	168 ± 4
Tup1 (E463N)	115 ± 10
Tup1 (Y489H)	61 ± 20
Tup1 (Y580H)	99 ± 7
Tup1 (L634S)	86 ± 2
Tup1 (K650N)	135 ± 10
Tup1 (N673S)	174 ± 20
Tup1 (S674P)	139 ± 10
Tup1 (1676T)	41 ± 10
Tup1 (I676V)	62 ± 6

Table 2-2. Point mutations do not affect repression by Tup1-lexA. The Tup1-lexA expression plasmids were cotransformed with pJK1621 into either a *TUP1* or *tup1Δ* strain and the transformants were assayed for β-galactosidase activity. Reporter JK1621 is *cyc1:lacZ* with four lexA sites upstream of the UAS (46).

	β-galactosidase activity	
Tup1-lexA plasmid	*TUP1* strain	*tup1Δ* strain
vector	529 ± 80	129 ± 50
wild-type	17 ± 10	9 ± 3
C348K	20 ± 3	19 ± 4
S448P	32 ± 10	28 ± 20
Y489H	22 ± 6	n.d.*
Y580H	19 ± 8	29 ± 2
L634S	32 ± 13	n.d.
I676V	22 ± 10	n.d.

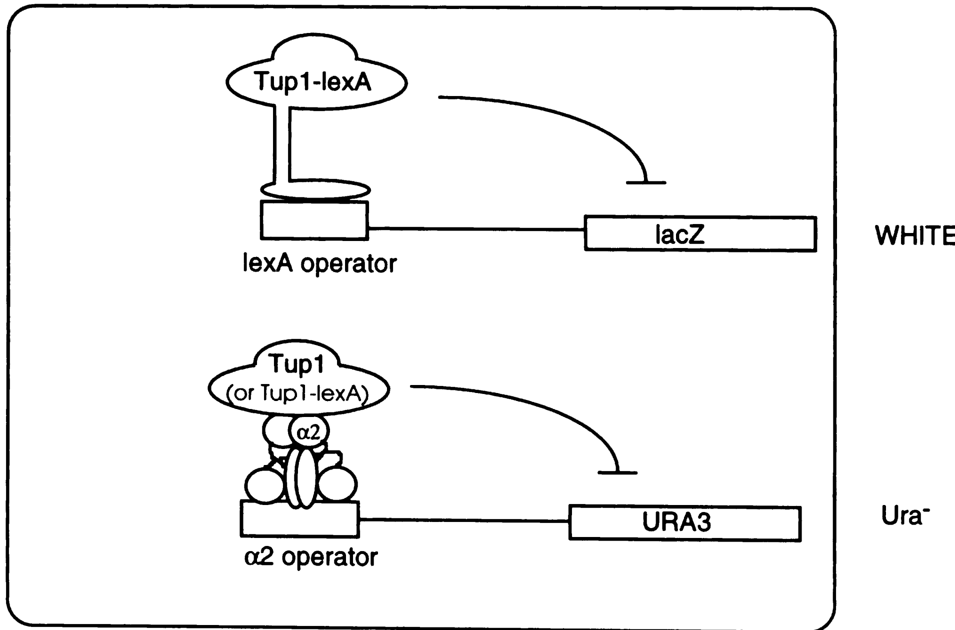
* n.d. = not determined

Table 2-3. Effect of Tup1 point mutations on repression of other Tup1/Ssn6-regulated reporters. KKY103 (*MATα tup1Δ*) carrying an *anb1:lacZ*, *suc2:lacZ*, or *rnr2:lacZ* reporter was transformed with a wild-type or mutant *TUP1* plasmid and assayed for β-galactosidase activity. The Δ255-713 mutant contains a complete deletion of the WD repeats and is known to be able to partially repress *ANB1* and *SUC2* (97). Relevant numbers from Table 2-1 are reproduced in the right-most column for ease of comparison.

	β-galactosidase activity			
TUP1 allele	*anb1:lacZ*	*suc2:lacZ*	*rnr2:lacZ*	*mfa2:lacZ*
vector	350 ± 60	130 ± 22	10.5 ± 2	170 ± 20
wild-type	18 ± 2	8 ± 1	2.6 ± 0.3	20 ± 6
C348K	107 ± 40	19 ± 2	6.0 ± 1	177 ± 20
S448P	49 ± 8	18 ± 3	8.3 ± 2	168 ± 4
Y489H	32 ± 5	14 ± 3	2.8 ± 0.2	61 ± 20
Y580H	54 ± 16	22 ± 6	4.5 ± 1	99 ± 7
L634S	66 ± 17	18 ± 3	4.6 ± 0.7	86 ± 2
I676V	46 ± 12	11 ± 3	4.8 ± 0.6	41 ± 10
Δ255-713	74 ± 12	22 ± 5	8.2 ± 1	176 ± 10

Figure 2-1. Screen for mutations in Tup1-lexA that affect interaction with α2. (A) Wild-type Tup1-lexA binds to the lexA operator and represses the *lacZ* reporter; either Tup1 or Tup1-lexA binds to α2 and represses the *URA3* reporter. Hence, *MATα TUP1* colonies expressing wild-type Tup1-lexA are white and Ura^-. (B) A Tup1-lexA mutant that cannot bind to α2 is able to repress from a lexA operator but interferes with α2-mediated repression, possibly by titrating some downstream repression component away from the α2. Thus, colonies expressing a mutant fusion are white and Ura^+. For simplicity, we have shown the mutant Tup1-lexA binding to endogenous Tup1 and forming heteromers that are incompetent for α2-binding, but the mutant could be titrating some other protein such as Ssn6. A Tup1-lexA fusion rather than Tup1 itself was used in order to screen against mutations that merely destabilize, unfold, or truncate Tup1 or affect its ability to interact with downstream components of the repression machinery since such mutations whould presumably cause derepression of the both the *lacZ* and *URA3* reporters.

A. Wild-type Tup1-lexA



B. Mutant Tup1-lexA

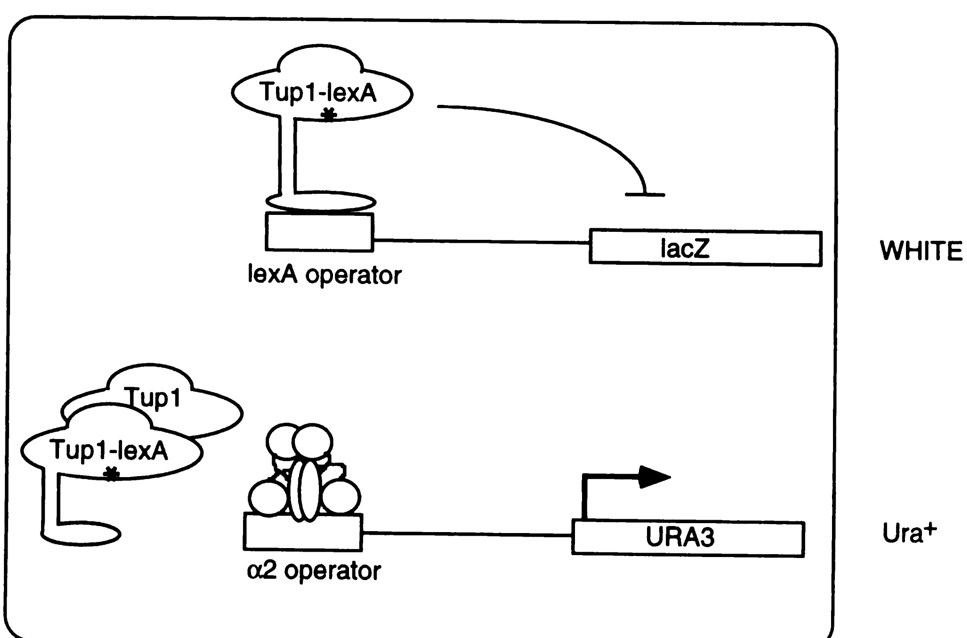


Figure 2-2. Binding of wild-type and mutant GST-Tup1 fusions to α2. Shown are Coomassie stained gels of fractions of a bacterial extract containing α2 that has been passed over a column containing glutathione-agarose beads bearing (**A**) GST-Tup1(wild-type), (**B**) GST-Tup1 (C348K), (**C**) GST-Tup1 (L634S), or (**D**) GST-Tup1 (I676V). The load is the same for all of the experiments and is only shown in panel A (L). The flowthrough fractions are labeled f1 through f4; the wash fractions, w1 through w4; the eluate, e. Depletion of α2 from the flowthrough fractions indicates binding to the column. Recovery of α2 in the high salt eluate is usually incomplete, making comparisons of the eluate fractions from different experiments difficult. The truncated form of α2 does not bind well to Tup1 and is included as a negative control to show that the α2-Tup1 interaction is specific.

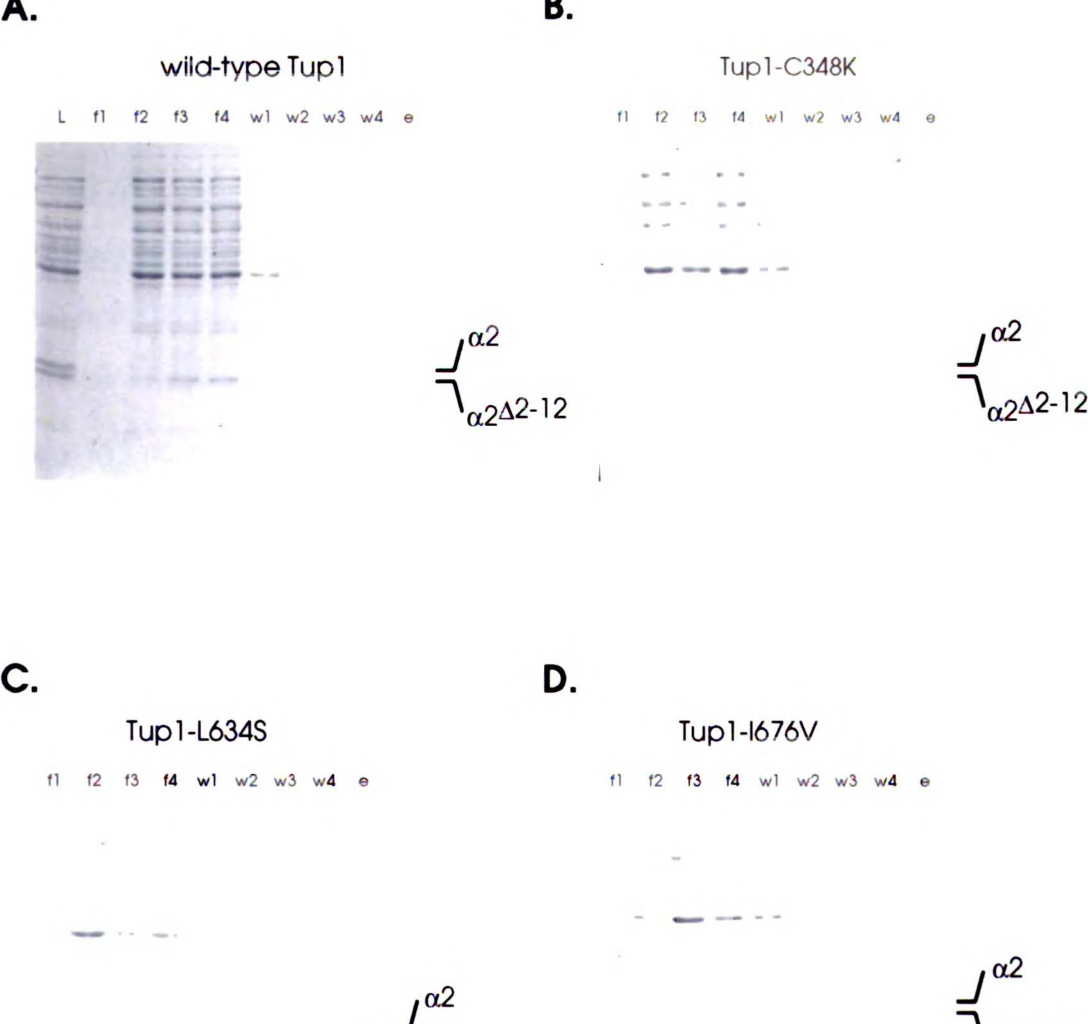

A.
wild-type Tup1
L f1 f2 f3 f4 w1 w2 w3 w4 e
α2
α2Δ2-12
B.
Tup1-C348K
f1 f2 f3 f4 w1 w2 w3 w4 e
α2
α2Δ2-12
C.
Tup1-L634S
f1 f2 f3 f4 w1 w2 w3 w4 e
α2
α2Δ2-12
D.
Tup1-I676V
f1 f2 f3 f4 w1 w2 w3 w4 e
α2
α2Δ2-12

Figure 3-3. Similarity between the region of Tup1 that binds $\alpha 2$ and the region of $G\beta$ that binds G_{α}. (**A**) Sequence of the Tup1 WD repeats, with the amino acids that are changed in mutants defective for $\alpha 2$ binding printed in bold type. The asterisk represents a stretch of seven amino acids that disrupts the spacing of WD7 and presumably forms a loop on the bottom surface of Tup1. (**B**) Sequence of the $G\beta$ WD repeats, with the amino acids that contact G_{α} printed in bold type (52, 88, 104). The seven rows in (A) and (B) are preceded by the positions of the amino-terminal residues of each repeat. The conserved WD (or WD-like) sequence at the end of each repeat is underlined. The solid lines above the repeats indicate the amino acids that form the four β-strands (labeled A through D) which make up each propeller blade in $G\beta$. The dotted lines below the repeats indicate the inter-strand loops that form the upper surface of $G\beta$. (**C**) Model for the structure of the WD repeats of Tup1. The backbone is drawn in white, using the coordinates for the structure of $G\beta$; the amino acids of $G\beta$ that are in the same position as the amino acids of Tup1 involved in $\alpha 2$-binding are highlighted in purple. (**D**) The structure of $G\beta$, with the backbone drawn in white and the amino acids that contact $G\alpha$ highlighted in green (52, 88, 104). Structures were drawn using Rasmol with coordinates provided by Stephen Sprang.

A. TUP1

	D strand		A strand	B strand	C strand

	Sequence
330	PREIDVELHKSLDH..TSVV**C**CVKFSNDGEYLATGC.NKTTQVYR
429	KDVENLNTSSSPSS..DL**Y**IR**S**VCFSPDGKFLATGAEDRLIRIWD
472	IENRKIVMILQGH..EQDI**Y**SLDYFPSGDKLVSGSGDRTVRIWD
516	TGQCSLTLSIEDGV..TTVAVSPG...DGKYIAAGSLDRAVRVWD
572	VERLDSENESGTGH..KDSV**Y**SVVFTRDGQSVVSGSLDRSVKLWN
616	TPNSGTCEVTYTGH..KDFV**L**SVATTQNDEYILSGS**K**DRGVLFWD
658	KKSGNPLLMLQGH..R**NS**V**I**SVAVANG*NVFATGSGDCKARIWK

D-A loop B-C loop

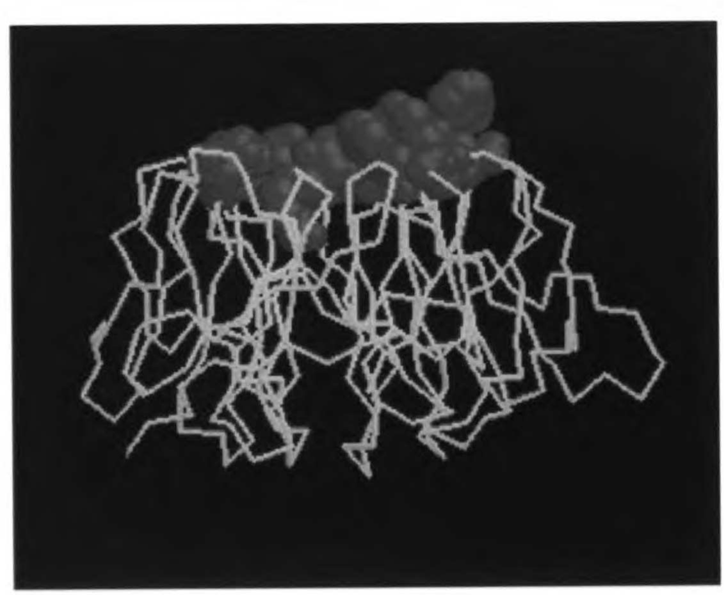
B. Gβ

	D strand		A strand	B strand	C strand

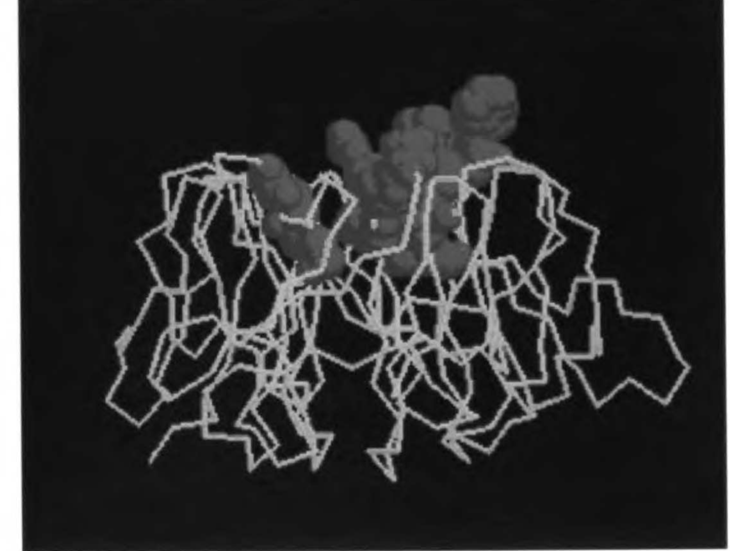
	Sequence
45	MRTRRTLRGH..LA**K**IYAMHWGTDSRLLLSAS**Q**DGKLIIWD
84	SYTTNKVHAIPLR..SS**W**VMTCAYAPSGNYVACGG**L**DNICSIYN
126	LKTREGNVRVSRELSGH..**T**G**Y**LSCCRFL.DDNQIVTSSG**D**TTCALWD
171	IETGQQTTTFTGH..TG**D**V**M**SLSLAPDTRLFVSGA**C**DASAKLWD
213	VREGMCRQTFTGH..ES**D**INAICFFPNGNAFATGS**D**DATCRLFD
255	LRADQELMTYSHDNIICGITSVSFSKSGRLLLAGYDDFNCNVWD
299	ALKADRAGVLAGH..DNRVSCLGVTDDGMAVATGS**W**DSFLKIWN

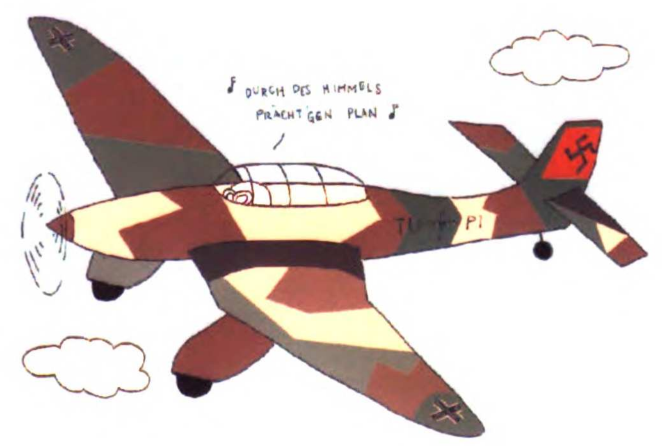
D-A loop B-C loop

C.



D.





APPENDICES TO CHAPTER TWO

Appendix F. Tup1-lexA represses transcription from a lexA operator

A lexA-Ssn6 fusion will repress transcription by 7- to 35-fold from lexA operators placed upstream of the *CYC1* UAS (46). This repression is decreased to about 3.5 -fold in a strain lacking *TUP1*, suggesting that Tup1 might act downstream of Ssn6 in the repression pathway. Initial attempts to test whether a lexA-Tup1 fusion could repress transcription from a lexA operator were unsuccessful in that the fusion did not complement a *TUP1* deletion for defects in growth, mating or repression of an *mfa2:lacZ* reporter, did not repress transcription from a lexA operator, did not interact with an Ssn6-Gal4 activation domain fusion by two-hybrid assay, and inhibited growth of both *TUP1* and *tup1Δ* strains (M.J. Redd and K. Komachi, unpublished observations). In addition, the fusion did not derepress an *mfa2:lacZ* fusion in wild-type strains, indicating that Tup1-lexA does not behave as a dominant negative allele of *TUP1*. Curiously, the poor growth of strains carrying the lexA-Tup1 fusion was suppressed by a plasmid bearing a TATA-binding-protein-GAL4 activation domain (TBP-Gal4) fusion, but the lexA-Tup1 and TBP-Gal4 fusions did not interact by two-hybrid assay.

