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Analysis of the SIN1 and SIN2 gene products and their role in transcriptional regulation in Saccharomyces Cerevisiae

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Analysis of the *SIN1* and *SIN2* gene products and their role in transcriptional regulation in *Saccharomyces cerevisiae*

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DEDICATION

To the memory of John Coleman Kruger
who first introduced me to the
concept of recombinant DNA.

PREFACE AND ACKNOWLEDGEMENTS

The work described in the following chapters has occupied a good deal of my life over the last four and one-quarter years. It would not have been possible, however, without the generous help of many colleagues here at U.S.C.F. who helped teach me how to do experimental science. The knowledge and training which I have received during this period I am sure will prove invaluable in the future.

I wish to thank Craig Peterson and Brenda Andrews for their valuable advice about experimental design, discussions on HO regulation, and material assistance in many of the experiments described within. I especially wish to thank Craig in this regard, whose energy and zeal inspired me. I also wish to thank the members of the Herskowitz lab for creating an intellectually stimulating environment and putting up with me when I was in a bad mood.

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ANALYSIS OF THE SIN1 AND SIN2 GENE PRODUCTS AND THEIR ROLE IN TRANSCRIPTIONAL REGULATION IN SACCHAROMYCES CEREVISIAE

by Warren David Kruger

ABSTRACT

The SIN1 and SIN2 genes of Saccharomyces cerevisiae were initially identified as negative regulatory genes involved in the precise transcriptional control of HO. Mutation in either SIN1 or SIN2 allows HO to be transcribed in the absence of certain SWI activator genes. I show here that SIN1 and SIN2 mutations also allow transcription from other enfeebled promoters. I have cloned SIN1 and found it to be identical to a previously identified gene SPT2. I analyzed the sequence of SIN1/SPT2 and found that the predicted protein has similarity to mammalian HMG1, a non-histone component of chromatin. The SIN1 protein is concentrated in the nucleus and binds to DNA with no detectable sequence specificity, thus exhibiting properties similar to HMG1. I have also cloned and determined the sequence of SIN2 and discovered that it is identical to HHT1, one of the two yeast genes which code for histone H3. These studies indicate that chromatin proteins are involved in regulation of transcription in yeast.

Null mutations in either SIN1 or SIN2 have less of an effect on HO transcription than do certain point mutations. These point mutations, but not null mutations, are semi-dominant. These findings suggest that an altered form of these proteins can interfere with wild-type protein function. Since SIN2, and probably SIN1, are

members of functionally redundant gene families, these observations suggest that dominant interfering mutations affecting chromatin components can be used to assign in vivo function to these products.

We have examined in detail the role of SIN1 in regulating HO transcription. HO is transcribed only in mother cells during the late G1 phase of the cell-cycle. This transcriptional control is achieved by the interaction of two large cis-acting regulatory modules, URS1 and URS2, which provide signals necessary for mother/daughter and cell-cycle control, respectively. I show here that mutations in SIN1 cause deregulated HO transcription by allowing activation of URS2 in the absence of URS1 activation. Based on these observations, I propose that SIN1 function is normally required for proper integration of the mother/daughter and cell-cycle control information at HO.

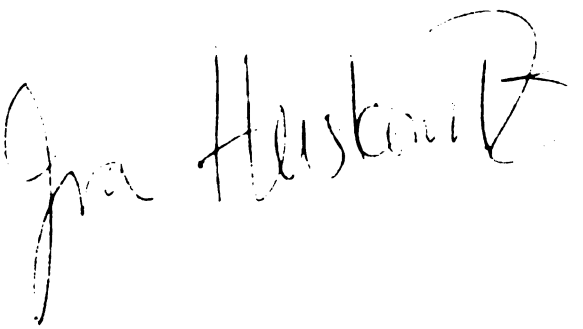

TABLE OF CONTENTS

LIST OF TABLES

Chapter 2

Chapter 3

Chapter 4

LIST OF FIGURES

Appendix

CHAPTER 1

INTRODUCTION

Introduction

The work described in the following chapters concerns the analysis of SIN1 and SIN2, two genes that were initially identified as negative regulators of HO transcription in Saccharomyces cerevisiae (Sternberg et al., 1987). At the beginning of these studies, essentially all that was known about SIN1 and SIN2 was that mutations in either gene allowed transcription of HO in circumstances in which it was normally not transcribed. My goal was to determine how these negative regulators functioned. At this time we entertained the hypothesis that the SIN1 and SIN2 gene products acted to repress transcription by being part of or affecting site-specific DNA-protein complexes at HO. As our studies progressed a second possible hypothesis emerged: the SIN1 and SIN2 gene products could negatively regulate transcription by being part of or influencing chromatin structure. It should be noted that these two models are not mutually exclusive: site-specific DNA binding proteins could repress transcription by altering chromatin structure in the local vicinity. In this introduction I will set the stage for the later chapters by discussing in more detail illustrations of both types of negative regulation of transcription, emphasizing examples from Saccharomyces cerevisiae.