Although Tzamarius and Struhl eventually constructed a lexA-Tup1 fusion that represses from a lexA site, the peculiar behavior of our lexA-Tup1 fusion persuaded us to fuse lexA to the C-terminal end of Tup1, creating a Tup1-lexA (as opposed to lexA-Tup1) fusion. Here we show that a Tup1-lexA fusion complements a *TUP1* deletion for repression of an **a**-specific gene and represses from a lexA sites positioned either upstream or downstream of a UAS. This repression does not require wild-type Tup1 but may be partially dependent on Ssn6. Smaller fragments of Tup1 fused to lexA repress to approximately the same degree as has been described for comparable versions of lexA-Tup1 (97).

In order to show that the Tup1-lexA fusion complements a *TUP1* deletion, we transformed KKY110 (*MATα tup1Δ mfa2:lacZ*) with a 2μm vector expressing Tup1, the Tup1-lexA fusion, or no protein . The Tup1 and Tup1-lexA expression plasmids both corrected the slow growth, clumpiness and sterility of KKY110, whereas the vector did not (data not shown). In addition, repression of the *mfa2:lacZ* reporter was restored by Tup1 and Tup1-lexA to approximately the same level (Table 1).

We then showed that the Tup1-lexA fusion is capable of repressing transcription from a lexA site by transforming the Tup1-lexA plasmid into a strain carrying a *cyc1:lacZ* reporter with lexA sites either upstream or downstream of the UAS and assaying the transformants for β-galactosidase activity. Both reporters were repressed by the Tup1-lexA fusion, indicating that the fusion is capable of 30-fold repression from upstream of the UAS and 140-fold repression from between the UAS and TATA (Table 2).

In order to determine whether or not repression by Tup1-lexA requires wild-type *TUP1* and *SSN6*, we transformed the Tup1-lexA plasmid into *tup1Δ* and *ssn6Δ* strains carrying the appropriate *cyc1:lacZ* reporters and assayed the transformants for β-galactosidase activity. The 20-fold repression by Tup1-lexA from upstream of the UAS in wild-type strains was decreased to 14-fold in a *tup1Δ* strain and to 3-fold in an *ssn6Δ* strain (Table 3). Hence, repression by Tup1-lexA does not require wild-type Tup1 but may require Ssn6 to some degree. Because *ssn6Δ* strains are sicker than wild-type or *tup1Δ* strains, though, it is possible that the loss of repression in the *ssn6Δ* strain was due to a nonspecific effect such as lower expression of Tup1-lexA.

Because mutations in genes encoding proteins associated with the RNA polymerase II holoenzyme affect Tup1/Ssn6-mediated repression (50, 102, 103), we also tested the ability of Tup1-lexA to repress in *srb10* and *srb8* strains and found that repression was not significantly decreased in either of the mutant strains (Table 4). The *srb8* strain used in these experiments, however, carries an allele that is partially suppressed by overexpression of *TUP1* (102). Since Tup1-lexA is being expressed from a 2 μm plasmid, it is possible

that the *srb8* phenotype is being suppressed and that the ability of Tup1-lexA to repress in this strain may not reflect an ability of the fusion to function in the absence of *SRB8*.

Finally, we attempted to identify fragments of Tup1 that are sufficient for repression when fused to lexA. Deletion analysis of lexA-Tup1 by Tzamarius and Struhl revealed that there are two nonoverlapping fragments of Tup1 that will repress transcription as lexA-Tup1 fusions: one spanning amino acids 1-200 and another spanning amino acids 288-713 (97). We showed that the Tup1-lexA fusion behaves in much the same manner as the lexA-Tup1 fusion by constructing a variety of deleted derivatives and testing them for their ability to repress from a lexA site (Figure 1). We also showed that both Tup1(1-253)-lexA and Tup1(254-713)-lexA are able to repress somewhat in the absence of *SSN6*, although repression in *ssn6Δ* strains is not as efficient as that which is observed in wild-type strains (Table 5).

In conclusion, a Tup1-lexA fusion is able to repress expression of a *cyc1:lacZ* reporter containing lexA sites, suggesting that Tup1 is transcriptional repressor. Tup1-lexA is able to repress in the absence of Ssn6, but the level of repression is greater in *SSN6* strains; thus, Ssn6 may play a role in repression beyond simply securing the interaction between Tup1 and DNA-binding proteins. Finally, in accordance with Tzamarius and Struhl, we have found that Tup1 appears to have two separate domains capable of repression when fused to lexA. Curiously, fusions containing less than seven WD repeats are expressed (M.J. Redd, unpublished data) but are unable to repress despite the presence of the full (1-253) repression domain, suggesting that WD repeats inhibit the repression domain unless the full septet is present. These results imply that the amino and carboxy termini of Tup1 somehow interact and modulate the net efficacy of the protein as a repressor.

Table F-1. Tup1-lexA complements a *tup1Δ* strain for repression of *mfa2:lacZ*. Yeast strain KKY110 (*MATα tup1Δ mfa2:lacZ*) was transformed with the indicated plasmids and assayed for β-galactosidase activity. Repression from the *TUP1* plasmids is incomplete compared to that obtained with chromosomally-expressed Tup1, possibly because of plasmid loss.

Plasmid	β-galactosidase activity
vector (pKK412)	128 ± 25
Tup1 (pKK448)	18 ± 5
Tup1-lexA (pKK631)	31 ± 4

Table F-2. Tup1-lexA represses from a lexA operator. Yeast strain 246-1-1 was cotransformed with either pCK30 or pAJ212 and either a vector or the Tup1-lexA expression plasmid and assayed for β-galactosidase activity. Plasmid pCK30 is a 2μm *cyc1:lacZ* reporter with one lexA operator between the UAS and TATA; pAJ212 is an integrating *cyc1:lacZ* reporter with four lexA operators upstream of the UAS.

reporter	plasmid	β-galactosidase activity	fold repression
pCK30	vector	300 ± 25	
pCK30	Tup1-lexA	2.1 ± 0.4	143
pAJ212	vector	40 ± 2	
pAJ212	Tup1-lexA	1.4 ± 0.2	29

Table F-3. Repression by Tup1-lexA does not require wild-type *TUP1* but may be partially dependent on *SSN6*. Yeast strains 246-1-1 (*TUP1 SSN6*), KKY103 (*tup1Δ SSN6*), and AJY159 (*TUP1 ssn6Δ9*) were cotransformed with either pCK30 or pAJ201 and either a vector or theTup1-lexA expression plasmid and assayed for β-galactosidase activity. Plasmid pAJ201 is a 2 μm *cyc1:lacZ* reporter with four lexA operators upstream of the UAS.

		β-galactosidase activity	
genotype	plasmid	pCK30	pAJ201
TUP1 SSN6	vector	896 ± 94	873 ± 120
	Tup1-lexA	19.8 ± 7	42 ± 11
tup1 SSN6	vector	233 ± 63	129 ± 52
	Tup1-lexA	8.7 ± 4	9 ± 3
TUP1 ssn6	vector	106 ± 22	105 ± 13
	Tup1-lexA	11 ± 6	42 ± 9

Table F-4. Repression by Tup1-lexA does not require *SRB8* or *SRB10*. Reporter plasmids pAJ212 or pCK30 were transformed into 246-1-1, MWY10, or MWY15 (103); the resulting strains were transformed with either a Tup1-lexA expressing plasmid (pKK546), or the vector (pKK361) and assayed for β-galactosidase activity.

			β-galactosidase activity	
strain	genotype	plasmid	pAJ212	pCK30
AJY82	*SRB10 SRB8*	vector	39.5 ± 2	300 ± 30
	SRB10 SRB8	Tup1-lexA	1.4 ± 0.2	2.1 ± 0.4
MWY10	*srb10 SRB8*	vector	10.2 ± 0.6	150 ± 20
	srb10 SRB8	Tup1-lexA	1.2 ± 0.01	2.1 ± 0.5
MWY15	*SRB10 srb8*	vector	20.2 ± 2	
	SRB10 srb8	Tup1-lexA	1.3 ± 0.2	

Figure F-1. Summary of Tup1-lexA and LexA-Tup1 fusions tested for their ability to repress *cyc1:lacZ* reporters containing lexA sites. Yeast strain EG123 was transformed with a Tup1-lexA plasmid and a reporter and assayed for β-galactosidase activity. In the leftmost column of the figure are graphic representations of the Tup1-lexA chimeras, with lexA in gray, Tup1 in white, the WD repeats as numbered boxes and deleted amino acids as a black line. The second column indicates the amino acids of Tup1 that are deleted or present in the fusions from the fusions, except in the case of the lexA-Tup1 fusion, where the amino acids present are indicated. The third and fourth columns display the β-galactosidase activity of strains carrying the indicated fusion and the reporter pCK30 and pAJ210, respectively.

plasmid	β-galactosidase activity pCK30	β-galactosidase activity pAJ201
vector	1110 ± 200	873 ± 120
Tup1-lexA	39 ± 10	42 ± 10
Tup1(Δ201-713)-lexA	9 ± 6	215 ± 100
Tup1(Δ333-431)-lexA	853 ± 130	
Tup1(Δ333-713)-lexA	544 ± 93	
Tup1(Δ670-713)-lexA	318 ± 44	
Tup1(Δ1-253)-lexA	8.5 ± 0.5	187 ± 40
vector	300 ± 30	
lexA-Tup1(282-340)	415 ± 70	

Table F-5. Tup1(1-713), Tup1(1-253)-lexA and Tup1(254-713)-lexA repress transcription in the absence of *SSN6*. AJY159 (*MATα ssn6Δ9*) was cotransformed with pCK30 and plasmids expressing Tup1(1-713)-lexA, Tup1(1-253)-lexA, Tup1(254-713)-lexA, or no fusion and assayed for β-galactosidase activity.

Tup1-lexA plasmid	β-galactosidase activity
vector	65.4 ± 9
Tup1(1-713)-lexA	12.6 ± 4
Tup1(1-253)-lexA	18.4 ± 13
Tup1(254-713)-lexA	15.8 ± 5

Appendix G. Dominance of the *TUP1* mutants is suppressed by overexpression of Ssn6

Overexpression of Tup1 mutants unable to bind to α2 causes derepression of the *mfa2:lacZ* reporter in *MATα TUP1* strains. We have found that deletion of the Ssn6-binding domain in the amino terminus of Tup1 destroys the ability of two of the mutants to derepress the *mfa2:lacZ* reporter (Table 1), suggesting that the mutant Tup1 proteins interfere with repression by binding to Ssn6 and preventing formation of wild-type Tup1/Ssn6 complexes . Since raising the concentration of Ssn6 in the cell should increase the levels of wild-type Tup1/Ssn6 complexes, we expected that overexpression of Ssn6 might suppress the dominance of the *TUP1* mutants. In order to test this idea, we transformed high copy *TUP1* or *SSN6* plasmids into *MATα mfa2:lacZ* strains carrying the mutant *TUP1* plasmids and assayed the transformants for β-galactosidase activity. In all of the strains tested, repression was restored by overexpression of either Tup1 or Ssn6 (Table2).

We also tested whether the repression defect of strains expressing only the mutant Tup1 proteins could be overcome by overexpression of Ssn6. KKY110 (*MATα tup1Δ mfa2:lacZ*) was cotransformed with a mutant *TUP1* plasmid and a high copy plasmid containing *TUP1* or *SSN6* and assayed for β-galactosidase activity. Overexpression of Ssn6 did not suppress the defect of either mutant (Table 3).

The Tup1 mutants were isolated on the basis of their ability to (1) derepress *mfa2:lacZ* and (2) repress as a lexA fusion. In principle, mutants defective in binding to either α2 or Ssn6 could have emerged from the screen. These results suggest that the dominant phenotype of the Tup1 mutants is due to sequestration of Ssn6 from wild-type Tup1, in which case the mutants must be able to bind Ssn6.

Table G-1. Deletion of the amino terminus of two of the Tup1 mutants destroys their ability to derepress *mfa2:lacZ*. Mutations K650N and N673S were introduced into Tup1(1-713) and Tup1(254-713) expression plasmids which were then transformed into SM1196 (*MAT*α *mfa2:lacZ*). The transformants were assayed for β-galactosidase activity by filter assay.

Tup1-expression plasmid	β-galactosidase activity
Tup1(1-713)-wild-type	white
Tup1(1-713)-K650N	blue
Tup1(1-713)-N673S	blue
Tup1(254-713)-wild-type	white
Tup1(254-713)-K650N	white
Tup1(1-713)-N673S	white

Table G-2. Dominance of the *TUP1* mutants is suppressed by overexpression of Tup1 or Ssn6. SM1196 (*MATα mfa2:lacZ*) was transformed with a mutant *TUP1* plasmid and a high copy plasmid carrying *TUP1* or *SSN6*, and the transformants were assayed for β-galactosidase activity.

TUP1 mutant	high copy plasmid	β-galactosidase activity
C348K	vector	37.5 ± 9
C348K	*TUP1*	8.4 ± 1
C348K	*SSN6*	15.9 ± 0.9
Y445C	vector	55.8 ± 7
Y445C	*TUP1*	9.0 ± 0.8
Y445C	*SSN6*	18.7 ± 1
Y489H	vector	14.2 ± 0.7
Y489H	*TUP1*	2.5 ± 0.6
Y489H	*SSN6*	2.5 ± 0.2
Y580H	vector	27.8 ± 1.5
Y580H	*TUP1*	7.0 ± 0.8
Y580H	*SSN6*	7.6 ± 0.5

Table 2. continued

L634S	vector	30.2 ± 3
L634S	*TUP1*	4.7 ± 0.6
L634S	*SSN6*	5.5 ± 0.5
I676T	vector	17.1 ± 2
I676T	*TUP1*	1.6 ± 0.5
I676T	*SSN6*	2.2 ± 0.5

Table G-3. The *TUP1* mutants are not suppressed by overexpression of Ssn6 in the absence of wild-type *TUP1*. KKY110 (*MATα tup1Δ mfa2:lacZ*) was cotransformed with a mutant *TUP1* plasmid and a high copy *TUP1* or *SSN6* plasmid and assayed for β-galactosidase activity.

TUP1 mutant	high copy plasmid	β-galactosidase activity
C348K	vector	150 ± 20
C348K	*TUP1*	9 ± 5
C348K	*SSN6*	113 ± 39
S448P	vector	155 ± 10
S448P	*TUP1*	6 ± 2
S448P	*SSN6*	113 ± 6

Appendix H. Fragments of Tup1 smaller than Tup1(254-713) bind to both $\alpha2$ and $\alpha2^{\Delta2-10}$

The interaction between a fragment of Tup1 containing amino acids 254 to 713 [Tup1(254-713)] and $\alpha2$ is sensitive to mutations in either $\alpha2$ or Tup1 that are thought to decrease binding in vivo. Tup1(254-713) contains all seven WD repeats as well as an upstream region that is conserved among functional Tup1 homologs from other fungi (B. Braun, unpublished observations). Here, we show that a fragment of Tup1 containing the seven WD repeats but lacking the conserved upstream region binds to both $\alpha2$ and $\alpha2^{\Delta2-10}$, a deleted version of $\alpha2$ that does not bind to Tup1(254-713). In addition, we show that although fragments of Tup1 as small as a single WD repeat will bind to $\alpha2$, the interaction is debilitated by neither a deletion of the $\alpha2$ amino terminus nor by point mutations in Tup1 that decrease binding to $\alpha2$ when in the context of Tup1(254-713).

We tested the ability of various Tup1 derivatives to bind to wild-type and mutant $\alpha2$ by passing bacterial extracts containing $\alpha2$ and $\alpha2^{\Delta2-10}$ over columns bearing different fragments of Tup1 fused to GST. Whereas GST-Tup1(254-713) bound only $\alpha2$, Tup1(340-713) bound both $\alpha2$ and $\alpha2^{\Delta2-10}$ (Figure 1). GST-Tup1(439-713), GST-Tup1(526-713), GST-Tup1(572-713), and GST-Tup1(626-713) also bound both $\alpha2$ and $\alpha2^{\Delta2-10}$ in much the same manner as did GST-Tup1(340-713) (data not shown).

We noted previously that WD2 of Tup1 will bind to both $\alpha2$ and $\alpha2^{\Delta2-10}$ and that an aspartate to cysteine mutation in the WD does not affect the interaction. In order to further characterize the interaction of $\alpha2$ with a single WD repeat, we introduced a variety of deletions and point mutations into GST-WD2 and tested the resulting constructs for their ability to bind to $\alpha2$ and $\alpha2^{\Delta2-10}$. A GST-WD2 fragment lacking the C-terminal WD residues [GST-WD2(ΔWD)] was able to bind to both $\alpha2$ and $\alpha2^{\Delta2-10}$, but deletion of the amino terminus of the repeat or further deletion of the carboxy terminus destroyed the ability of the single repeat to interact with $\alpha2$ (Figure 2). Mutations Y445C, E463N, and

S448P, which decrease binding of Tup1(254-713), had no discernible effect on the interaction between WD2 and α2 (data not shown).

In short, none of the fragments of Tup1 smaller than Tup1(254-713) were able to distinguish between α2 and $\alpha2^{\Delta2\text{-}10}$, nor were any of the smaller fragments affected by point mutations in the WD repeats (summarized in Figure 3). Given that a single WD repeat does not comprise a discrete stuctural unit (52, 88, 104), it is perhaps surprising that the WD2 and WD6-7 proteins bound to α2 at all, and one possiblity is that the interaction between α2 and all of the fragments smaller than Tup1(254-713) is nonspecific and artifactual. Since it is difficult to compare the strength of the interaction between α2 and different GST fusions, another possibility is that the binding of α2 to the smaller fragments is weaker than the column experiments would indicate; perhaps the single WD repeat fusions are able to self-associate and form a propeller-like structure that α2 is able to recognize, albeit weakly. If such is the case, we surmise that Tup1(254-713) must contain a "masking" domain that prevents this weak non-specific interaction between the WD repeats and the α2 carboxy terminus since Tup1(254-713) does not bind to α2Δ2-10 and mutant versions of Tup1(254-713) do not bind to α2.

Figure H-1. GST-Tup1(340-713) binds to both $\alpha 2$ and $\alpha 2^{\Delta 2\text{-}10}$ in vitro. Coomassie stained gels of fractions of a bacterial extract containing $\alpha 2$ and $\alpha 2^{\Delta 2\text{-}10}$ that has been passed over columns containing glutathione agarose beads bearing GST-Tup1(254-713) (left panel) or GST-Tup1(340-713) (right panel). The columns were prepared and run as described in Chapter One. The load is the same for both columns and is shown only in the left panel. The flowthrough fractions are labeled F1 through 4; the wash fractions, W1 through 4; the eluate fraction, E.

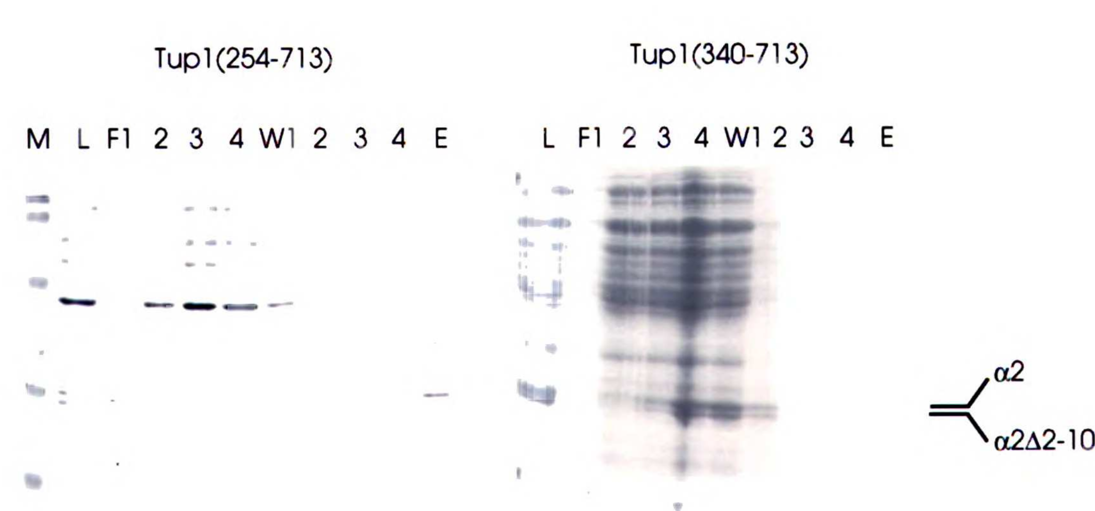
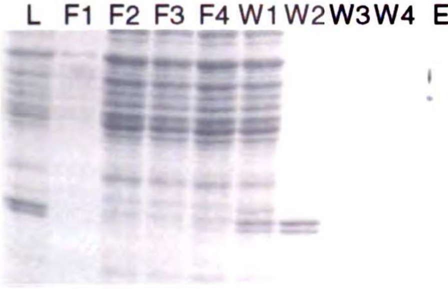

Tup1(254-713)
Tup1(340-713)
M L F1 2 3 4 W1 2 3 4 E
L F1 2 3 4 W1 2 3 4 E
α2
α2Δ2-10

Figure H-2. Deletions of GST-WD2 disrupt binding of both $\alpha 2$ and $\alpha 2^{\Delta 2\text{-}10}$. Coomassie stained gels of fractions of a bacterial extract containing $\alpha 2$ and $\alpha 2^{\Delta 2\text{-}10}$ that has been passed over columns containing glutathione agarose beads bearing (**A**) GST-WD2 (wild-type), (**B**) GST-WD2 (ΔWD), (**C**) GST-WD2 (ΔNT), or (**D**) GST-WD2 (ΔCT). The columns were prepared and run as described in Chapter One. The load is the same for all columns and is shown only in (A). The flowthrough fractions are labeled F1 through F4; the wash fractions, W1 through W4; the eluate fraction, E.

A.

GST-WD2 (wild-type)



B.

GST-WD2 (ΔWD)

F F F F W W W3 W E

α2

α2Δ2-10

C.

GST-WD2 (ΔNT)

F F F F W W W W E
1 2 3 4 1 2 3 4

D.

GST-WD2 (ΔCT)

F F F F W W W3 W E
1 2 3 4 1 2 4

α2

α2Δ2-10

Figure H-3. Summary of GST-Tup1 fusions tested for their ability to bind to $\alpha 2$ by the column assay used in Figures 1 and 2. On the left are graphic depictions of the GST-Tup1 fusions with GST in gray, Tup1 in white, and the WD repeats as numbered boxes. Binding to $\alpha 2$ or $\alpha 2^{\Delta 2\text{-}10}$ is as indicated in the columns on the right.

Construct	Tup1 residues	mutation	α2	$\alpha2^{\Delta 2\text{-}10}$
GST 1 2 3 4 5 6 7	254-713		+	-
GST 1 2 3 4 5 6 7	340-713		+	+
GST 2 3 4 5 6 7	439-713		+	+
GST 4 5 6 7	526-713		+	+
GST 5 6 7	572-713		+	+
GST 6 7	626-713		+	+
GST 6	626-659		-	-
GST 7	668-713		-	-
GST 1 2 3 4 5 6	254-659		+	+
GST 1 2	254-471		+	+
GST 6 7	(254-340) + (668-713)		+	+
GST	254-340		-	-
GST 1 2 3 4 5 6 7*	254-713	N673S	-	-
GST 1 2 3 4 5 6* 7	254-713	K650N	-	-
GST 6* 7	626-713	N673S	+	+
GST 6 7*	626-713	K650N	+	+
GST 2	440-471		+	+
GST 2 (ΔWD)	440-469		+	+
GST 2 (ΔNT)	451-471		-	-
GST 2 (ΔCT)	440-465		-	-
GST 2	440-471	Y445C	+	+
GST 2	440-471	E463N	+	+
GST 2	440-471	S448P	+	+

Appendix I. Tup1(363-713)-N673S, F632S causes derepression of *mfa2:lacZ* when overexpressed

The ability of Tup1(363-713) to restore mating to a *MATα tup1Δ ssn6Δ* strain suggested that a transcriptional repression domain resides in this C-terminal fragment of Tup1. We reasoned that mutants having defects in this domain would be able to bind to α2 but unable to repress transcription and hence would have a dominant phenotype. In an attempt to isolate such mutants, we transformed yeast strain SM1196 (*MATα mfa2:lacZ*) with a randomly mutagenized plasmid expressing Tup1(363-713) and screened the transformants for β-galactosidase activity by filter assay. Plasmid DNA was isolated from blue colonies and retransformed into SM1196. Of 18,000 transformants screened, 34 colonies were blue by filter assay, and 1 of these blue colonies yielded a plasmid that derepressed the *mfa2:lacZ* reporter when retransformed into SM1196. This plasmid was sequenced and found to contain two mutations causing the amino acid substitutions F632S and N673S.

In order to distinguish which mutation was responsible for the phenotype, we introduced the single mutations into the Tup1(363-713) expression plasmid. In addition, we introduced the single and double mutations into the Tup1(1-713) and Tup1(254-713) expression plasmids. All of the resulting plasmids were then transformed into SM1196, and the transformants were assayed for β-galactosidase activity by filter assay. None of the mutations were dominant within the context of Tup1(254-713); both N673S and N673S/F632S were dominant within the context of Tup1(1-713); only the double mutant was dominant within the context of Tup1(363-713) (Table 1).

Since the dominance of many *TUP1* mutants is suppressed by overexpression of Ssn6, we examined whether the derepression of *mfa2:lacZ* in strains carrying the Tup1(363-713)-F632S, N673S plasmid was alleviated by overexpression of Ssn6 and

found that the dominance of Tup1(1-713)-F362S, N673S but not of Tup1(363-713)-F632S, N673S was suppressed by a high copy SSN6 plasmid (Table 2).

We are presently unable to explain the behavior of the Tup1(363-713) mutants. The simplest explanation--that amino acid residues 632 and 673 both lie in the repression domain and must both be mutated to cause a defect--is contradicted by the observation that the N673S mutation impairs binding of Tup1 to $\alpha2$ in vitro and by the premise that a Tup1 mutant that can neither bind $\alpha2$ nor interact with the repression machinery should not be dominant. However, since binding of $\alpha2$ to fragments of Tup1 smaller than Tup1(254-713) appears to be unaffected by mutations in the WD repeats, it is also possible that the N673S mutation does not prevent Tup1(363-713) from binding $\alpha2$ in vivo and that the combined mutations actually improve binding of the shorter fragment to $\alpha2$. Since Tup1(363-713) is not as effective a repressor as full-length Tup1, the double mutant would cause derepression by displacing full-length Tup1; wild-type Tup1(363-713) would not cause derepression because it lacks the Ssn6-binding domain and thus does not bind $\alpha2$ as well as does full length Tup1. This explanation is somewhat appealing since residue 632 is predicted to lie on the flat surface of the Tup1 propeller where other residues thought to be involved in $\alpha2$ binding lie.

Table I-1. Tup1(254-713)-F632S, N673S derepresses *mfa2:lacZ*. Yeast strain SM1196 (*MATα mfa2:lacZ*) was transformed with plasmids expressing Tup1(1-713), Tup1(254-713), Tup1(363-713), or mutant derivatives thereof and assayed for β-galactosidase activity by filter assay. Since expression of the C-terminal fragments of Tup1 is driven by the *GAL10* promoter, strains were grown on plates containing galactose as the carbon source.

Tup1 fragment	mutation(s)	color by filter assay
Tup1(1-713)	none (wild-type)	white
Tup1(1-713)	F632S	white
Tup1(1-713)	N673S	blue
Tup1(1-713)	F632S, N673S	blue
Tup1(254-713)	none (wild-type)	white
Tup1(254-713)	F632S	white
Tup1(254-713)	N673S	white
Tup1(254-713)	F632S, N673S	white
Tup1(363-713)	none (wild-type)	white
Tup1(363-713)	F632S	white
Tup1(363-713)	N673S	white
Tup1(363-713)	F632S, N673S	blue

Table I-2. Dominance of *TUP1*-(363-713)-F632S, N673S is not suppressed by overexpression of Ssn6. SM1196 (*MATα mfa2:lacZ*) was cotransformed with a plasmid expressing Tup1(1-713)-F632S, N673S or Tup1(363-713)-F632S, N673S and a 2 μm plasmid bearing *TUP1* or *SSN6* and assayed for β-galactosidase activity by filter assay.

TUP1 plasmid	2 μm plasmid	color by filter assay
TUP1(1-713)-F632S, N673S	vector	blue
TUP1(1-713)-F632S, N673S	*TUP1*	white
TUP1(1-713)-F632S, N673S	*SSN6*	white
TUP1(1-713)-F632S, N673S	*TUP1* + *SSN6*	white
TUP1(363-713)-F632S, N673S	vector	blue
TUP1(363-713)-F632S, N673S	*TUP1*	white
TUP1(363-713)-F632S, N673S	*SSN6*	blue
TUP1(363-713)-F632S, N673S	*TUP1* + *SSN6*	white

Appendix J. Ste4 can interact with $\alpha 2$ in vitro but does not appear to do so in vivo

The ability of $\alpha 2$ to bind nonspecifically to the WD repeats of Tup1 prompted us to investigate whether or not $\alpha 2$ can bind to a functionally unrelated protein containing WD repeats with little homology to those in Tup1. For this purpose, we chose to examine the interaction between $\alpha 2$ and Ste4, the β subunit of the yeast heterotrimeric G protein involved in pheromone response (105), and found that Ste4 will bind to $\alpha 2$ in vitro. This interaction differs from the Tup1-$\alpha 2$ interaction in that it does not require the amino terminus of $\alpha 2$. For reasons discussed below, we then looked for evidence of an interaction between $\alpha 2$ and Ste4 in vivo but found none.

In order to determine whether or not $\alpha 2$ can bind to Ste4, we passed a bacterial extract containing $\alpha 2$ and $\alpha 2^{\Delta 2\text{-}10}$ over columns bearing either GST-Tup1(254-713) or GST-Ste4. Whereas GST-Tup1(254-713) bound only $\alpha 2$, GST-Ste4 bound both $\alpha 2$ and $\alpha 2^{\Delta 2\text{-}10}$ (Figure 1). When the columns were loaded and washed at 200 mM KCl instead of 50 mM KCl, the interaction between GST-Ste4 and both $\alpha 2$ and $\alpha 2^{\Delta 2\text{-}10}$ was weakened, whereas the interaction between GST-Tup1(254-713) and $\alpha 2$ was unaffected (data not shown). Hence, Ste4 can bind to $\alpha 2$ in vitro, but the interaction does not show the same specificity or stability to salt as does the Tup1-$\alpha 2$ interaction.

Two observations suggested that $\alpha 2$ might be able to interact with Ste4 in vivo as well. First, overexpression of $\alpha 2$ suppresses the lethality caused by either deletion of *GPA1* (the gene encoding the G_{α} subunit) (89) or overexpression of *STE4* (data not shown). In the pheromone response pathway, Gpa1 prevents $G\beta\gamma$ from stimulating cell cycle arrest in the absence of pheromone (10). Deletion of *GPA1* or overexpression of Ste4 therefore leads to constitutive cell cycle arrest and death (14, 65, 106). We postulated that $\alpha 2$ might suppress this lethality by binding to Ste4 and mimicking the inhibitory effect of Gpa1. The second observation suggesting an in vivo interaction between $\alpha 2$ and Ste4 is

that a chimera of the N-terminal amino acids of α2 fused to lacZ is mislocalized to the cytoplasm and is lethal to **a** or α but not **a**/α cells when overexpressed . One possibility is that this α2-lacZ fusion binds to Ste4 and disrupts its interaction with Gpa1 but is unable to prevent Ste4 from activating cell cycle arrest. Since the components of the signal transduction pathway are repressed in a/α cells, the α2-lacZ fusion would only be lethal in the haploid cell types.

In order to address whether the suppression of Ste4 overexpression by α2 is due to an α2-Ste4 interaction, we tested the ability of two α2 mutants to restore growth to a strain carrying a *STE4* plasmid. The mutant α2Δ188 is defective for binding to **a**1/α2 sites but binds to and represses from α2/Mcm1 operators (58); the mutant α2-H3-3 is defective for binding to α2/Mcm1 sites but binds to and represses from **a**1/α2 operators (99). Since both of these mutants are capable of repressing from sites that they are able to occupy, both presumably are able to interact with Tup1 and are expected to be able to interact with Ste4 in vitro. However, the mutant α2-H3-3 is unable to suppress the lethality of Ste4 overexpression (Table 1). Although these results do not prove that α2 is not binding to and inhibiting excess Ste4, they do suggest that the suppression brought about by α2 overexpression involves repression of an unknown gene whose upstream regulatory sequences contain a weakened α2/Mcm1 operator.

In order to test whether the toxicity of the α2-lacZ fusion involves the signal transduction pathway, we examined the effect of the fusion in a strain lacking *STE4*. If the α2-lacZ fusion kills cells by releasing Ste4 from its inhibitory association with Gpa1, then strains lacking *STE4* should be immune to the toxic effects of the α2-lacZ fusion. We transformed an α2-lacZ/*LEU2* 2μm plasmid or a *LEU2* 2μm vector into KKY161 (*MATa/MATα ste4/STE4 leu2/leu2*), sporulated the transformants, and dissected tetrads onto either rich plates (YEPD plates) or plates lacking leucine (-Leu plates). Four-spored tetrads were recovered from the vector-transformed strain on both YEPD and -Leu plates and from the α2-lacZ fusion-transformed strain on YEPD plates. In contrast, no spores

were recovered from the α2-lacZ fusion-transformed strain on -Leu plates, indicating that the plasmid is lethal to both *STE4* and *ste4* haploids (Table 2). All of the four-spored tetrads from the α2-lacZ fusion-transformed strain that grew on YEPD plates contained 2 fertile spores and 2 sterile spores, and all of the colonies were Leu^-, indicating loss of the α2-lacZ fusion plasmid. Hence, the toxic effect of the α2-lacZ fusion does not require *STE4* and probably does not involve an α2-Ste4 interaction.

In order to beat a dead horse, we tested whether α2 and Ste4 or Tup1 and Gpa1 could interact in two-hybrid assays (18). All of the Ste4 fusions were able to interact with the Gpa1 fusions, but no interaction could be detected between α2 and Tup1, α2 and Ste4, or Gpa1 and Tup1 (Figure 1). In addition, we tested whether a variety of truncated and chimeric Tup1 derivatives could activate expression of *fus1:lacZ*, a reporter whose transcription is induced by the signal transduction pathway, or whether a variety of chimeric Ste4 derivatives could affect regulation of *mfa2:lacZ*. As expected, the *STE4* plasmid activated *fus1:lacZ*; in contrast, the Tup1 derivatives had no effect on *fus1:lacZ* expression (Figure 2). Likewise, the Ste4 derivatives neither restored repression of *mfa2:lacZ* in a *tup1Δ* strain nor interfered with repression of *mfa2:lacZ* in a *TUP1* strain (Figure 3).

In summary, we have found no evidence for an interaction between α2 and Ste4 in vivo. Although the two proteins will bind to each other in vitro, the interaction does not require the amino terminus of α2 and may simply be an artifact. Another possibility, however, is that the in vitro interaction between α2 and Ste4--and between α2 and subfragments of Tup1(254-713)--may reflect an ability of α2 to recognize some feature conserved among WD repeats in general.

Figure J-1. GST-Ste4 binds to $\alpha2$ and $\alpha2^{\Delta2-10}$ in vitro. Coomassie stained gels of fractions of a bacterial extract containing $\alpha2$ and $\alpha2^{\Delta2-10}$ that has been passed over columns containing glutathione agarose beads bearing either GST-Tup1(254-713) (top) or GST-Ste4 (bottom). The columns were prepared and run as described in Chapter One. The load (L) is the same for both columns; the flowthrough fractions are labelled F1 through F4; the wash fractions, W1 through W4; the eluate fractions, E.

Table 2. The α2-lacZ fusion is toxic to both *ste4* and *STE4* haploids.

Plasmid	Medium	# of tetrads dissected	# of 4-spored tetrads
vector	YEPD	14	12
α2-lacZ	YEPD	14	11
vector	-Leu	14	10
α2-lacZ	-Leu	56	1

TableJ-1. Ste4 overexpression is not suppressed by α2 mutants unable to bind DNA.

STE4 plasmid	α2 plasmid	growth
vector	vector	+++
vector	*MATα2*	+++
vector	*MATα2*$^{\Delta 188\text{-}210}$	+++
vector	*MATα2-H3-3*	+++
STE4	vector	+/-
STE4	*MATα2*	+++
STE4	*MATα2*$^{\Delta 188\text{-}210}$	+++
STE4	*MATα2-H3-3*	+/-

GST-Tup1(254-713)

L F1 F2 F3 F4 W1 W2 W3 W4 E

α2

α2Δ2-10

GST-Ste4

F1 F2 F3 F4 W1 W2 W3 W4 E

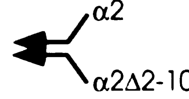
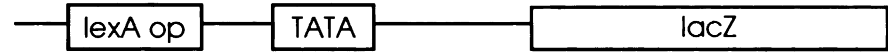


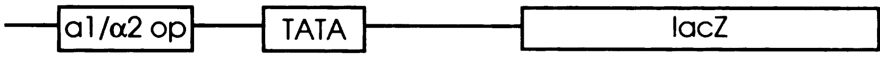
Figure J-2. Tup1 does not interact with Gpa1, nor does α2 interact with Ste4 in two-hybrid type assays. (**A**) Yeast strain CTY10-5d (Stan Fields), which contains a *cyc1:lacZ* reporter in which the UAS has been replaced by four lexA operators, was transformed with a lexA fusion plasmid and a Gal4 activation domain plasmid and assayed for β-galactosidase activity by filter assay. Blueness by filter assay indicates activation of the reporter. (**B**) Yeast strain AJY87 (*MATa/MATα*) or KKY104 (*MATa/MATα tup1Δ/tup1Δ*)was transformed with the reporter pCG21 and the indicated Gal4 activation domain fusion plasmids and assayed for β-galactosidase assays by filter assay. pCG21 was provided by Caroline Goutte and is a *cyc1:lacZ* reporter in which the UAS has been replaced with an **a**1/α2 operator. (**C**) Yeast strain 246-1-1 (*MATα*) or KKY103 (*MATα tup1Δ*) was transformed with the reporter pAJ8 and the indicated Gal4-activation domain fusion plasmids and assayed for β-galactosidase activity by filter assay. pAJ8 is a *cyc1:lacZ* reporter in which the UAS has been replaced with an α2/Mcm1 operator.

REPORTER



LexA fusion	GAL4 activation domain fusion	color by filter assay
LexA-GPA1	GAL4AD-STE4(1-423)	blue
LexA-GPA1	GAL4AD-STE4(84-423)	blue
LexA-GPA1	GAL4AD-TUP1 (340-713)	white
LexA-GPA1	GAL4AD	white
LexA-STE4(1-423)	GAL4AD-GPA1	blue
LexA-STE4(1-423)	α2-GAL4AD	white
LexA-STE4(1-423)	GAL4AD	white
LexA-STE4 (84-423)	GAL4AD-GPA1	blue
LexA-STE4 (84-423)	α2-GAL4AD	white
LexA-STE4 (84-423)	GAL4AD	white
Tup1(1-713)-lexA	GAL4AD-GPA1	blue
Tup1(1-713)-lexA	α2-GAL4AD	white
Tup1(1-713)-lexA	GAL4AD	white

REPORTER



genotype	GAL4 activation domain fusion	color by filter assay
a/α TUP1/TUP1	GAL4 AD-TUP1(340-713)	white
a/α TUP1/TUP1	GAL4 AD-STE4	white
a/α TUP1/TUP1	GAL4 AD	white
a/α tup1Δ/tup1Δ	GAL4 AD-TUP1(340-713)	white
a/α tup1Δ/tup1Δ	GAL4 AD-STE4	white
a/α tup1Δ/tup1Δ	GAL4 AD	white

REPORTER

genotype	GAL4 activation domain fusion	color by filter assay
α TUP1	GAL4 AD-TUP1(340-713)	white
α TUP1	GAL4 AD-STE4	white
α TUP1	GAL4 AD	white
α tup1Δ	GAL4 AD-TUP1(340-713)	light blue
α tup1Δ	GAL4 AD-STE4	light blue
α tup1Δ	GAL4 AD	light blue

Figure J-3. WD repeats of Tup1 and Ste4 are not interchangeable in vivo. The Tup1/Ste4 chimeras illustrated in the left-hand column were tested for their ability to complement the mating defect of a *ste4* mutant, to induce the signal-transduction-pathway-regulated reporter *fus1:lacZ* in wild-type cells in the absence of pheromone, to complement the mating defect of a *tup1Δ* mutant, and to cause derepression of *mfa2:lacZ* in wild-type strains.

Construct	STE4 function	fus1:lacZ induction	TUP1 function	mfa2:lacZ derepression
Tup1(1-713)	-	-	+	-
Ste4(1-423)	+	+	-	-
	-	-	-	-
	-	-	-	-
	+	nd	-	+
	-	nd	-	+
	-	nd	-	+
	+	nd	-	+
	+	+	-	-
Tup1(254-713)	-	-	+	-
Tup1(363-713)	-	+/-	+/-	-
Tup1(1-707)	-	-	+	-
Tup1(254-707)	-	-	+	-
Tup1(363-707)	-	-	+/-	-

Moral #1: Possibly it could have been worse; probably it could not (79). When I started this work, I wanted to know how α2 actively represses transcription, and in a sense, I got an answer: α2 represses by binding to Tup1. However, since Tup1 seems to be the actual repressor and since nothing I've done addresses how Tup1 interferes with the transcription of the genes to which it is recruited, I'm basically back where I started.