How transcription works: the current view

Before discussing negative regulation of transcription, I shall first discuss the transcription process in the absence of negative

regulation. The key player in the transcription process is the RNA polymerase enzyme. Transcriptional regulation is the process of getting this protein to initiate transcription on the correct DNA sequence only during proper conditions at the right time. How is this done?

In prokaryotes, this process is in part determined by the polymerase molecule itself in combination with an auxiliary subunit known as a sigma factor (see Stragier and Losick, 1990). The polymerase-sigma complex recognizes specific DNA sequences at the promoter, binds, undergoes a conformational change, and then begins to transcribe. Different sigma factors control the ability of the polymerase-sigma complex to recognize specific DNA sequences at the promoter. Thus the availability of different sigma factors and the promoter sequence determines what genes are transcribed.

Site-specific DNA binding proteins also play a role in the regulation of prokaryotic transcription. For example, the catabolic gene activator protein-cAMP complex (CAP-cAMP) binds to specific DNA sequences in several bacterial promoters and stimulates transcription (see Reznikoff et al., 1985). The mechanism by which CAP-cAMP stimulates transcription seems to depend on the promoter context. At the galP1 promoter, CAP-cAMP increases the rate of the conformational change in polymerase from the open to the closed form (Herbert et al., 1986), whereas at the lac promoter, CAP-cAMP increases the binding affinity of polymerase to the promoter (Straney et al., 1989). There are also many examples of negative regulation of transcription by site-specific DNA binding proteins in prokaryotes which are described in the next section

Site-specific DNA binding proteins also play an important role in transcriptional control in eukaryotes. The sites to which these proteins bind are found in transcriptional control regions know as enhancers in higher eukaryotes or upstream activation sequences (UAS) in yeast. Enhancers and UAS elements are functionally defined by their ability to increase transcription from nearby promoters. It is thought that the proteins which bind to these sites somehow interact with other proteins in the general transcription machinery. This general machinery (for genes transcribed by RNA polymerase II (Pol II)) consists of the RNA polymerase II enzyme as well as at least six other auxiliary transcription factors (see Kadonaga, 1990). Among these auxiliary factors is TFIID, the so-called TATA binding protein. TFIID binds A-T rich sequences that are found near the transcriptional initiation point for most Pol II transcribed genes. Studies show that the first step in creating an initiation complex for RNA polymerase II is binding of TFIID to the TATA sequences. Later, TFIIA, TFIIB, Pol II, and TFIIE/F bind respectively (Buratowski et al., 1989).

Because TFIID binding is rate limiting in vitro, it has been proposed that enhancer- or UAS-binding proteins may work by aiding TFIID binding in vivo (Meisterernst and Roeder, 1990). One idea is that the DNA sequences between the UAS and the TATA are looped out in order for factors bound upstream to help stabilize TFIID binding by direct or indirect contact. Recent experiments have shown that one upstream activator protein, VP16, binds to TFIID with high affinity in vitro, lending support to this model (Stringer and Greenblatt, 1990). However, as VP16 also binds with high

affinity to TFIIB in vitro, it is unclear which contact is important for activation (Lin and Green, 1991). It should be noted that in vitro most genes do not require upstream activators to initiate transcription, but in vivo they do. This observation suggests the presence of some inhibitory factors in vivo.

In the following sections this overview will provide a framework to help understand some of the potential mechanisms of negative regulation.

Negative regulation by site-specific DNA binding proteins.

There are many examples of negative regulation by site-specific DNA binding proteins in bacteria. In these cases negative regulation is thought to occur by steric hindrance of the polymerase binding site at the promoter. Binding sites (operators) for repressor proteins are located within promoters. When the repressor protein is present RNA polymerase cannot bind; therefore transcription can not occur. Examples of this mechanism occur at the P_R promoter by λ repressor and cro (see Ptashne 1987; Ptashne et al., 1980; Gussin et al., 1983), in the regulation of SOS genes by LexA (see Gottesman 1984), and in the regulation of lac P_2 promoter by cAMP receptor protein (Reznikoff 1978; Malan and Mclure, 1984).