Moral #2: In this world, that which is square is not round (72) Although I can offer no pearls of wisdom regarding the function of Tup1, I did collect a few pebbles regarding its structure. To be fair, I doubt that the mutations I isolated would have allowed me to fold Tup1 into a propeller without divine intervention, chemical inspiration, or the publication of the structure of G_{β}. I also imagine that most people need no further evidence than the G_{β} structure to be convinced that all WD proteins are folded into propellers. Still, the only way to know for sure what Tup1 looks like is to solve its structure, and until such data is available, the mutations are a fairly good indication that Tup1's molecular origami mimics G_{β}'s.

Moral #3: The hole is greater than the sum of the parts. The realization that the Tup1 C-terminus is a donut frosted on one side by α2 is by no means the end of the story, since the WD repeats alone are insufficient for strong repression. That the Tup1(363-713) fragment containing only WD repeats is able to repress at all suggests that this fragment is able to fold into a structure that binds α2; but the 90-amino-acid fragment upstream of the presumptive propeller clearly contributes to repression and/or α2 binding since Tup1(254-713) represses quite well in comparison to Tup1(363-713). Furthermore, the fragment containing only WD repeats binds both α2 and $\alpha2^{\Delta2\text{-}10}$ in vitro, whereas Tup1(254-713) binds only α2, suggesting that the amino acids from 254 to 363 are involved in both repression of transcription and inhibition of nonspecific binding of the

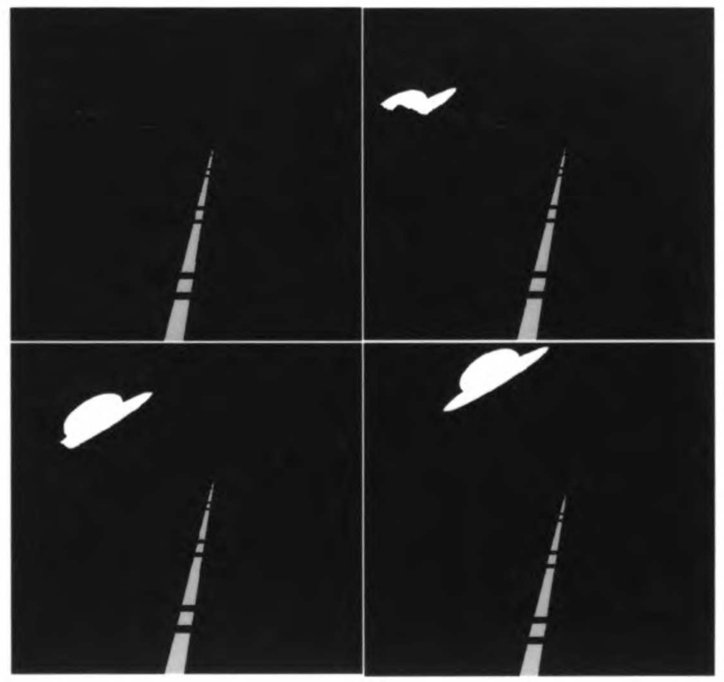
WD repeats to α2. What does this mean? Quite possibly, nothing, but a slightly more interesting possibility is that α2 binding induces some sort of conformational change in Tup1 which enhances its efficacy as a repressor. Such a mechanism would presumably prevent Tup1 from interacting well with its downstream targets until after it has been recruited by a DNA-binding protein.

Moral #4: It ain't over 'til the fat lady sings. The big question that remains, of course, is how Tup1 represses transcription, and although I personally feel no desire to get into it, at UCSF the traditional way to wrap things up is to hallucinate over models and prophesy the course of future research. The three basic models of active repression--interference with activators, inhibition of the basal transcription machinery, and restructuring of chromatin--have been the subject of many recent reviews, both excellent and otherwise (12, 30, 40, 76), which readers famished for further details are advised to devour at their own risk. The three mechanisms are not mutually exclusive, and none has been ruled out entirely for Tup1.

Although the accepted dogma seems to be that Tup1 represses by interacting with both the general transcription machinery and nucleosomes, the evidence for these assertions is still indirect. For instance, α2 will inhibit both activated and basal transcription 3- to 4-fold in a crude in vitro system that presumably lacks nucleosomes; but how this repression takes place and whether it would be stronger in the presence of nucleosomes are both unknown. Tup1 can be made to interact directly with histones H3 and H4 in vitro (16); but this binding may be a reflection of α2/Tup1's supposed ability to position nucleosomes (77, 83), a phenomenon which does not correlate well with repression (see Appendix O and reference(78)). Mutations in subunits of the holoenzyme or in histone H3 will partially derepress Tup1-regulated genes (27, 50, 103), but such mutations are pleiotropic and may be affecting repression indirectly. Sorting out which gene products are directly involved in repression will require an in vitro system reconstituted from purified components.

In addition, the Tup1/Ssn6 complex is quite large (73, 98), and there is, as of yet, no version of full-length Tup1 that fails to repress transcription once recruited to the DNA. So Tup1 may also turn out to be a passive repressor, which would amuse me to no end. After all, if a decade here has taught me anything, it's taught me never to underestimate repressors that inhibit just by showing up for work.

So, as Homer Simpson would say, "Is that one fat enough for you, son?" (100)

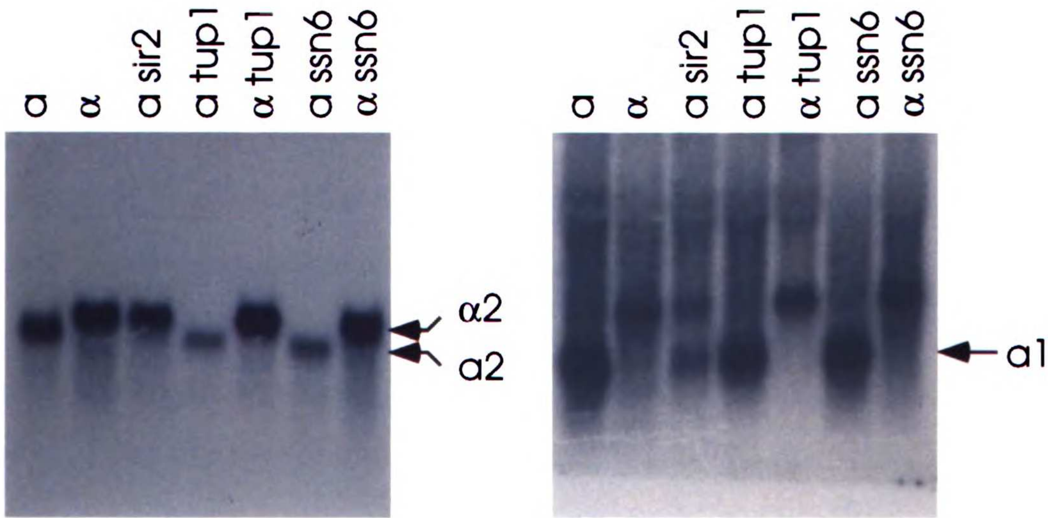


MISCELLANEOUS APPENDICES

Appendix K. *TUP1* and *SSN6* are not required for silencing of the mating-type loci

Most of the early genetic screens and selections for mutants defective in silencing of the mating-type loci required that the mutants be proficient for α2-mediated repression and therefore would have failed to identify genes involved in both silencing and repression (reviewed in (53)). Because the mating of ***MATa*** strains is not significantly affected by mutations in *TUP1* and *SSN6*, it has been assumed that these two genes are not required for silencing *HMLα*. Given that *HMLα* is the more easily derepressed of the two silent loci, it seems unlikely that *TUP1* and *SSN6* are required for silencing of ***HMRa***. However, any silencing defect in *MATα tup1* or *MATα ssn6* strains would be undetectable by mating assays, since such strains are already rendered sterile by their inability to repress the **a**-specific genes. In order to show that silencing is indeed intact in *tup1* and *ssn6* mutants, we performed Northern blots on *tup1Δ* and *ssn6Δ* strains of both mating types and found that neither the *HMLα* locus in mutant ***MATa*** strains nor the ***HMRa*** locus in mutant *MATα* strains was derepressed (Figure 1), indicating that *TUP1* and *SSN6* do not play a significant role in silencing.

Figure K-1. Northern blot of *tup1Δ* and *ssn6Δ* strains probed for *MATα2* or *MATa1* RNA. RNA was extracted from yeast strains EG123 (*MAT**a***), 246-1-1 (*MATα*), JRY3010 (*MATα sir1Δ*), KKY102 (*MAT**a** tup1Δ*), KKY103 (*MATα tup1Δ*), AJY158 (*MAT**a** ssn6Δ*), and AJY159 (*MATα ssn6Δ*) using the RNeasy protocol (QIAgen, Inc.), run out on a 1% agarose gel, transferred to a nitrocellulose membrane and hybridized to a probe recognizing either *MATα2* (left panel) or *MATa1* (right panel). The *MATα2* probe also hybridizes to the ***a**2* message which is encoded by *MAT**a*** and *HMR**a***. Each lane contains 25 μg RNA total and approximately the same amount of rRNA relative to one another as estimated from ethidium staining (data not shown). Yeast strain JRY3010 was provided by Lorraine Pillus.


a
α
a sir2
a tup1
α tup1
a ssn6
α ssn6
α2
a2
a
α
a sir2
a tup1
α tup1
a ssn6
α ssn6
a1

Appendix L. Inhibition of α2 synthesis leads to rapid derepression of the **a**-specific gene *MFA2*.

The α2 protein is extremely short-lived, having a half-life of approximately 5 minutes at 30°C (36). The rapid turnover of α2 presumably facilitates mating-type switching, since the swift conversion of an α cell to an **a** cell requires the prompt expression of the **a**-specific genes. Here we show that the **a**-specific gene *MFA2* is derepressed in α cells when α2 expression is inhibited. Attempts to determine whether or not this derepression requires progression through the cell cycle were inconclusive (data not shown). Establishment of α2-mediated repression, on the other hand, appears to occur in both dividing and arrested cells.

In order to test whether *MFA2* is derepressed upon removal of α2, we treated α cells with the protein synthesis inhibitor cycloheximide, and checked samples removed at various times after treatment for the presence of *MFA2* RNA. No *MFA2* transcript was detected in untreated α cells; however, significant levels of *MFA2* RNA were detected after 20 minutes of cycloheximide treatment (Figure 1A). When this experiment was repeated using an **a**/α strain, *MFA2* RNA was detected after 10 minutes of cycloheximide treatment (data not shown). Because cycloheximide treatment may affect levels of other proteins required for repression, we also placed α2 under the control of the *GAL1* promoter and examined the rate at which the *MFA2* transcript appeared when the cells were shifted from galactose to glucose. No *MFA2* transcript was detected in a *matα2* + pGAL-α2 strain grown in galactose; however, *MFA2* RNA was detectable 45 minutes after shifting the cells to glucose (Figure 1B). This derepression was not as rapid as that observed with cycloheximide treatment, possibly because the starting level of α2 is higher in strains carrying the pGAL-α2 plasmid.

In order to test whether the establishment of α2 repression requires progression through the cell cycle, we examined the ability of cells arrested at START to establish repression of *MFA2*. A *matΔ* strain carrying a galactose-inducible α2-expression plasmid and growing in glucose contains no α2 protein and therefore expresses *MFA2*. This strain was arrested at START with α-factor, and the arrested cells were then shifted into galactose to induce expression of α2 in the presence or absence of α factor. Samples were taken every hour and examined by Northern blot for the presence of the *MFA2* transcript. Upon induction of α2, the level of *MFA2* RNA dropped in both the presence and absence of α factor (Figure 2). Hence, establishment of repression can take place in both arrested and dividing cells, although achievement of complete repression was slightly slower in the α factor-arrested cells. *MFA2* RNA levels remained high in cells that were not shifted into galactose (data not shown).

In summary, we have shown that *MFA2* is rapidly derepressed by depletion of α2 via cycloheximide treatment. This rapid derepression suggests that derepression does not require progression through the cell cycle since the yeast cell cycle is longer than the time required for derepression of *MFA2*. In addition, we have shown that the establishment of repression clearly can take place in arrested cells. Since the establishment of silencing of *HML* and *HMR* is known to require progression through the cell cycle (63), these results are further evidence that α2-mediated repression is mechanistically distinct from silencing.

Figure L-1. Inhibition of α2 synthesis leads to derepression of the **a**-specific gene *MFA2*. (**A**) Northern blot showing the induction of *MFA2* RNA in α cells treated with cycloheximide. Yeast strain 246-1-1 (*MATα*) was grown to an OD_{600} of approximately 0.5 in YEPD. At time zero, cycloheximide was added to a concentration of 0.1 mg/mL to half of the culture. Samples were taken every 10 minutes. RNA was extracted from the cells by resuspending the cells in lysis buffer (0.5 M NaCl, 0.2 M Tris-HCl, pH7.4, 10 mM EDTA, 1% SDS), adding equal volumes of 1:1 (v/v) phenol/chloroform and glass beads, vortexing twice for 5 minutes, and precipitating the RNA with ethanol. RNA was then run out on a 1% agarose gel and transferred to a nylon membrane which was hybridized to a probe that recognizes the *MFA2* transcript. The blot was then stripped and rehybridized to a *URA3* probe, as shown in the lower part of the panel. (**B**) Northern blot showing induction of *MFA2* RNA when α2 expression is turned off. Yeast strain AJY85 (*matΔ*) was transformed with pAV88 (pGAL-α2/2 μm *URA3*) and grown in SGAL-URA medium to an OD_{600} of approximately 0.5. At time zero, the cells were pelleted, washed with water, and resuspended in either SD-URA(D) or SGAL-URA (G). Samples were taken at 0, 45, and 90 minutes. RNA was extracted and blotted as described above. Each lane contains 25 μg total RNA and approximately the same amount of rRNA, as estimated by ethidium staining (data not shown). Plasmid pAV88 was provided by Andrew Vershon.

A.

-CYCLOHEXIMIDE +CYCLOHEXIMIDE

0 10 20 30 40 50 60 | 0 10 20 30 40 50 60 minutes

MFA2

URA3

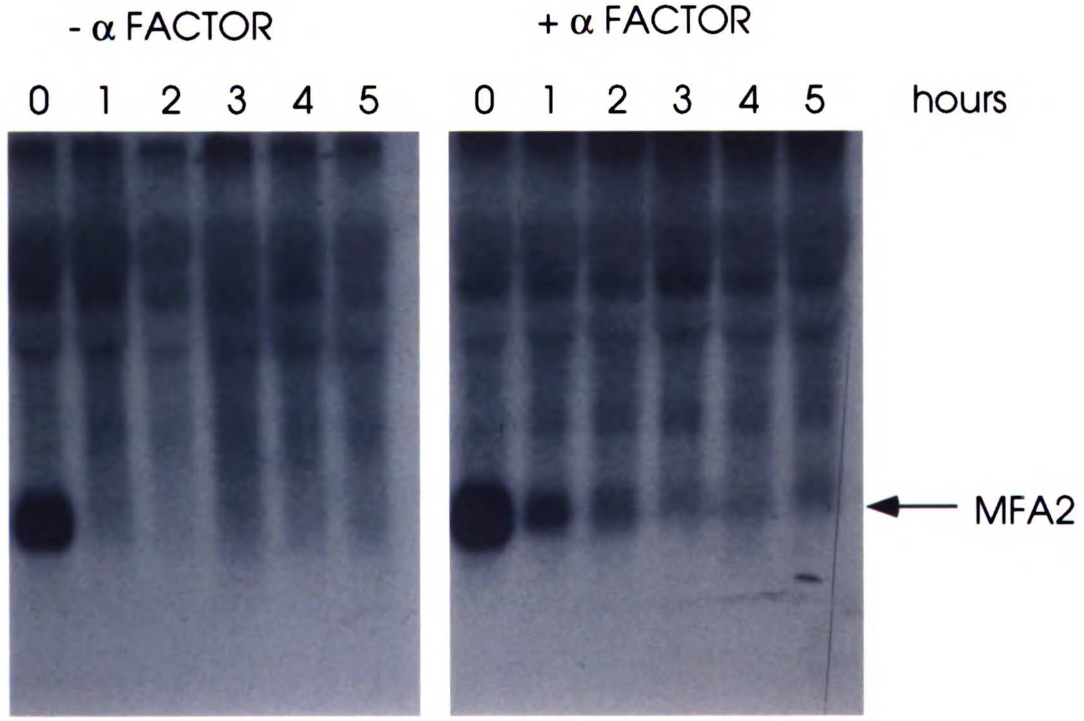
B.

0' 45' 90' time

D G D G D G media

MFA2

Figure 2. Establishment of α2-mediated repression occurs in α factor-arrested cells. AJY85 (*matΔ*) was transformed with pAV88 (pGAL-α2/URA3) and grown to an OD_{600} of approximately 0.5 in SD-URA medium. α factor was added to a final concentration of 2 μg/mL. After 2 hours, the cells were pelleted, washed with water, and resuspended in SGAL-URA medium with or without α factor. Samples were taken every hour, and RNA was extracted and blotted as described above.


- α FACTOR
+ α FACTOR
0 1 2 3 4 5
0 1 2 3 4 5
hours
MFA2

Appendix M. Multimerization of the $\alpha2$ operator increases repression

Many transcriptional activators are said to act synergistically in that the presence of more than one activator binding site increases transcription to a level that is greater than the sum of the levels of expression obtained with each site individually (29, 55). Here, we show that a similar phenomenon is observed in $\alpha2$-mediated repression when the $\alpha2$ operator is multimerized. We assayed repression of a *cyc1:lacZ* reporter containing either one or three $\alpha2$ operators upstream of the *CYC1* UAS With one operator, 17-fold repression was observed; with three operators, 370-fold repression was observed (Table 1). Because 100-fold repression is obtained when one $\alpha2$ operator is placed between the UAS and TATA (41) and because $\alpha2$ leaves a strong in vivo footprint on a single $\alpha2$ operator (46), it is thought that a single operator is fully bound at endogenous levels of $\alpha2$; hence, it is unlikely that the increase in repression caused by multimerization of the operator is attributable to an increase in operator occupancy via cooperative interactions between adjacent $\alpha2$/Mcm1 complexes.

Table M-1. Greater repression is observed when the α2 operator is multimerized. AJY126 (*matΔ*) and 246-1-1 (*MATα*) were transformed with *cyc1:lacZ* reporter pAJ5 or pKK77 and assayed for β-galactosidase activity. Reporter pAJ5 contains one α2 operator upstream of the *CYC1* UAS; pKK77 contains three α2 operators upstream of the UAS.

MAT allele	# of operators	β-galactosidase activity	fold repression
matΔ	one	539 ± 250	
MATα	one	31 ± 18	17
matΔ	three	808 ± 270	
MATα	three	2.2 ± 1.5	370

Appendix N. Dominant negative *SSN6* mutants

Ssn6, a protein containing ten copies of the tetratrichopeptide repeat (TPR) motif interacts directly with $\alpha 2$ and is required for $\alpha 2$-mediated repression (87). We were interested in isolating mutations in *SSN6* that decrease the Ssn6-$\alpha 2$ interaction in an attempt to determine which of the TPRs bind $\alpha 2$ in vivo. Because *TUP1* mutants that fail to interact with $\alpha 2$ are able to derepress $\alpha 2$-repressed genes in wild-type strains, it seemed likely that *SSN6* mutants defective for binding to $\alpha 2$ would also have a dominant negative phenotype. Here we describe the isolation and partial characterization of several dominant negative *SSN6* mutants.

We screened for dominant negative *SSN6* mutants by cotransforming a gapped 2 μm plasmid and a PCR-mutagenized fragment of DNA containing a complementing fragment of *SSN6* [*SSN6* (1-595)] into yeast strain SM1196 (*MATα mfa2:lacZ*) and assaying the transformants for β-galactosidase activity by filter assay. Plasmid DNA recovered from reproducibly blue colonies was retransformed into SM1196, and the transformants were tested for β-galactosidase activity. Of 36,000 transformants screened, 31 were blue by filter assay, and 5 yielded plasmids that reproducibly derepressed the *mfa2:lacZ* reporter in SM1196.

In order to determine if any of the plasmids could complement a deletion of *SSN6*, we transformed the plasmids into AJY159 (*MATα ssn6Δ9*) and examined the transformants, all of which remained sterile, clumpy, and slow-growing (data not shown). Thus, none of the dominant negative *SSN6* mutants are functional.

The *SSN6* mutants (designated as *SSN6-a* through *SSN6-e*) were then tested for their ability to derepress three Ssn6/Tup1-regulated reporters by transforming the plasmids into a wild-type strain carrying an *mfa2:lacZ*, *anb1:lacZ*, or *suc2:lacZ* reporter and assaying the transformants for β-galactosidase activity. All of the mutant plasmids caused

derepression of the three reporters (Table 1). We do not know why overexpression of the wild-type Ssn6 fragment causes slight derepression; because high copy plasmids carrying wild-type full-length *SSN6* also derepress the reporters to some degree (data not shown and R. Smith, personal communication), the derepression is not the result of using Ssn6(1-595) rather than full-length Ssn6.

We next tested whether overexpression of Tup1 or Ssn6 would suppress the dominance of the *SSN6* mutants by cotransforming each mutant into a *MAT*α *mfa2:lacZ* strain with 2 µm plasmids bearing *TUP1*, *SSN6*, or no insert. Repression was partially restored in all cases by both the *TUP1* and *SSN6* plasmids (Table 2).

Finally, we tested the ability of the strongest mutant, Ssn6-d, to bind α2 in vitro. Wild-type and mutant Ssn6 were expressed as GST fusions in E. coli, purified and immobilized on glutathione agarose beads. A bacterial extract containing α2 was passed over the columns which were then washed and eluted with high salt. α2 bound to both the GST-Ssn6 and GST-Ssn6-d columns, as indicated by depletion of α2 from the first flowthrough fractions and by the presence of α2 in the eluate from both columns (Figure 1). Binding to GST-Ssn6-d may be lower than binding to wild-type GST-Ssn6, but the difference is very slight.

The *SSN6* open reading frame from each of the plasmids was partially sequenced, but when it became apparent from the large number of mutations (>5) present in each clone that determining which mutation was responsible for the phenotype would be unfeasible, the sequencing was pursued no further.

In short, we have isolated *SSN6* mutants capable of derepressing an α2-regulated reporter and a hypoxic reporter in wild-type strains when overexpressed. The dominance of these mutants is suppressed by overexpression of either Ssn6 or Tup1 in much the same way that the dominance of *TUP1* mutants defective in binding α2 is suppressed by overexpression of either Tup1 or Ssn6. Hence we think it likely that the mutation or mutations responsible affect the Ssn6-α2 and Ssn6-Rox1 interactions. Although the one

Plasmids

Plasmid pKK413 is the PvuII fragment of pLN113-3 cloned into the PvuII site of pKK412. pKK412 is pΔSJ (42) from which the PvuII fragment has been removed.

GST-SSN6-d is a derivative of GST-SSN6 (87) and was constructed by inserting a PCR fragment made using oligos (5'- AAATTA GGA TCC ATG AAT CCG GGC -3') and (5'- GGC TGA ATT TCT AGT GTT CAA AGG -3') as primers and pKK413-d as template into the BamHI and EcoRI sites of pGEX2T (86). Oligos and the GST-SSN6 plasmid were provided by Michael Redd.

Mutagenesis

A PCR fragment containing *SSN6* coding sequences was generated under mutagenic conditions as described (66), using oligos (5'- AGA TAA TGG GGC TCT TTA CAT TTC -3') and (5'- AGC ACG CTT ATC GCT CCA ATT TCC -3') as primers and plasmid pKK413 as template. For the screen, PvuII digested pKK412 and the mutagenized PCR fragment were cotransformed into SM1196 (*MATα mfa2:lacZ*).

Table N-1. Overexpression of *SSN6* mutants derepresses *mfa2:lacZ*, *anb1:lacZ*, and *suc2:lacZ* in wild-type strains. Plasmids containing wild-type or mutant *SSN6* were transformed into SM1196 (*MATα mfa2:lacZ*) or 246-1-1 (*MATα*) carrying either an *anb1:lacZ* or *suc2:lacZ* reporter plasmid and assayed for β-galactosidase activity.

plasmid	*mfa2:lacZ*	*anb:lacZ*	*suc2:lacZ*
pKK412 (vector)	1.4 ± 0.7	0.08 ± 0.03	0.77 ± 0.09
pKK413 (wild-type)	2.3 ± 0.2	4.1 ± 0.3	3.2 ± 0.2
SSN6-a	12.5 ± 1	14.1 ± 3	9.1 ± 1
SSN6-b	15.8 ± 1		
SSN6-c	7.2 ± 0.3	22.1 ± 4	5.5 ± 0.8
SSN6-d	54.7 ± 3	70.8 ± 13	22.1 ± 4
SSN6-e	22.6 ± 0.3	14.7 ± 5	14.7 ± 5

Table N-2. Derepression of *mfa2:lacZ* caused by the dominant *SSN6* mutants is suppressed by overexpression of Ssn6 or Tup1. The mutant *SSN6* plasmids and a 2 μm plasmid bearing no insert, *SSN6* or *TUP1* were cotransformed into SM1196, and the transformants were assayed for β-galactosidase activity.

SSN6 mutant	2 μm plasmid	β-galactosidase activity
SSN6 (wild-type)	vector	0.9 ± 0.1
SSN6-a	vector	9.1 ± 0.6
	TUP1	0.8 ± 0.3
	SSN6	2.8 ± 0.2
SSN6-b	vector	12.1 ± 1
	TUP1	1.7 ± 0.3
	SSN6	3.5 ± 0.1
SSN6-c	vector	6.5 ± 0.4
	TUP1	0.7 ± 0.1
	SSN6	5.3 ± 0.6
SSN6-d	vector	57.9 ± 6
	TUP1	8.1 ± 1
	SSN6	18.5 ± 2.6

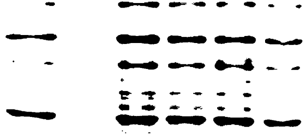
SSN6-e	vector	12.9 ± 1.6
	TUP1	1.3 ± 0.1
	SSN6	3.4 ± 0.3

Figure N-1. Ssn6 and Ssn6-d both bind to $\alpha2$ in vitro. Coomassie stained gels of fractions of a bacterial extract containing $\alpha2$ that has been passed over a column containing glutathione agarose beads bearing (**A**) GST-Ssn6 (wild-type) or (**B**) GST-Ssn6-d. The columns were prepared and run as previously described (87), except that the extract contains both $\alpha2$ and $\alpha2^{\Delta 2\text{-}10}$, a mutant of $\alpha2$ that is able to bind to Ssn6. The load (L) is the same for both columns; flowthrough fractions are labeled F1 through F4; wash fractions, W1 through W4; elution fractions, E.

A.

GST-SSN6 (wild-type)

L F1 F2 F3 F4 W1 W2 W3 W4 E



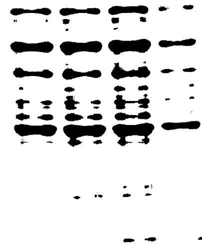
$\alpha2$

$\alpha2^{\Delta2-12}$

B.

GST-SSN6d

F1 F2 F3 F4 W1 W2 W3 W4 E



$\alpha2$

$\alpha2^{\Delta2-12}$

Appendix O. Effect of histone mutations on α2-mediated repression

Several lines of evidence indicate that the α2/Ssn6/Tup1 complex is able to alter chromatin structure by positioning nucleosomes. First, an α2 operator induces a nuclease protection pattern consistent with nucleosomes being positioned onto the DNA surrounding the operator (77, 83). Second, the absence of α2, Tup1, Ssn6, or the amino terminus of histone H4 will disrupt this nucleosome positioning (11, 78). Third, Tup1 binds to purified histones H3 and H4 in vitro (16). Fourth, mutations in histone H4 that disrupt nucleosome positioning also disrupt the Tup1-histone H4 interaction (16, 78). Hence, it is plausible that an α2-bound operator positions nucleosomes by recruiting Tup1 which interacts directly with histones.

The correlation between α2's ability to position nucleosomes and its ability to repress transcription, however, is tenuous. Complete (200- to 800-fold) repression by α2 can take place in the absence of nucleosome positioning (74), and mutations in histone H4 that eliminate both the histone H4-Tup1 interaction and nucleosome positioning result in only a 2- to 3-fold increase in the expression of an α2-repressed reporter(16, 78). Interpretation of this slight derepression is complicated by the fact that strains bearing a mutant histone H4 aberrantly express the normally silent mating cassettes and therefore behave as **a**/α cells; repression by α2 is usually about 2-fold lower in **a**/α cells than in α cells, probably because α2 is slightly repressed by **a**1/α2 (24). Finally, although combined mutations in histone H3 and histone H4 lead to a 10- to 13-fold decrease in α2-mediated repression, the mutant strains used to demonstrate this effect were not bona fide α strains and may not have been expressing α2 at wild-type levels (16). Here we quantitate the effect of histone H3 and H4 mutations on α2 repression more accurately by using isogenic sets of yeast strains that allow us to perform controls that were missing from previous studies.

Effect of histone H4 mutations on α2/Mcm1-mediated repression

In order to control for the effect of silent mating cassette derepression in histone H4 mutants we constructed an isogenic set of **a**/α strains deleted for both histone H4 genes (*HHF1* and *HHF2*) and carrying either wild-type or mutant *HHF2* on a CEN plasmid. These strains also carry an integrated *cyc1:lacZ* reporter that has no α2/Mcm1 operator, one operator between the UAS and TATA, or one operator upstream of the UAS. As summarized in Table 1, the histone H4 mutations caused a decrease in lacZ expression from all of the reporters; thus, in terms of absolute level of expression, the histone H4 mutations do not cause derepression of the reporter with the α2/Mcm1 operator. However, if the repression ratio is considered to be the expression of the reporter without the operator divided by the expression of the reporter with the operator, there is a 2- to 3-fold decrease in repression in the histone H4 mutants relative to the wild-type strain.

Because the *cyc1:lacZ* reporter contains an artificial promoter which may differ from other yeast promoters with respect to nucleosome structure (9), we also examined the effect of the *hhf2Δ4-23* mutation on an *mfa2:lacZ* reporter. In order to control for the effect of silent mating cassete derepression in *hhf* mutants, we disrupted *HMRa* in a *MATα hhf1Δ hhf2Δ mfa2:lacZ* strain and *HMLα* in a *MAT**a** hhf1Δ hhf2Δ mfa2:lacZ* strain; in all strains, wild-type *HHF2* or *hhf2Δ4-23* was carried on a plasmid. Expression of *mfa2:lacZ* was virtually the same in *hhf2Δ4-23* and wild-type strains (Table 2), indicating that repression of *mfa2:lacZ* is not affected by deletion of the amino terminus of histone H4.

Effect of histone H3 mutations on α2/Mcm1-mediated repression

In order to determine the effect of histone H3 mutations on α2 repression, we constructed a set of isogenic **a** and α strains deleted for both histone H3 genes (*HHT1* and *HHT2*) and carrying either wild-type or mutant *HHT2* on a CEN plasmid. These strains also carried an integrated *cyc1:lacZ* reporter with either no α2/Mcm1 operator or one

operator between the UAS and TATA. Expression of the reporter with the operator was approximately 4-fold higher in *MATα hht2Δ1-30* strains than in *MATα HHT2* strains (Table 3). Since expression of the reporters in the absence of α2 and/or the α2 operator is also decreased by the *hht2Δ1-30* mutation, the level of repression may be decreased by as much as 14-fold, depending on how the repression ratio is calculated.

We next examined the effect of the *hht2Δ1-30* mutation on repression of an *mfa2:lacZ* reporter by transforming ***MATa*** and *MATα* strains deleted for *hht1* and *hht2* with an integrating *mfa2:lacZ* plasmid and a plasmid carrying either *hht2Δ1-30* or *HHT2*. Expression of the reporter was approximately 2-fold higher in *MATα hht2* strain than in the *MATα HHT2* strain and approximately 3-fold lower in the ***MATa** hht2* strain than in the ***MATa** HHT2* strain. The level of repression, therefore, may be decreased as much as 6-fold by deletion of the histone H3 amino terminus.

Effect of histone H4 or H3 mutations on **a**1/α2-mediated repression

Since both **a**1/α2- and α2/Mcm-mediated repression require α2 and Tup1/Ssn6, both types of repression should be affected by mutations in histone H4 or histone H3. We tested the effect of the *hhf2Δ4-23* mutation on **a**1/α2-mediated repression by constructing a *MATα hhf1Δ hhf2Δ* strain carrying an integrated ***MATa*** plasmid, an integrated *cyc1:lacZ* reporter with either no operator or two **a**1/α2 operators between the UAS and TATA, and a wild-type or mutant *HHT2* plasmid. Expression of the **a**1/α2-repressible reporter was 2-fold higher in the *hht2Δ4-23* strain than in the *HHT2* strain (Table 5). Since expression of the reporter without the operator was slightly decreased in the mutant strain, **a**1/α2-mediated repression may be decreased by as much as 2.6-fold in the *hht2Δ4-23* strain.

We tested the effect of the *hht2Δ1-30* mutation on **a**1/α2-mediated repression by constructing a ***MATa**/MATα (hhf1, hht1)Δ/(hhf1, hht1)Δ (hhf2, hht2)Δ/(hhf2, hht2)Δ* strain carrying an integrated *cyc1:lacZ* reporter with either no operator or two **a**1/α2 operators between the UAS and TATA and carrying either *HHT2* or *hht2Δ1-30* on a

HHF2/CEN plasmid. The repression ratio was decreased by less than two-fold in the *hht2Δ1-30* strain (Table 6).

Hence, mutations in histone H4 or H3 affect **a**1/α2-mediated repression to an even lesser degree than than they do α2/Mcm1-mediated repression.

Effect of mutations in both histone H3 and histone H4 on α2/Mcm1-mediated repression

In order to determine the effect of mutations in both histone H3 and histone H4 on α2 repression, we constructed a set of **a**/α strains that are deleted for the genes encoding histone H3 and histone H4 and that carry wild-type or mutant *HHF2* and wild-type or mutant *HHT2* on a CEN plasmid. These strains also carried an integrated *cyc1:lacZ* reporter that has either no α2/Mcm1 operator or one operator between the UAS and TATA. Expression of the reporter with the operator was approximately equal in *MATα hhf2 hht2* and *MATα HHT2* strains (Table 7). However, since expression of the reporter without the operator was significantly decreased by the histone mutations, the level of repression may be decreased by up to 15-fold in the double mutant. In either case, the double mutant does not appear to have a significantly greater repression defect than does the *hht2* single mutant.

Effect of histone mutations on *matα2:lacZ* expression

Because mutations in either histone H3 or H4 decreased the expression of most of the reporters and because a 3- to 5- fold decrease in α2 expression leads to measurable derepression of *BAR1* and *STE2* (23), we tested the effect of the histone mutations on the expression of a *matα2:lacZ* reporter and found that *matα2:lacZ* expression was slightly decreased in the *hhf2Δ4-19* and *hht2Δ1-30* mutants (Table 8). We also examined the levels of α2, Ssn6, and Tup1 in *hht2* and *HHT2* strains by Western blot and found no dramatic decrease in the levels of these three proteins in the *hht2Δ1–30* mutant strains, although it is unlikely that we would have been able to detect a two-fold difference in

protein levels (data not shown). High copy plasmids carrying *TUP1*, *SSN6*, *TUP1+SSN6*, or *MATα2* did not suppress the slight repression defect of the *hht2Δ1-30* strain (Table 9).

In short, we have found that α2-mediated repression is decreased less than 3-fold by mutations in histone H4 and 4- to 14-fold by mutations in histone H3. Our results differ from those reported earlier(16, 78) in several respects. First, our results were obtained using congenic strains and integrated reporters in all cases where the level of repression was being compared. Second, we found that the histone H4 mutations have almost no effect on α2/Mcm1-repression of *mfa2:lacZ* and neither histone H3 nor histone H4 mutations have an appreciable effect on **a**1/α2-mediated repression. Third, we observed that much of the effect of the histone mutations on the repression is due to a decrease in transcription under nonrepressing conditions (i.e., the absence of α2 and/or its operator). If one looks only at the level of expression of the reporter under repressing conditions, the histone H3 mutations cause at most a 4-fold increase in expression relative to wild-type strains, the histone H4 mutations either decrease expression or have no effect, and the double mutation has no effect. Hence, though histone H3 and H4 mutations do lead to defects in α2 repression, the magnitude and significance of the effect is a matter of interpretation and opinion.

Plasmids

Plasmids pKK8, pKK10, and pKK794 were constructed by deleting the 2 μm-containing HindIII fragment from plasmids pAJ1 (pLG-Δ312S), pAJ3 (pS1-19), and pAJ5 (pS1-85) (41), respectively.

pKK561, 563 and 564 were constructed by inserting the BamHI-EcoRI fragments from pMH310, pPK613, and pPK618, respectively, into BamHI-EcoRI-digested pRS313 (84).

pKK799 and pKK803 were constructed by inserting the BamHI-EcoRI fragments from pPK613 and pPK618, respectively, into BamHI-EcoRI-digested pRM200 (59).

pKK826 and pKK830 were constructed by replacing the BamHI-EcoRI fragment of pRM200 and pRM430 (59), respectively, with the BamHI-EcoRI fragment of pKK824. pKK824 is the EcoRI-HindIII fragment of pKK822 inserted into the EcoRI and HindIII sites of pPK613. pKK 822 was constructed by inserting the double stranded oligo 5'-GAT CTA AAG GTG GTA AAG GTC TAG GTC AAG GTG GTG CCC AGC GTC ACA-3'/ 5'-GAT CTG TGA CGC TGG GCA CCA CCT TGA CCT AGA CCT TTA CCA CCT TTA-3' into the BglII site of pKK549. pKK549 is the EcoRI fragment of pKK548 ligated into pRS304 (84). pKK548 was constructed by ligating BglII-HindIII-cut PCR fragment 1 and and BglII-EcoRI-cut PCR fragment 2 into HindIII-EcoRI-cut pUC18 (110). PCR fragment 1 was generated using the oligos 5'-AGA TAA TGG GGC TCT TTA CAT TTC-3' and 5'-TTT ACC ACC TTT AGA TCT ACC GGA CAT TAT TTT ATT GTA-3' as primers and pKK541 as template; PCR fragment 2 was generated using the oligos 5'-AGC ACG CTT ATC GCT CCA ATT TCC-3' and 5'-AAG CGT CAC AGA TCT ATT CTA AGA GAT AAC ATC CAA GCT-3' as primers and pKK541 as template. pKK541 is the HindIII fragment from pMH310 inserted into HindIII-digested pΔSJ (42).

pKK792 and pKK793 were constructed by inserting the BamHI fragment from pR490 into the BglII site of pKK833 and pKK834, respectively. pKK833 and pKK834

are pAJ1 and pAJ3, respectively, in which a BglII linker has been inserted between the HindIII and SmaI sites.

pKK795 was constructed by inserting the BamHI-SalI fragment from pRM200 into pPK618.

pKK797 was constructed by replacing the BamHI-EcoRI fragment of pKK795 with the BamHI-EcoRI fragment of pKK561.

pKK836, pKK838, and pKK839 were constructed by replacing the EcoRI-SalI fragment of pPK613 with the EcoRI-SalI fragments of pKK826, pKK830, and pRM430, respectively.

pKK840 and pKK841 were constructed by inserting the BamHI fragment from pR490 into pKK561 and pKK563, respectively.

pKK806 was constructed by inserting the *mfa2:lacZ*-containing HindIII fragment from CYp246 (61) into the HindIII site of pBR328-*LYS2*. pBR328-*LYS2* was obtained via Andrew Vershon and is the EcoRI-ClaI fragment containing *LYS2* in pBR328 (2).

pKK807 contains the upstream regulatory sequences and sequences coding for the first thirteen amino acids of α2 fused in frame to the lacZ coding sequence and was constructed by inserting the HindIII fragment containing *LYS2* from pDP6+DAM (Dan Gottschling) into HindIII-cut pKK723. pKK723 is the BglII-SmaI fragment from pAV116 ligated into BglII-SmaI-cut pKK720. pAV116 was constructed by Andrew Vershon and is the same as pAV115 (58) except that the HindIII fragment is in the reverse orientation. pKK720 is the double stranded oligo (5'-TCG ACA GAT CTT TTA AAT CCA CAA G-3'/5'-GAT CCT TGT GCA TTT AAA AGA TCT G-3') ligated into SalI-BamHI-digested pAJ1.

pKK49 is the HindIII fragment containing *MATα* cloned into the HindIII site of pGEM3 (Promega).

pKK789 is the EcoRI-BglII fragment containing *TRP1* cloned into the EcoRI and BglII sites of pKK334. pKK334 is the HindIII fragment of pJR154 cloned into pGEM3.

pJR154 is the HindIII fragment containing *HMRa* cloned into YCp50 and was provided by Frank McNally/Jasper Rine. pJR866 was provided by Lorraine Pillus and contains the BamHI-BamHI fragment of *HML* in which sequences between the XhoI sites have been replaced with *TRP1*.

Strain Constructions

Yeast strains were constructed as described in Table 7, with the following specifications.

Replacement of the *MAT* locus with *matΔ::URA3* was performed by transforming the parent strain with HindIII-cut pKK143 (103). Replacement of *matΔ::URA3* with *MATα* was performed by transforming the parent strain with HindIII-cut pKK49 and selecting for 5-FOA resistant transformants.