In eukaryotes there are two potential targets for negative regulation: the RNA polymerase and associated proteins (i.e. general transcription machinery) and the upstream activator proteins. An example of negative regulation of an upstream activator protein occurs in the interaction between the GAL4 and GAL80 proteins of yeast. The GAL4 activator, which binds to a 17-basepair sequence

located upstream of many galactose inducible genes, is active only when cells are grown in galactose (West et al., 1984; Giniger et al., 1985). In the absence of galactose, negative regulation of GAL4 is brought about by interaction with the negative regulator GAL80. Mutations in the GAL80 gene lead to constitutive activity of GAL4 (Nogi et al., 1977; Perlman and Hopper, 1979). The GAL80 protein has been shown to bind to the C-terminal end of GAL4 (Lue et al., 1987; Johnston et al., 1987; Ma and Ptashne, 1987). The GAL80 protein appears constitutively bound to GAL4 but inhibits GAL4 activity only in the absence of galactose, possibly by undergoing a conformational change (Parthun and Jaehning 1990; Chasman and Kornberg, 1990). GAL80 can negatively regulate the UAS activity of GAL4 binding sites but not other UAS sequences located nearby (see Struhl, 1985, Table 2; E. Giniger, unpublished). These observations suggest that GAL80 works by binding to GAL4 and "masking" its activation sequences, thus preventing them from contacting their normal targets.

In the above example the negative regulation is specific to a single activator protein. Other repression mechanisms can act on several different activators. An example of this occurs in the repression of a-specific gene transcription by the MATα2 gene product in yeast. The MATα2 gene product represses transcription through a 32-basepair operator sequence found upstream of all a-specific genes (Johnson and Herskowitz, 1985; Wilson and Herskowitz, 1986). This operator sequence can repress transcription from many different UAS sequences when placed either between UAS and TATA sequences, or when placed as far as a few

hundred basepairs upstream of a UAS (Johnson and Herskowitz, 1985; Caroline Goutte, personal communication). The ability of the α2 operator to function from a variety of locations suggests that the α2 protein does not act by sterically hindering RNA polymerase binding.

The α2 protein binds to the α2 operator cooperatively with the MCM1 protein in vitro (Johnson and Herskowitz, 1985; Keleher and Johnson, 1987; Keleher et al., 1989). Mutations in either MATα2 or MCM1 abolish repression by these operator sequences in vivo, indicating that both proteins are required for repression (Hall and Johnson, 1987; Elble et al., submitted). Studies examining the effects of α2-MCM1 on the binding of the site-specific activator protein GAL4 show that the operator does not interfere with GAL4 binding in vivo (Micheal Redd, personal communication). Thus α2-MCM1 does not appear to work by preventing site-specific activators from binding. This observation implies that α2-MCM1 affects some step downstream of activator binding in the transcription process. For example, it may interfere with the ability of the activator to contact the general transcription machinery or the ability of the general transcription machinery to bind to DNA.

Although repression by the α2 operator can work from a short distance away from a UAS, repression by the silencers at HMR and HML can function over much greater distances. Site-specific DNA binding proteins play an important role in this type of repression. Normally, genes located at HMR or HML are not expressed due to the actions of cis-acting silencer DNA sequences located nearby called E (Brand et al., 1985). The E sequences are able to repress

transcription of heterologous genes from distances as far as 2.5kb away (Schnell and Rine, 1986; Brand et al., 1985; Buchman et al., 1987; Shore and Nasmyth, 1987; Shore et al., 1987). The E region is composed of three distinct regulatory elements, called A, E, and B (Brand, 1986; Brand et al., 1987). These three elements appear to be functionally redundant: mutation in any one does not destroy silencer activity, but mutation in any two does. Site-specific DNA binding proteins have been detected binding to all three of these elements (Shore et al., 1987; Kimmerly et al., 1988). One of these site-specific binding factors has been cloned and codes for an essential protein named RAP1. An allele of RAP1 has been described which causes derepression of the silent loci, indicating that RAP1 is important for silencing (Kurtz and Shore, 1991). Thus at least one site-specific DNA binding protein is essential for silencing. I will discuss silencing further in the next section.

Negative regulation by chromatin structure: overview.

Chromatin refers to the complex of DNA and proteins found in the chromosomes of eukaryotes. The main protein components of chromatin are the histone proteins which form the nucleosome particle, and HMG proteins which are non-histone components of chromatin. I distinguish between chromatin proteins and site-specific transcription factors by the former's lack of site-specific DNA binding, relatively low DNA-binding affinity, and high abundance (see Alberts et al.). It is thought that the primary role of chromatin proteins is to "package" the DNA so it will fit into the confining

space of the nucleus. As described below, a second function may be to help regulate transcription.