Replacement of *HMRa* in *MATα hhf2* strains with *hmrΔ::TRP1* was performed by transforming the parent strain with XhoI-SalI-cut pKK789 and screening the Trp^+ transformants for α-maters. Replacement of *HMLα* in *MATa hhf2* strains with *hmlΔ::TRP1* was performed by transforming the parent strain with BamHI-cut pJR866 and screening the Trp^+ transformants for **a**-maters.

Table 1. Repression of *cyc1:lacZ* reporters by α2 in wild-type and *hhf2* strains. *MATa/MATα hhf1Δ/hhf1Δ hhf2Δ/hhf2Δ* strains carrying an integrated *cyc1:lacZ* reporter (pKK8, pKK10, or pKK794) and wild-type or mutant *HHF2* on a CEN ARS plasmid were constructed and assayed for β-galactosidase activity. Reporters pKK 8, pKK10, and pKK794 contain no α2 operator, one operator between the UAS and TATA, and one operator upstream of the UAS, respectively.

Strain	*HHF2* allele	Position of α2 operator	β-galactosidase activity
KKY183	wild-type	no α2 operator	34.8 ± 0.8
KKY184	Δ4-23	no α2 operator	4.5 ± 0.7
KKY185	Δ4-19	no α2 operator	4.6 ± 0.8
KKY186	wild-type	between UAS and TATA	0.37 ± 0.03
KKY187	Δ4-23	between UAS and TATA	0.17 ± 0.03
KKY188	Δ4-19	between UAS and TATA	0.13 ± 0.04
KKY189	wild-type	upstream of UAS	1.5 ± 0.1
KKY190	Δ4-23	upstream of UAS	0.6 ± 0.1
KKY191	Δ4-19	upstream of UAS	0.38 ± 0.08

Table 2. Repression of the *mfa2:lacZ* reporter in wild-type and *hhf2* strains. A *MATα hmrΔ::TRP1 hhf1Δ hhf2Δ mfa2:lacZ/LYS2* strain and a ***MATa*** *hmlΔ::TRP1 hhf1Δ hhf2Δ mfa2:lacZ/LYS2* strain were cotransformed with a plasmid bearing *HHF2* or *hhf2Δ4-23* and assayed for β-galactosidase activity.

Strain	*MAT* allele	*HHF2* allele	β-galactosidase activity
KKY 241	α	wild-type	0.23 ± 0.01
KKY242	α	Δ4-23	0.37 ± 0.03
KKY284	**a**	wild-type	92.3 ± 2
KK285	**a**	Δ4-23	136 ± 4

Table 3. Repression of *cycl:lacZ* reporters by α2 in wild-type and *hht2* strains. *MATa hhfl, hht1Δ::LEU2 hhf2, hht2Δ::HIS3* and *MATα hhfl, hht1Δ::LEU2 hhf2, hht2Δ::HIS3* strains carrying an integrated *cycl:lacZ* reporter (pKK792 or pKK793) and wild-type or mutant *HHT2* on an *HHF2*/ CEN ARS plasmid were constructed and assayed for β-galactosidase activity. Reporters pKK792 and pKK793 have no α2 operator and one operator between the UAS and TATA, respectively.

Strain number	Mating type	*HHT2* allele	Reporter	β-galactosidase activity
KKY202	**a**	wild-type	no α2 site	62.2 ± 0.6
KKY203	**a**	Δ 4-20	no α2 site	22.0 ± 2
KKY204	**a**	Δ 4-30	no α2 site	18.5 ± 2
KKY205	**a**	wild-type	+ α2 site	201 ± 20
KKY206	**a**	Δ 4-20	+ α2 site	95 ± 10
KKY207	**a**	Δ 4-30	+ α2 site	102 ± 20
KKY208	α	wild-type	no α2 site	85.2 ± 10
KKY209	α	Δ 4-20	no α2 site	16.1 ± 1.5
KKY210	α	Δ 4-30	no α2 site	79.5 ± 3
KKY211	α	wild-type	+ α2 site	0.07 ± 0.02
KKY212	α	Δ 4-20	+ α2 site	0.10 ± 0.02
KKY213	α	Δ 4-30	+ α2 site	0.29 ± 0.07

Table 4. Repression of an *mfa2:lacZ* reporter in wild-type and *hht2* strains.

Strain	*MAT* allele	*HHT2* allele	β-galactosidase activity
KKY344	**a**	wild-type	161 ± 10
KKY345	**a**	Δ1-30	48.9 ± 0.6
KKY346	α	wild-type	0.19 ± 0.01
KKY347	α	Δ1-30	0.37 ± 0.01

Table 5. Repression of an **a**1/α2-repressible reporter in wild-type and *hhf2* strains. A *MATα hhf1Δ hhf2Δ* strain carrying an integrated *MATa/URA3* plasmid and either pKK792 or pNH165 was transformed with a plasmid bearing either *HHF2* or *hhf2Δ4-23*; and the transformants were assayed for β-galactosidase activity. pNH165 is an integrating *cyc1:lacZ* reporter with two consensus **a**1/α2 operators between the UAS and TATA.

Strain	reporter	*HHF2* allele	β-galactosidase activity
KKY292	no operator	wild-type	15.1 ± 0.5
KKY293	no operator	Δ4-23	12.4 ± 1
KKY290	**a**1/α2 operators	wild-type	0.007 ± 0.002
KKY291	**a**1/α2 operators	Δ4-23	0.015 ± 0.003

Table 6. Repression of an **a**1/α2-repressible reporter in wild-type and hht2Δ1-30 strains. A *MATa/MATα (hhf1, hht1)Δ/(hhf1, hht1)Δ (hhf2, hht2)Δ/(hhf2, hht2)Δ* strain carrying reporter pKK792 or pNH165 and a *HHF2*-containing plasmid bearing either *HHF2* or *hht2Δ1-30* was assayed for β-galactosidase activity.

Strain	Reporter	*HHT2* allele	β-galactosidase activity	fold-repression
KKY336	no operator	wild-type	5.8 ± 0.4	
KKY337	no operator	Δ1-30	2.1 ± 0.1	
KKY338	**a**1/α2 operators	wild-type	0.02 ± 0.008	340
KKY339	**a**1/α2 operators	Δ1-30	<0.01	>210

Table 7. Repression of *cyc1:lacZ* reporters by α2 in wild-type strains and in strains having mutations in *HHF2* and/or *HHT2*. *MATa/MATα hhf1, hht1Δ::LEU2/ hhf1, hht1Δ::LEU2 hhf2, hht2Δ::HIS3 / hhf2, hht2Δ::HIS3* strains carrying an integrated *cyc1:lacZ* reporter (pKK792 or pKK793) and wildtype or mutant *HHF2* and wild-type or mutant *HHT2* on a CEN ARS plasmid were constructed and assayed for β-galactosidase activity.

Strain	*HHF2* allele	*HHT2* allele	reporter	β-galactosidase activity
KKY222	wild-type	wild-type	no α2 site	131.8 ± 6
KKY223	wild-type	Δ1-30	no α2 site	53.4 ± 3
KKY224	K12Q, K16Q	wild-type	no α2 site	34.8 ± 0.3
KKY225	K12Q, K16Q	Δ1-30	no α2 site	12.4 ± 0.1
KKY226	wild-type	wild-type	+ α2 site	0.12 ± 0.03
KKY227	wild-type	Δ1-30	+ α2 site	0.47 ± 0.1
KKY228	K12Q, K16Q	wild-type	+ α2 site	0.03 ± 0.005
KKY229	K12Q, K16Q	Δ1-30	+ α2 site	0.18 ± 0.1

Table 8. Effect of histone mutations on expression of *MAT*α*2:lacZ*. *MAT*α *hhf1, hht1*Δ*::LEU2 hhf2, hht2*Δ*::HIS3* strains carrying an *MAT*α*2:lacZ* reporter integrated at *LYS2* and wild-type or mutant *HHF2* and wild-type or mutant *HHT2* on a CEN ARS plasmid were constructed and assayed for β-galactosidase activity.

Strain	*HHF2* allele	*HHT2* allele	β-galactosidase activity
KKY274	wild-type	wild-type	6.3 ± 0.3
KKY275	Δ4–19	wild-type	1.8 ± 0.01
KKY276	wild-type	Δ1-30	2.6 ± 0.3
KKY277	K12Q, K16Q	wild-type	5.0 ± 0.8

Table 9. High copy plasmids carrying *TUP1, SSN6, TUP1+SSN6*, or *MATα2* do not suppress the repression defect of a *hht2Δ1–30* strain. *MATα hhf1, hht1Δ::LEU2 hhf2, hht2Δ::HIS3* strains carrying pKK792 or pKK793 and wild-type or mutant *HHT2* on a *HHF2*/CEN ARS plasmid were transformed with 2 μm plasmids carrying *TUP1*, *SSN6*, *TUP1* + *SSN6*, or *MATα2* and assayed for β-galactosidase activity.

Strain	Reporter	*HHT2* allele	high copy plasmid	β-galactosidase activity
KKY249	no operator	wild-type	vector	119 ± 22
KKY250	no operator	wild-type	*TUP1*	110 ± 4
KKY251	no operator	wild-type	*SSN6*	49 ± 2
KKY252	no operator	wild-type	*TUP1* + *SSN6*	43 ± 2
KKY253	no operator	wild-type	*MATα2*	110 ± 8
KKY254	no operator	Δ1-30	vector	140 ± 23
KKY255	no operator	Δ1-30	*TUP1*	145 ± 50
KKY256	no operator	Δ1-30	*SSN6*	83 ± 45
KKY257	no operator	Δ1-30	*TUP1* + *SSN6*	76 ± 22
KKY258	no operator	Δ1-30	*MATα2*	147 ± 18
KKY259	+ α2 operator	wild-type	vector	0.08 ± 0.01
KKY260	+ α2 operator	wild-type	*TUP1*	0.06 ± 0.01
KKY261	+ α2 operator	wild-type	*SSN6*	0.20 ± 0.04
KKY262	+ α2 operator	wild-type	*TUP1* + *SSN6*	0.05 ± 0.02

KKY263	+ α2 operator	wild-type	*MATα2*	0.14 ± 0.05
KKY264	+ α2 operator	Δ1-30	vector	0.51 ± 0.03
KKY265	+ α2 operator	Δ1-30	*TUP1*	0.34 ± 0.05
KKY266	+ α2 operator	Δ1-30	*SSN6*	0.45 ± 0.01
KKY267	+ α2 operator	Δ1-30	*TUP1* + *SSN6*	0.20 ± 0.01
KKY268	+ α2 operator	Δ1-30	*MATα2*	0.69 ± 0.02

Table 9. Yeast strains

Strain name	Genotype/Construction	Reference
UKY403	*MATa ade2-101 (och) arg4-1 his3Δ200 leu2-3 leu2-112 lys2-801 (amb) trp1-Δ901 ura3-52 thr*$^-$ *tyr*$^-$ *hhf1Δ::HIS3 hhf2Δ::LEU2* /pUK421	(43)
PKY903	*MATα ade2-101 (och) arg4-1 his3Δ200 leu2-3 leu2-112 lys2-801 (amb) trp1-Δ901 ura3-52 thr*$^-$ *tyr*$^-$ *hhf1Δ::HIS3 hhf2Δ::LEU2 /* pUK421	(43)
KKY165	UKY403 transformed with pKK8	
KKY166	UKY403 transformed with pKK10	
KKY167	UKY403 transformed with pKK794	
KKY168	KKY165 transformed with pKK561	
KKY169	KKY165 transformed with pKK563	
KKY170	KKY165 transformed with pKK564	
KKY171	KKY166 transformed with pKK561	
KKY172	KKY166 transformed with pKK563	
KKY173	KKY166 transformed with pKK564	
KKY174	KKY167 transformed with pKK561	
KKY175	KKY167 transformed with pKK563	
KKY176	KKY167 transformed with pKK564	
KKY177	PKY903 transformed with pKK 8	
KKY178	PKY903 transformed with pKK 10	
KKY179	PKY903 transformed with pKK 794	
KKY183	KKY177 x KKY168 and cured of pUK421	

KKY184	KKY177 x KKY169 and cured of pUK421	
KKY185	KKY177 x KKY170 and cured of pUK421	
KKY186	KKY178 x KKY171 and cured of pUK421	
KKY187	KKY178 x KKY172 and cured of pUK421	
KKY188	KKY178 x KKY173 and cured of pUK421	
KKY189	KKY179 x KKY174 and cured of pUK421	
KKY190	KKY179 x KKY175 and cured of pUK421	
KKY191	KKY179 x KKY176 and cured of pUK421	
RMY200	*MATa ade2-101 (och) his3Δ201 lys 2-801 (amb) trp1Δ901 ura3-52 hht1, hhf1Δ::LEU2 hht2, hhf2Δ::HIS3/pRM200*	(59)
KKY192	RMY200 in which *MATa* has been replaced with *matΔ::URA3*	
KKY193	KKY192 in which *matΔ::URA3* has been replaced with *MATα*	
KKY194	RMY200 transformed with pKK792	
KKY195	RMY200 transformed with pKK793	
KKY196	KKY193 transformed with pKK792	
KKY197	KKY193 transformed with pKK793	
KKY198	KKY194 transformed with pKK795 and cured of pRM200	
KKY199	KKY195 transformed with pKK795 and cured of pRM200	
KKY200	KKY196 transformed with pKK795 and cured of pRM200	
KKY201	KKY197 transformed with pKK795 and cured of pRM200	
KKY202	KKY198 transformed with pRM200 and cured of pKK795	
KKY203	KKY198 transformed with pRM420 and cured of pKK795	
KKY204	KKY198 transformed with pRM430 and cured of pKK795	
KKY205	KKY199 transformed with pRM200 and cured of pKK795	
KKY206	KKY199 transformed with pRM420 and cured of pKK795	
KKY207	KKY199 transformed with pRM430 and cured of pKK795	

KKY208	KKY200 transformed with pRM200 and cured of pKK795
KKY209	KKY200 transformed with pRM420 and cured of pKK795
KKY210	KKY200 transformed with pRM430 and cured of pKK795
KKY211	KKY201 transformed with pRM200 and cured of pKK795
KKY212	KKY201 transformed with pRM420 and cured of pKK795
KKY213	KKY201 transformed with pRM430 and cured of pKK795
KKY214	KKY202 transformed with pKK797 and cured of pRM200
KKY215	KKY205 transformed with pKK797 and cured of pRM200
KKY216	KKY214 x KKY196
KKY217	KKY215 x KKY197
KKY218	KKY216 cured of pRM200
KKY219	KKY217 cured of pRM200
KKY222	KKY218 transformed with pRM200 and cured of pKK797
KKY223	KKY218 transformed with pRM430 and cured of pKK797
KKY224	KKY218 transformed with pKK826 and cured of pKK797
KKY225	KKY218 transformed with pKK830 and cured of pKK797
KKY226	KKY219 transformed with pRM200 and cured of pKK797
KKY227	KKY219 transformed with pRM430 and cured of pKK797
KKY228	KKY219 transformed with pKK826 and cured of pKK797
KKY229	KKY219 transformed with pKK830 and cured of pKK797
KKY238	PKY903 transformed with pKK559 and cured of pUK421
KKY239	KKY238 that is *hmlΔ::TRP1* instead of *HMLα*
KKY240	KKY239 transformed with pKK806 (*mfa2:lacZ/LYS2*)
KKY241	KKY240 transformed with pKK840 and cured of pKK559
KKY242	KKY240 transformed with pKK841 and cured of pKK559

KKY243	PKY903 that is *matΔ::URA3* instead of *MATα*
KKY244	KKY243 transformed with pKK841 and cured of pRM421
KKY245	KKY244 that is *hmlΔ::TRP1* instead of *HMLα*
KKY246	KKY245 transformed with pKK806 (*mfa2:lacZ/LYS2*)
KKY247	KKY243 transformed with pKK840
KKY248	KKY247 transformed with pKK806 (*mfa2:lacZ/LYS2*)
KKY249	KKY208 transformed with YEp24
KKY250	KKY208 transformed with *TUP1*/YEp24
KKY251	KKY208 transformed with *SSN6*/YEp24
KKY252	KKY208 transformed with *TUP1+SSN6*/YEp24
KKY253	KKY208 transformed with *α2*/Yep24
KKY254	KKY210 transformed with YEp24
KKY255	KKY210 transformed with *TUP1*/YEp24
KKY256	KKY210 transformed with *SSN6*/YEp24
KKY257	KKY210 transformed with *TUP1+SSN6*/YEp24
KKY258	KKY210 transformed with *α2*/Yep24
KKY259	KKY211 transformed with YEp24
KKY260	KKY211 transformed with *TUP1*/YEp24
KKY261	KKY211 transformed with *SSN6*/YEp24
KKY262	KKY211 transformed with *TUP1+SSN6*/YEp24
KKY263	KKY211 transformed with *α2*/Yep24
KKY264	KKY213 transformed with YEp24
KKY265	KKY213 transformed with *TUP1*/YEp24
KKY266	KKY213 transformed with *SSN6*/YEp24
KKY267	KKY213 transformed with *TUP1+SSN6*/YEp24

KKY268	KKY213 transformed with *α2*/Yep24
KKY269	RMY200 transformed with pKK807 (*matα2:lacZ/LYS2*)
KKY270	KKY269 transformed with pKK797 and cured of pRM200
KKY274	KKY270 transformed with pKK836 and cured of pRM200
KKY275	KKY270 transformed with pKK799 and cured of pKK797
KKY276	KKY270 transformed with pRM430 and cured of pKK797
KKY277	KKY270 transformed with pKK826 and cured of pKK797
KKY281	KKY243 transformed with pKK 795 and cured of pUK421
KKY282	KKY281 that is *hmlΔ::TRP1* instead of *HMLα*
KKY283	KKY282 transformed with pKK806 (*mfa2:lacZ/LYS2*)
KKY284	KKY283 transformed with pKK840
KKY285	KKY283 transformed with pKK841
KKY286	PKY903 transformed with pKK492
KKY287	PKY903 transformed with pKK792
KKY288	KKY286 transformed with *MATa*/YIp5
KKY289	KKY287 transformed with *MATa*/YIp5
KKY290	KKY288 transformed with pKK840
KKY291	KKY288 transformed with pKK841
KKY292	KKY289 transformed with pKK840
KKY293	KKY289 transformed with pKK841
KKY300	RMY200 transformed with pKK792
KKY302	RMY200 transformed with pNH165
KKY332	KKY300 x KKY308 cured of pRM200

KKY333	KKY302 x KKY308 cured of pRM200
KKY336	KKY332 transformed with pRM200 and cured of pKK842
KKY337	KKY332 transformed with pRM430 and cured of pKK842
KKY338	KKY333 transformed with pRM200 and cured of pKK842
KKY339	KKY333 transformed with pRM430 and cured of pKK842
KKY342	RMY200 transformed with pKK806 (integrating *mfa2:lacZ* reporter)
KKY343	KKY193 transformed with pKK806 (integrating *mfa2:lacZ* reporter)
KKY344	KKY342 transformed with pKK797 and cured of pRM200
KKY345	KKY342 transformed with pKK839 and cured of pRM200
KKY346	KKY343 transformed with pKK797 and cured of pRM200
KKY347	KKY343 transformed with pKK839 and cured of pRM200

Table 10. Plasmids

Plasmid	Description	Source/ Reference
pUK421	p*GAL-HHF2*/*TRP1* ARS CEN	(47)
pKK8	*cyc1:lac*Z (no α2/Mcm1 operator)/*URA3* integrating vector	
pKK10	*cyc1:lac*Z + α2/Mcm1 operator between UAS and TATA/*URA3* integrating vector	
pKK794	*cyc1:lac*Z + α2/Mcm1 operator upstream of UAS /*URA3* integrating vector	
pKK561	*HHF2*/*HIS3* ARS CEN	
pKK563	*hhf2Δ4-23*/*HIS3* ARS CEN	
pKK564	*hhf2Δ4-19*/*HIS3* ARS CEN	
pR490	*ADE2*/pBR322	Beth Rockmill
pRM200	*HHF2 HHT2*/ *TRP1* ARS CEN	(59)
pRM420	*HHF2 hht2Δ4-20*/*TRP1* ARS CEN	(59)
pRM430	*HHF2 hht2Δ4-30*/*TRP1* ARS CEN	(59)
pKK798	*hhf2Δ4-23 HHT2*/ *TRP1* ARS CEN	
pKK803	*hhf2Δ4-19 HHT2*/ *TRP1* ARS CEN	

pKK826	*hhf2 (K12Q, K16Q) HHT2/ TRP1* ARS CEN	
pKK830	*hhf2 (K12Q, K16Q) hht2Δ4-30/ TRP1* ARS CEN	
pKK792	*cyc1:lacZ* (no α2/Mcm1 operator)/*ADE2* integrating vector	
PKK793	*cyc1:lacZ* + α2/Mcm1 operator between UAS and TATA/*ADE2* integrating vector	
pNH165	*cyc1:lacZ* + two **a**1/α2 operators between UAS and TATA/*ADE2* integrating vector	Nancy Hollingsworth
pKK795	*hhf2Δ4-19 HHT2 /URA3* ARS CEN	
pKK797	*HHF2 HHT2 /URA3* ARS CEN	
pKK836	*hhf2 (K12Q, K16Q) HHT2/ URA3* ARS CEN	
pKK838	*hhf2 (K12Q, K16Q) hht2Δ4-30/ URA3* ARS CEN	
pKK839	*HHF2 hht2Δ4-30/ URA3* ARS CEN	
pKK840	*HHF2/ HIS3 ADE2* ARS CEN	
pKK841	*hhf2Δ4-23/ HIS3 ADE2* ARS CEN	
pKK806	*mfa2:lacZ/ LYS2* integrating plasmid	
pKK807	*matα2:lacZ/ LYS2* integrating plasmid	
YEp24	2 μm URA3 vector	(5)
pFW28	*TUP1*/YEp24	(107)
pLN113-3	*SSN6*/YEp24	(82)
pKK371	*TUP1* + *SSN6*/YEp24	

References

1. **Astell, C. R., L. Ahlstrom-Jonasson, M. Smith, K. Tatchell, K. A. Nasmyth, and B. D. Hall.** 1981. The sequence of the DNAs coding for the mating-type loci of Saccharomyces cerevisiae. Cell **27**.