In thinking about the role of chromatin proteins in transcription it is important to realize that the most of the DNA in the cell is complexed with chromatin proteins. One might imagine two mechanisms by which chromatin proteins could negatively influence transcription: (1) they could interfere or compete with DNA binding by activator proteins or the general transcription machinery; or (2) they could interfere with protein-protein contacts between bound upstream activators and the general transcription machinery. In the following sections I will describe evidence which supports the idea that chromatin proteins, in particular histones, negatively regulate transcription.

The absence of chromatin proteins correlates with increased transcription

In eukaryotes changes in chromatin structure of individual genes have been correlated with expression. In Drosophila it has been noted that genes which are transposed by chromosomal rearrangement to locations in or near heterochromatin have reduced expression (Baker, 1968; Spofford, 1983). Heterochromatin refers to the regions of the chromosome which are highly condensed or have a more compacted chromatin structure. Similarly, mammalian genes located on the highly condensed X-chromosome in females, known as the Barr body, are not expressed (see Gartler et al.,1983). These observations suggest that certain higher ordered chromatin structures can repress transcription.

It is possible to examine the chromatin structure of individual genes by examining their nuclease sensitivity. Nucleases, such as micrococcal nuclease, are only able to cleave naked DNA and not DNA complexed with other proteins. In general, it has been observed that genes that are not expressed have a more micrococcal nuclease resistant chromatin structure then expressed genes. In yeast it has been observed that chromatin at promoters of repressed genes is more resistant to cleavage by micrococcal nuclease than chromatin at promoters of transcribed genes (see Perez-Ortin, 1989). One example of this occurs at the PHO5 promoter in yeast. The PHO5 gene, which codes for a secreted acid phosphatase, is transcribed only in cells grown in media containing low levels of inorganic phosphate (Bergman et al., 1986). In repressing conditions (high levels of inorganic phosphate), there are six precisely positioned nucleosomes covering the upstream regulatory sequences of PHO5. Upon induction, four of these nucleosomes are removed (Almer et al., 1986). Two site specific activator proteins, PHO2 and PHO4, are required for this change in chromatin structure to occur (Fascher et al., 1990). However, these observations do not allow determination of whether changing chromatin structure allows transcription or whether transcription causes changes in chromatin structure.

Chromatin represses transcription in vivo

Recently, in vivo evidence has emerged that supports the view that chromatin proteins negatively regulate transcription. The first example of this was described for a HIS4 gene in yeast in which transcription had been reduced by the insertion of a δ element in its

upstream regulatory sequence. The δ element insertion, resulting from Ty insertion followed by subsequent excision, causes a large reduction in transcription from the normal promoter of the HIS4 gene. In the labs of Gerry Fink and Fred Winston, mutations were isolated in genes (called SPT genes) which restored transcription of HIS4 (Winston et al., 1984; Fassler and Winston, 1988). Two of these genes, SPT11 and SPT12, were shown to be the same as HTA1 and HTB1, two of the structural genes coding for histone proteins H2A and H2B (Clark-Adams et al., 1988). Experiments examining the dosage effects of the various histone genes suggested that altering the ratio of H2A-H2B dimers to H3-H4 dimers was sufficient to account for the SPT phenotype (Clarke-Adams et al., 1988; Sherwood and Osley, submitted). Additional studies showed that chromatin structure, as judged by nuclease sensitivity, was indeed altered in cells carrying either deletions or duplications of genes coding for H2A and H2B (Norris et al., 1988). These studies indicate that alterations of chromatin structure can cause derepression of transcription from from some promoters in yeast.

The mechanism of repression of the silent mating loci, HMR and HML, also suggests an important role for chromatin. Nuclease sensitivity studies have suggested that the chromatin structure at HML is different than the chromatin structure of the same DNA sequences at MAT (Nasmyth, 1982). Recently genetic experiments have confirmed that chromatin plays an important role in silencing. In cells which produce a histone H4 protein lacking its N-terminal residues, HML and HMR are derepressed (Kayne et al., 1988). Further studies have specifically implicated lysine residues in this region

as being critical for repression (Johnson et al., 1990). Four genes, SIR1-SIR4, have been shown to be required for silencing, but their biochemical role is still unknown (Rine and Herskowitz, 1987). Mutations in the SIR genes do not affect any of the site-specific DNA binding activities described earlier (Buchman et al., 1988). One of the SIR genes, SIR3, may be involved in influencing chromatin structure as extragenic suppressors of the lysine mutations in histone H4 described above map to SIR3 (Johnson et al., 1990). Thus studies on silencing indicate that both site-specific DNA binding proteins and chromatin structure are involved. It is thought that the site-specific proteins somehow demarcate a "chromatin domain", which has a unique chromatin structure. How these site specific proteins influence the surrounding chromatin is unknown.