2. **Barnes, D. A., and J. Thorner.** 1986. Genetic manipulation of Saccharomyces cerevisiae by use of the LYS2 gene. Molecular and Cellular Biology **6:**2828-2838.

3. **Bender, A., and G. F. Sprague.** 1987. MAT alpha 1 protein, a yeast transcription activator, binds synergistically with a second protein to a set of cell-type-specific genes. Cell **50:**681-691.

4. **Biggin, M. D., and R. Tjian.** 1989. A purified Drosophila homeodomain protein represses transcription in vitro. Cell **58:**433-440.

5. **Botstein, D., S. C. Falco, S. E. Stewart, M. Brennan, S. Scherer, D. T. Stinchcomb, K. Struhl, and R. W. Davis.** 1979. Sterile host yeast (SHY): a eukaryotic system of biological containment for recombinant DNA experiments. Gene **8:**17-34.

6.**Braun, B., and A. D. Johnson.** 1997. The transcriptional repressor TUP1 controls filament formation in thepathogenic yeast Candida albicans. Science .

7. **Brown, H. G.** 1997. Step into my parlor, Cosmopolitan, vol. 222.

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

9. **Chen, J., M. ding, and D. S. Pederson.** 1994. Binding of TFIID to the CYC1 TATA boxes in yeast occurs independently of uupstream activating sequences. Proceeding of the National Academy of Sciences, U.S.A. **91:**11909-11913.

10. **Clark, K. L., D. Dignard, D. Y. Thomas, and M. Whiteway.** 1993. Interactions among the subunits of the G protein involved in Saccharomyces cerevisiae mating. Molecular and Cellular Biology **13:**1-8.

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

12. **Cowell, I. G.** 1994. Repression versus activation in the control of gene expression. Trends in Biochemical Science **19:**38-42.

13. **Damm, K., C. C. Thompson, and R. M. Evans.** 1989. Protein encoded by v-erbA functions as a thyroid-hormone receptor antagonist. Nature **339:**593-597.

14. **Dietzel, C., and J. Kurjan.** 1987. The yeast SCG1 gene: a G alpha-like protein implicated in the a- and alpha-factor response pathway. Cell **50:**1001-1010.

15. **Duronio, R. J., J. I. Gordon, and M. S. Boguski.** 1992. Comparative analysis of the beta transducin family with identification of several new members including PWP1, a nonessential gene of Saccharomyces cerevisiae that is divergently transcribed from NMT1. Proteins **13:**41-56.

16. **Edmondson, D. G., M. M. Smith, and S. Y. Roth.** 1996. Repression domain of the yeast global repressor Tup1 interacts directly with histones H3 and H4. Genes and Development **10:**1247-59.

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

18. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature **340:**245-246.

19. **Fong, H. K., J. B. Hurley, R. S. Hopkins, R. Miake-Lye, M. S. Johnson, R. F. Doolittle, and M. I. Simon.** 1986. Repetitive segmental structure of the transducin beta subunit: homology with the CDC4 gene and identification of related

mRNAs. Proceedings of the National Academy of Sciences of the United States of America **83:**2162-2166.

20. **Fujita, A., S. Matsumoto, S. Kuhara, Y. Misumi, and H. Kobayashi.** 1990. Cloning of the yeast SFL2 gene: its disruption results in pleiotropic phenotypes characteristic for tup1 mutants. Gene **89:**93-99.

21. **Garcia-Higuera, I., J. Fenoglio, Y. Li, C. Lewis, M. P. Panchenko, O. Reiner, T. F. Smith, and E. J. Neer.** 1996. Folding of proteins with WD-repeats: comparison of six members of the WD-repeat superfamily to the G protein beta subunit. Biochemistry **35:**13985-94.

22. **Gaudet, R., A. Bohm, and P. B. Sigler.** 1996. Crystal structure at 2.4 angstroms resolution of the complex of transducin betagamma and its regulator, phosducin. Cell **87:**577-588.

23. **Giesman, D., L. Best, and K. Tatchell.** 1991. The role of RAP1 in the regulation of the MAT alpha locus. Molecular and Cellular Biology **11:**1069-79.

24. **Goutte, C., and A. D. Johnson.** 1988. a1 protein alters the DNA binding specificity of alpha 2 repressor. Cell **52:**875-882.

25. **Goutte, C., and A. D. Johnson.** 1994. Recognition of a DNA operator by a dimer composed of two different homeodomain proteins. EMBO Journal **13:**1434-1442.

26. **Guarente, L., and M. Ptashne.** 1981. Fusion of Escherichia coli lacZ to the cytochrome c gene of Saccharomyces cerevisiae. Proceedings of the National Academy of Sciences, USA **78:**2199-2203.

27. **Gustafsson, C. M., L. C. Myers, Y. Li, M. J. Redd, M. Lui, H. Erdjument-Bromage, P. Tempst, and R. D. Kornberg.** 1997. Identification of Rox3 as a component of mediator and RNA polymerase II holoenzyme. Journal of Biological Chemistry **272:**48-50.

28. **Hall, M. N., and A. D. Johnson.** 1987. Homeo domain of the yeast repressor alpha 2 is a sequence-specific DNA-binding domain but is not sufficient for repression. Science **237:**1007-1012.

29. **Han, K., M. S. Levine, and J. L. Manley.** 1989. Synergistic activation and repression of transcription by Drosophila homeobox proteins. Cell **56:**573-583.

30. **Hanna-Rose, W., and U. Hansen.** 1996. Active repression mechanisms of eukaryotic transcription repressors. Trends in Genetics **12:**229-234.

31. **Harashima, S., A. M. Miller, K. Tanaka, K. Kusumoto, K. Tanaka, Y. Mukai, K. Nasmyth, and Y. Oshima.** 1989. Mating-type control in Saccharomyces cerevisiae: isolation and characterization of mutants defective in repression by a1-α 2. Molecular and Cellular Biology **9:**4523-4530.

32. **Herskowitz, I.** 1989. Life cycle of the budding yeast Saccharomyces cerevisiae. Microbiological Reviews **52:**536-553.

33. **Herskowitz, I.** 1989. A regulatory hierarchy for cell specialization in yeast. Nature **342:**749-757.

34. **Herskowitz, I., J. Rine, and J. Strathern.** 1992. Mating type determination and mating-type interconversion in Saccharomyces cerevisiae, p. 583-656, The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression, vol. 2. Cold Spring Harbor Laboratory Press.

35. **Ho, C., J. G. Adamson, R. S. Hodges, and M. Smith.** 1994. Heterodimerization of the yeast MATa1 and MATα2 proteins is mediated by two leucine zipper-like coiled-coil motifs. EMBO Journal **13:**1403-1413.

36. **Hochstrasser, M., and A. Varshavsky.** 1990. In vivo degradation of a transcriptional regulator: the yeast α2 repressor. Cell **61:**697-708.

37. **Jaynes, J. B., and P. H. O'Farrell.** 1991. Active repression of transcription by the engrailed homeodomain protein. Embo Journal **10:**1427-33.

38. **Jensen, R., G. F. Sprague, and I. Herskowitz.** 1983. Regulation of yeast mating-type interconversion: feedback control of HO gene expression by the mating-type locus. Proceedings of the National Academy of Sciences of the United States of America **80:**3035-3039.

39. **Johnson, A. D.** 1995. Molecular mechanisms of cell-type determination in budding yeast. Current Opinion in Genetics and Development **5:**552-8.

40. **Johnson, A. D.** 1995. The price of repression. Cell **81:**655-658.

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

42. **Johnson, S. L.** 1991. Ph.D. thesis. University of Washington, Seattle.

43. **Kayne, P. S., U. J. Kim, M. Han, J. R. Mullen, F. Yoshizaki, and M. Grunstein.** 1988. Extremely conserved histone H4 N terminus is dispensable for growth but essential for repressing the silent mating loci in yeast. Cell **55:**27-39.

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

45. **Keleher, C. A., S. Passmore, and A. D. Johnson.** 1989. Repressor α2 binds to its operator cooperatively with the yeast protein Mcm1. Molecular and Cellular Biology **9:**5228-5230.

46. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6-Tup1 is a general repressor of transcription in yeast. Cell **68:**pp. 709-719.

47. **Kim, U. J., M. Han, P. Kayne, and M. Grunstein.** 1988. Effects of histone H4 depletion on the cell cycle and transcription of Saccharomyces cerevisiae. EMBO Journal **7:**2211-2219.

48. **Klar, A. J. S., J. N. Strathern, J. R. Broach, and J. B. Hicks.** 1981. Regulation of transcription in expressed and unexpressed mating type cassettes of yeast. Nature **289:**239-244.

49. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD Repeats of Tup1 interact with the homeodomain protein α2. Genes and Development **8:**2857-2867.

50. **Kuchin, S., P. Yeghiayan, and M. Carlson.** 1995. Cyclin-dependent protein kinase and cyclin homologs SSN3 and SSN8 contribute to transcriptional control in yeast. Proceedings of the National Academy of Sciences of the United States of America **92:**4006-4010.

51. **Kunkel, T. A., J. D. Roberts, and R. A. Zakour.** 1987. Rapid and efficient site-specific mutagenesis without phenotypic selection. Methods in Enzymology **154:**367-382.

52. **Lambright, D. G., J. Sondek, A. Bohm, N. P.Skiba, H. E. Hamm, and P. B. Sigler.** 1996. The 2.0 A crystal structure of a heterotrimeric G protein. Nature **379:**311-319.

53. **Laurenson, P., and J. D. Rine.** 1992. Silencers, silencing, and heritable transcription states. Microbiological Reviews **56:**543-560.

54. **Licht, J. D., M. J. Grossel, J. Figge, and U. M. Hansen.** 1990. Drosophila Kruppel protein is a transcriptional repressor. Nature **346:**76-79.

55. **Lin, Y. S., M. F. Carey, M. Ptashne, and M. R. Green.** 1988. GAL4 derivatives function alone and synergistically with mammalian activators in vitro. Cell **54:**659-664.

56. **Lipke, P. N., and C. Hull-Pillsbury.** 1984. Flocculation of Saccharomyces cerevisiae tup1 mutants. Journal of Bacteriology **159:**797-799.

57. **MacKay, V. L., and T. R. Manney.** 1974. Mutations affecting sexual conjugation and related processes in Saccharomyces cerevisiae. II. Genetic analysis of nonmating mutants. Genetics **76:**272-288.

58. **Mak, A., and A. D. Johnson.** 1993. The carboxy-terminal tail of the homeo domain protein alpha 2 is required for function with a second homeo domain protein. Genes and Development **7:**1862-1870.

59. **Mann, R. K., and M. Grunstein.** 1992. Histone H3 N-terminal mutations allow hyperactivation of the yeast GAL1 gene in vivo. EMBO Journal **11:**3927-3306.

60. **Manney, T. R., P. Jackson, and J. Meade.** 1983. Two temperature-sensitive mutants of Saccharomyces cerevisiae with altered expression of mating-type functions. Journal of Cell Biology **96:**1592-1600.

61. **Michaelis, S., and I. Herskowitz.** 1988. The a-factor pheromone of Saccharomyces cerevisiae is essential for mating. Molecular and Cellular Biology **8:**1309-1318.

62. **Miller, A. M., V. L. MacKay, and K. A. Nasmyth.** 1985. Identification and comparison of two sequence elements that confer cell-type specific transcription in yeast. Nature **314:**598-603.

63. **Miller, A. M., and K. A. Nasmyth.** 1984. Role of DNA replication in the repression of silent mating type loci in yeast. Nature **312:**247-251.

64. **Miller, J. H.** 1972. Experiments in molecular genetics. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, New York.

65. **Miyajima, I., M. Nakafuku, N. Nakayama, C. Brenner, A. Miyajima, K. Kaibuchi, K. Arai, Y. Kaziro, and K. Matsumoto.** 1987. GPA1, a haploid-specific essential gene, encodes a yeast homolog of mammalian G protein which may be involved in mating factor signal transduction. Cell **50:**1011-1019.

66. **Muhlrad, D., R. Hunter, and R. Parker.** 1992. A rapid method for localized mutagenesis of yeast genes. Yeast **8:**79-82.

67. **Mukai, Y., S. Harashima, and Y. Oshima.** 1991. AAR1/TUP1 protein, with a structure similar to that of the beta subunit of G proteins, is required for a1-alpha 2 and

alpha 2 repression in cell type control of Saccharomyces cerevisiae. Molecular and Cellular Biology **11:**3773-3779.

68. **Nasmyth, K., and D. Shore.** 1987. Transcriptional regulation in the yeast life cycle. Science **237:**1162-1170.

69. **Neer, E. J., C. J. Schmidt, R. Nambudripad, and T. F. Smith.** 1994. The ancient regulatory-protein family of WD-repeat proteins. Nature **371:**297-300.

70. **Neer, E. J., and T. F. Smith.** 1996. G protein heterodimers: new structures propel new questions. Cell **84:**175-8.

71. **Neigeborn, L., and M. Carlson.** 1987. Mutations causing constitutive invertase synthesis in yeast: genetic interactions with snf mutations. Genetics **115:**247-53.

72. **Ponte, L. d.** 1787. Don Giovanni.

73. **Redd, M. J., M. B. Arnaud, and A. D. Johnson.** 1997. A complex composed of Tup1 and Ssn6 Represses Transcription. Journal of Biological Chemistry .

74. **Redd, M. J., M. R. Stark, and A. D. Johnson.** 1996. Accessibility of alpha 2-repressed promoters to the activator Gal4. Molecular and Cellular Biology **16:**2865-2869.

75. **Remer, S., A. Sherman, E. Kraig, and J. E. Haber.** 1979. Suppressor of deoxythmidine monophosphate uptake in Saccharomyces cerevisiae. Journal of Bacteriology **138:**638-641.

76. **Roth, S. Y.** 1995. Chromatin-mediated transcriptional repression in yeast. Current Opinion in Genetics and Development **5:**168-173.

77. **Roth, S. Y., A. Dean, and R. T. Simpson.** 1990. Yeast alpha2 repressor positions nucleosomes in TRP1/ARS1 chromatin. Molecular and Cellular Biology **10:**2247-2260.

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

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

79. **Sabatini, R.** 1921. Scaramouche. Grosset and Dunlop, New York.

80. **Sauer, R. T., D. L. Smith, and A. D. Johnson.** 1988. Flexibility of the yeast α2 repressor enables it to occupy the ends of its operator, leaving the center free. Genes and Development **2:**807-816.

81. **Schnell, R., and J. D. Rine.** 1986. A position effect on the expression of a tRNA gene mediated by the SIR genes in Saccharomyces cerevisiae. Molecular and Cellular Biology **6:**494-501.

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

83. **Shimizu, M., S. Y. Roth, C. Szent-Gyorgyi, and R. T. Simpson.** 1991. Nucleosomes are positioned with base pair precision adjacent to the alpha2 operator in Saccharomyces cerevisiae. EMBO Journal **10:**3033-3041.

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

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

86. **Smith, D. B., and K. S. Johnson.** 1988. Single-step purification of polypeptides expressed in Escherichia coli as fusions with glutathione S-transferase. Gene **67:**31-40.

87. **Smith, R. L., M. J. Redd, and A. D. Johnson.** 1995. The tetratricopeptide repeats of Ssn6 interact with the homeo domain of alpha 2. Genes and Development **9:**2903-2910.

88. **Sondek, J., A. Bohm, D. G. Lambright, H. E. Hamm, and P. B. Sigler.** 1996. Crystal structure of a GA protein beta gamma dimer at 2.1A resolution. Nature **379:**369-374.

89. **Spain, B. H., D. Koo, M. Ramakrishnan, B. Dzudzor, and J. Colicelli.** 1995. Truncated forms of a novel yeast protein suppress the lethality of a G protein alpha subunit deficiency by interacting with the beta subunit. Journal of Biological Chemistry **270:**25435-25444.

90. **Sprague, G. F., and J. W. Thorner.** 1992. Pheromone response and signal transduction during the mating process of Saccharomyces cerevisiae, p. 657-744, The Molecular and Cellular Biology of the Yeast Saccharomyces: Gene Expression, vol. 2. Cold Spring Harbor Laboratory Press.

91. **Strathern, J., J. Hicks, and I. Herskowitz.** 1981. Control of cell type in yeast by the mating type locus: the $\alpha 1$-$\alpha 2$ hypothesis. Journal of Molecular Biology **147:**357-372.

92. **Tatchell, K., K. A. Nasmyth, B. D. Hall, C. Astell, and M. Smith.** 1981. In vitro mutation analysis of the mating-type locus in yeast. Cell **27:**25-35.

93. **Thrash-Bingham, C., and W. L. Fangman.** 1989. A yeast mutation that stabilizes a plasmid bearing a mutated ARS1 element. Molecular and Cellular Biology **9:**809-816.

94. **Trumbly, R. J.** 1988. Cloning and characterization of the CYC8 gene mediating glucose repression in yeast. Gene **73:**97-111.

95. **Trumbly, R. J.** 1992. Glucose repression in the yeast Saccharomyces cerevisiae. Molecular Microbiology **6:**15-21.

96. **Trumbly, R. J.** 1986. Isolation of Saccharomyces cerevisiae mutants constitutive for invertase synthesis. Journal of Bacteriology **166:**1123-1127.

97. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature **369:**758-61.

98. **Varanasi, U. S., M. Klis, P. B. Mikesell, and R. J. Trumbly.** 1996. The Cyc8 (Ssn6)-Tup1 corepressor complex is composed of one Cyc8 and four Tup1 subunits. Molecular and Cellular Biology **16:**6707-6714.

99. **Vershon, A. K., Y. Jin, and A. D. Johnson.** 1995. A homeo domain protein lacking specific side chains of helix 3 can still bind DNA and direct transcriptional repression. Genes and Development **9:**182-192.

100. **Vitti, J.** 1990. "Bart the Genius 7G02," The Simpsons. cr. M. Groening, dir. D. Silverman, prod. M. Groening, J. L. Brooks, B. Oakley, J. Weismuller. Fox Broadcasting Company. aired 14 January 1990.

101. **Voorn, L. v. d., and H. L. Ploegh.** 1992. The WD-40 repeat. FEBS Letters **307:**131-134.

102. **Wahi, M.** 1996. Ph.D. thesis. University of California, San Francisco.

103. **Wahi, M., and A. D. Johnson.** 1995. Identification of genes required for alpha 2 repression in Saccharomyces cerevisiae. Genetics **140:**79-90.

104. **Wall, M. A., D. E. Coleman, E. Lee, J. A. Iniguez-Lluhi, B. A. Posner, A. G. Gilman, and S. R. Sprang.** 1995. The structure of the G protein heterotrimer Gi alpha 1 beta 1 gamma 2. Cell **83:**1047-1058.

105. **Whiteway, M., L. Hougan, D. Dignard, D. Y. Thomas, L. Bell, G. C. Saari, F. J. Grant, P. O'Hara, and V. L. MacKay.** 1989. The STE4 and STE18 genes of yeast encode potential beta and gamma subunits of the mating factor receptor-coupled G protein. Cell **56:**467-477.

106. **Whiteway, M., L. Hougan, and D. Y. Thomas.** 1990. Overexpression of the STE4 gene leads to mating response in haploid Saccharomyces cerevisiae. Molecular and Cellular Biology **10**.

107. **Williams, F. E., and R. J. Trumbly.** 1990. Characterization of TUP1, a mediator of glucose repression in Saccharomyces cerevisiae. Molecular and Cellular Biology **10:**6500-6511.

108. **Wilson, K. L., and I. Herskowitz.** 1984. Negative regulation of STE6 gene by the α2 gene product of yeast. Molecular and Cellular Biology **4:**2420-2427.

109. **Yamashiro, C. T., D. J. Ebbole, B. U. Lee, R. E. Brown, C. Bourland, L. Madi, and C. Yanofsky.** 1996. Characterization of rco-1 of Neurospora crassa, a pleiotropic gene affecting growth and development that encodes a homolog of Tup1 of Saccharomyces cerevisiae. Molecular and Cellular Biology **16:**6218-6228.

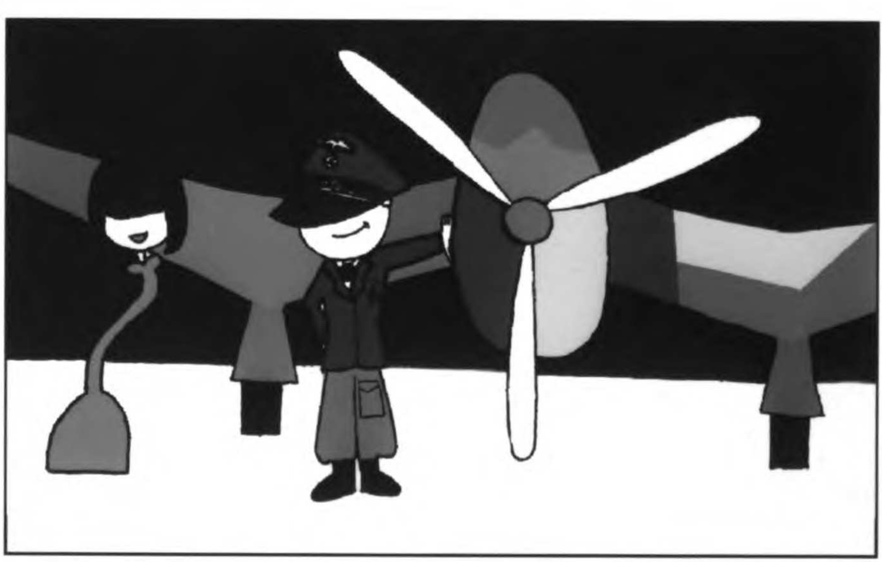
110. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103-119.

111. **Yuan, Y. O., I. L. Stroke, and S. Fields.** 1993. Coupling of cell identity to signal response in yeast: interaction between the α1 and STE12 proteins. Genes and Development **7:**1584-1597.

112. **Zhang, M., L. S. Rosenblum-Vos, C. V. Lowry, K. A. Boakye, and R. S. Zitomer.** 1991. A yeast protein with homology to the beta-subunit of G proteins is involved in control of heme-regulated and catabolite-repressed genes. Gene **97:**153-161.

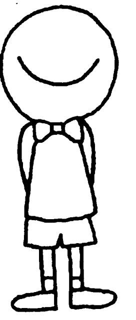
113. **Zhou, Z., and S. J. Elledge.** 1992. Isolation of crt mutants constitutive for transcription of the DNA damage inducible gene RNR3 in Saccharomyces cerevisiae. Genetics **131:**851-856.

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



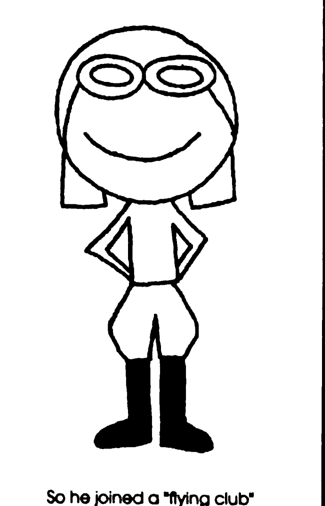
The Bomber of Civilians,
or
"The Useless Propeller"

There once was a boy named Siegfried Siegmund Notung Sachs. People felt sorry for him, but Siegfried knew that even if this isn't the best of all possible worlds, it can always get worse.



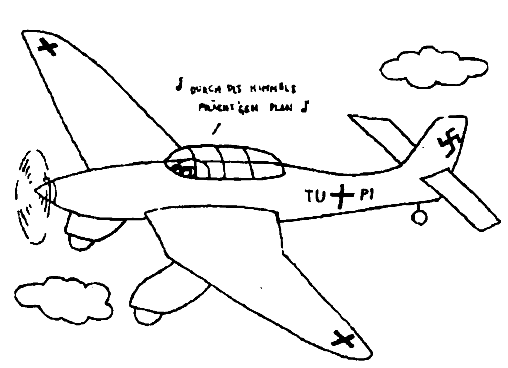
After all, his mother could have had a thing for Strauss.

It soon became obvious that Siegfried was no Heldentenor.



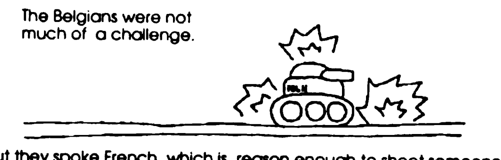
So he joined a "flying club"

and got a plane with bombs and wing guns and a cool air siren that he could use to scare the shit out of people on the ground.



Plus he got to blow things up, which was a lot of fun.

The Belgians were not much of a challenge.

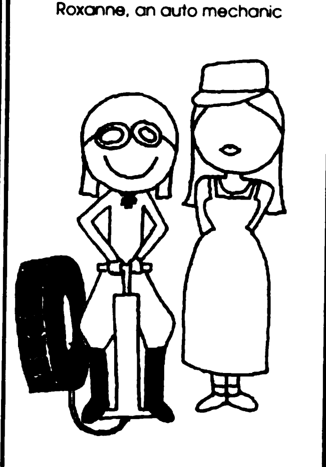


But they spoke French, which is reason enough to shoot someone.

Siegfried was quick to make his presence known among the female half of the occupied population.

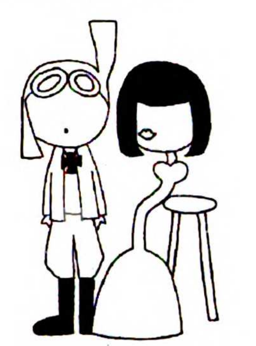
Three of the women who succumbed to his dubious charms were Mignon, the manager of a sweet shop,

Roxanne, an auto mechanic

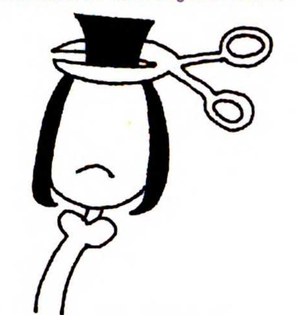


(apologies to Donna Barr)

and Eva, a Parisian Freudenmädchen who found her country to be highly overrated and in need of a good invasion.



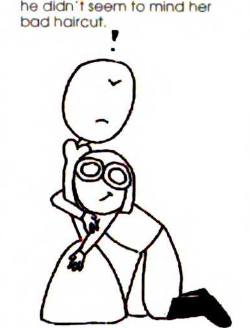
There was a war going on, though, and international relationships of this sort were frowned upon. When Allied officials found out about Siegfried and Eva,



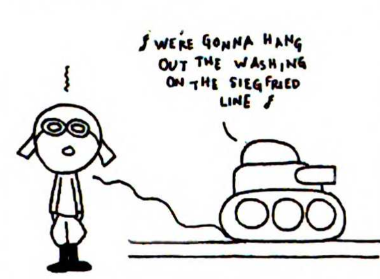
they cut off Eva's hair as punishment for collaborating with the enemy

and shot the propeller off of Siegfried's plane.

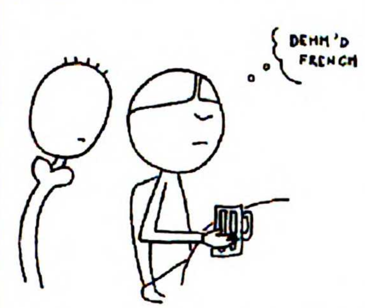
Since Siegfried had never paid all that much attention to any part of Eva above her neck, he didn't seem to mind her bad haircut.



But for Eva, the affair was over. Without his plane, Siegfried was a threat to no one and something of an embarrassment to boot.



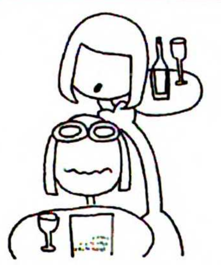
Eva had always thought the English were even more ridiculous than the French, but she found herself making eyes at a RAF pilot who flew a Spitfire.



Siegfried was bereft. Grounded and unloved, he took to hanging out in cheap cafes, singing cheesy cabaret tunes,

and writing sentimental drivel.

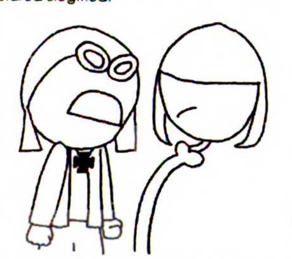
He was halfway through Chapter Two of his memoirs when a sultry voice behind him said,



"You don't expect anyone to publish that, do you?"

It was Eva, sporting a sneer and a strangely Aryan hair color, neither of which Siegfried found particularly attractive.

"Why, bless my soul, if it isn't Lionel Trilling!" blared Siegfried.

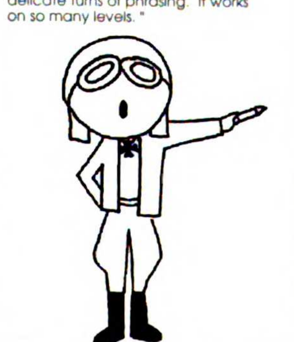


"Really, dear, you mustn't shout and speak German at the same time," said Eva, wiping her face. "It's most unpleasant."

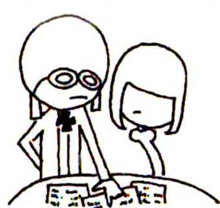
"Hmf," sniffed Siegfried, "Just wait. People are going to pay me to read about this. You think it's just a dumb story about an airplane. And it is. But it also happens to be very well written!"

"Yes, your penmanship is lovely," noted Eva earnestly.

"It's got irony, it's got foreshadowing, it's got nicely rounded vowels and delicate turns of phrasing. It works on so many levels."

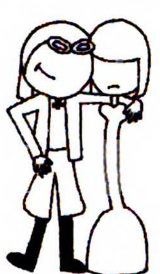


For instance," he continued, "which Panzer Division was the first to cross the Meuse?"
"The Seventh," answered Eva.
"How many years did it take Friedrich der Grosse to slap Europe into shape?"
"Seven."



"Can it possibly be a coincidence that there were seven blades on my plane's propeller before those damned Tommies shot it off? It's a sign: Götterdämmerung is approaching!"
Eva was silent.

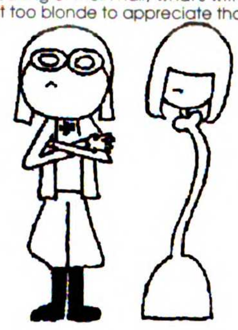
"Let me fill in that blank stare of yours," said Siegfried in his most smugly Teutonic manner. "Seven is a magic number. Seven days in the week, seven wonders of the world, seven seas to sail, seven continents to conquer."



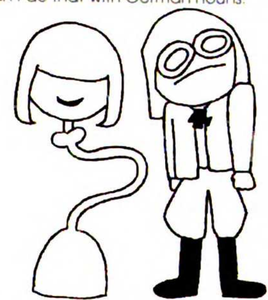
What's the highest level of heaven? How many tiers are in a ziggurat? How many sages laid the foundation for Gilgamesh's city? How many sins are deadly? For how many years does scabies make you itch? How many years bad luck do you get for breaking a mirror? How many doors are in Bluebeard's castle? How many veils are in Salome's dance? How many samurai does it take to save a village?

How many swans are a-swimming? What times six is forty two? Jawohl, mein Herr, you've got a seven-watt Leitmotiv blazing through this Lebenswerk of yours

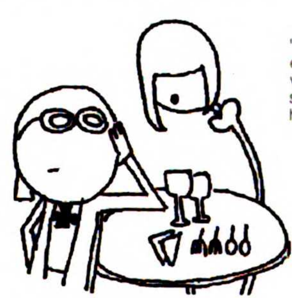
"Don't try to be clever," sulked Siegfried. "It's perfectly easy to be clever. And speaking of mein hair, what's with the Eva Braun 'do? I think you're just too blonde to appreciate that there's a Meaning here."



"Don't you love English?" mused Eva. "How you can give words significance just by capitalizing their first letter: God, Heaven, a Meaning. You can't do that with German nouns."



"I'll bet it's because those stupid Amis need to be told which nouns are proper. I mean, have you ever tried to take an American anywhere? No sense of propriety whatsoever.



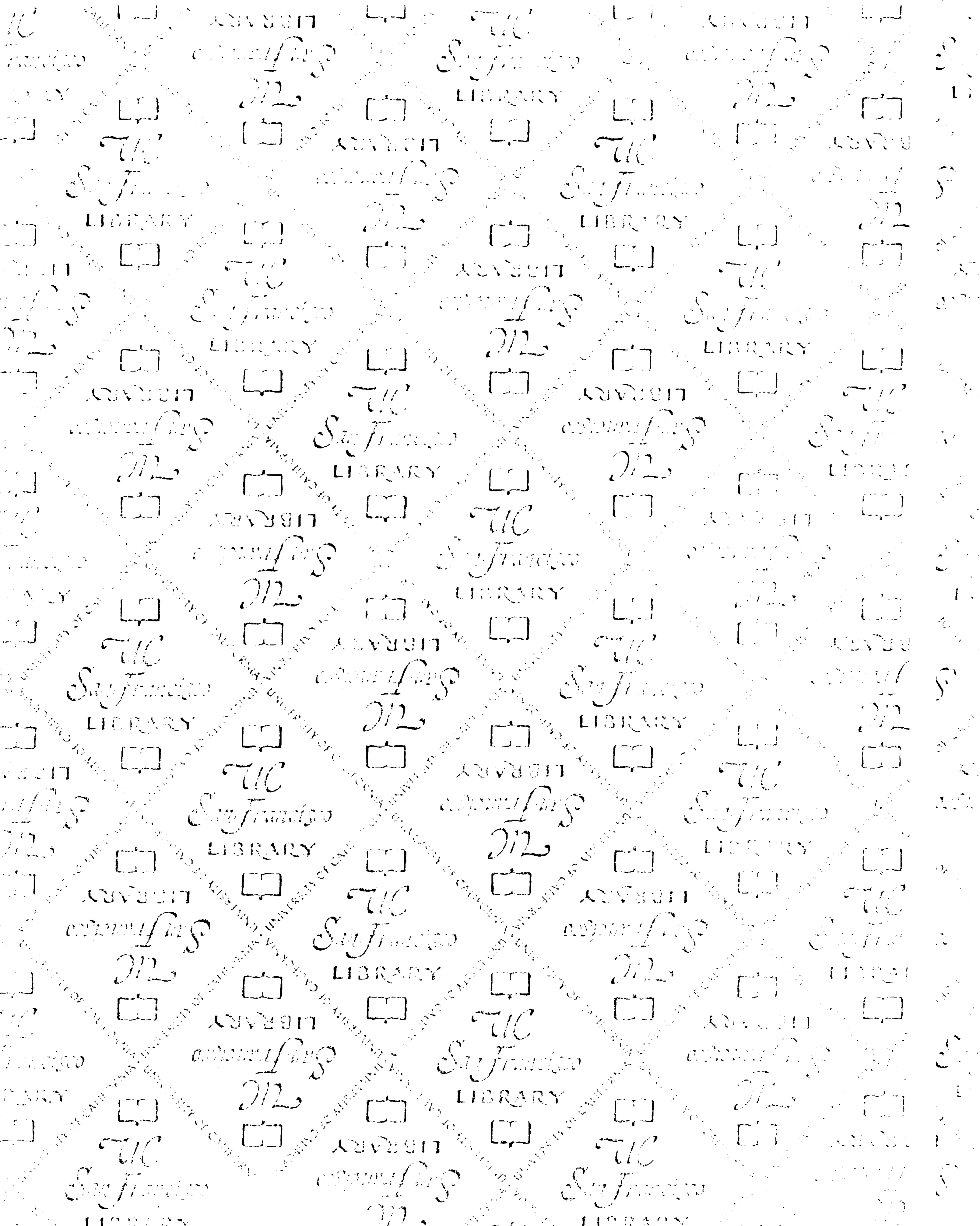
"Schiß, they can't even figure out which hand you're supposed to use to hold your fork."

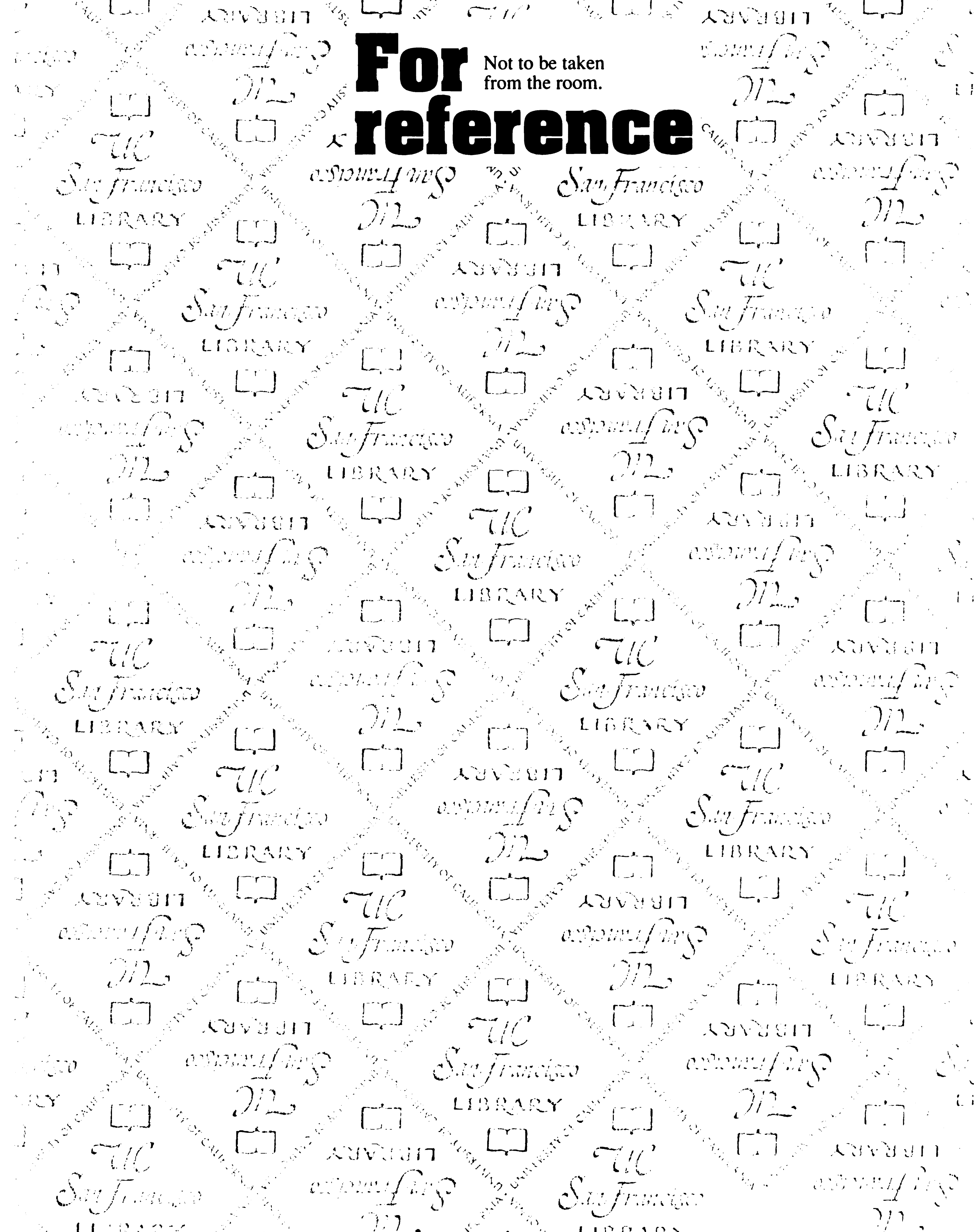
"Um Gottes willen, will you shut up?" shouted Siegfried. "I'm talking about life, the universe, and everything, and you're babbling about grammar and etiquette! This isn't just a story about you and my airplane, Eva. It has broad implications."

"Oh, I suppose you could say that," consoled Eva. "And you could probably even convince people that it's true. People will believe almost anything."

"But you know what I think?" she asked.
"Was?"

"Der Mond ist wie der Mond. Das ist alles.
Wir wollen hineingehen."




For reference
Not to be taken from the room.