Recent evidence suggests that negative regulation of transcription by chromatin occurs at almost all yeast promoters. Experiments in which nucleosomes have been depleted in cells by genetic means have shown that a wide variety of promoters are activated even when their upstream activating sequences have been deleted (Han and Grunstein, 1988; Han et al., 1988). These UASless genes are transcribed at about 10-15% of their fully induced level. These experiments suggest that basal level transcription is normally repressed by nucleosomes. These findings are consistent with the idea that one of the roles of UAS binding factors may be to overcome repression by nucleosomes.

Histones can repress transcription *in vitro*

Studies on the effect of histones on transcription in vitro are consistent with the in vivo studies. Specifically, nucleosomes repress transcription in vitro. Studies on both class II (transcribed by RNA polymerase II) and class III genes (transcribed by RNA polymerase III) have demonstrated that chromatin-associated DNA templates are unable to be transcribed unless first incubated with specific transcription factors. For example, transcription factor TFIID allows in vitro transcription of the adenovirus major late promoter only if added before the template is assembled into nucleosomes (Workman and Roeder, 1987). Analogous results have been observed for TFIIIA in the transcription of 5S RNA genes in vitro (see Brown, 1984). These results show that chromatin can repress transcription in vitro by competing for DNA binding sites with transcription factors. Using extracts in which transcription and nucleosome assembly occur simultaneously, it has been observed that USF, an upstream activator protein, stimulates transcription better in the presence of histones then in their absence. The above result is seen because induced transcription (i.e. in the presence of USF) is repressed less efficiently by histones then is basal transcription (Workman et al., 1990). This observation suggests one of the functions of upstream factors may be to overcome repression by chromatin.

Conclusion

Negative regulation of transcription can involve two different types of proteins; proteins involved in site-specific DNA binding and chromatin proteins. In the following chapters I will show an

analysis of the negative regulators encoded by SIN1 and SIN2 and their role in regulating transcription of a variety of genes in yeast, emphasizing their role in the regulation of HO. This analysis will demonstrate that these two proteins are chromatin proteins and that their normal function is required for proper transcriptional regulation. These studies reinforce the role of chromatin in the process of transcriptional regulation.

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CHAPTER 2

A NEGATIVE REGULATOR OF HO TRANSCRIPTION, SIN1 (SPT2), IS A NONSPECIFIC DNA-BINDING PROTEIN RELATED TO HMG1

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Abstract

The *SIN1* gene was initially identified because mutations in *SIN1* bypass the need for *SWI1* to activate transcription of the yeast *HO* gene. We show here that transcription of *HO* in *swi1*⁻ *sin1*⁻ cells efficiently utilizes the normal start site. We have cloned *SIN1* and found that it is identical to the previously identified gene *SPT2*, mutations in which allow transcription from certain mutated regulatory regions. The predicted SIN1/SPT2 protein has a distinctive amino acid composition (45% charged residues, 25% basic and 20% acidic) and has similarity to the mammalian HMG1 protein, a non-histone component of chromatin. We show that SIN1 is concentrated in the nucleus and binds to DNA with little or no sequence specificity *in vitro*. It thus exhibits properties of an HMG protein. Addition of random DNA segments to a test promoter alters regulation by *SIN1* in a manner similar to addition of a segment from the *HO* upstream region. Functional analysis of certain *SIN1* mutations suggests that SIN1 may be part of a multiprotein complex. Based on these results, we propose that SIN1 is a non-histone component of chromatin which creates the proper context for transcription. Because *sin1* mutants exhibit increased loss of chromosome III, SIN1 may also play a role in fidelity of chromosome segregation.

Introduction

The Saccharomyces cerevisiae HO gene provides an opportunity to study how multiple inputs regulate gene expression. HO encodes a site-specific endonuclease necessary for initiation of mating-type interconversion (20), the process by which yeast cells convert between **a** and α cell types. Regulation of this process is determined by transcriptional control of HO (for review see 13). HO is expressed only in **a** and α cells, only in mother cells, and only during the late G1 phase of the cell cycle (17, 31). Transcription of HO is controlled by a regulatory region that extends 1400 bp upstream of the transcription start site and is functionally divided into two sub-regions, URS1 and URS2 (32,33). URS1 is responsible for mother/daughter regulation of HO (32, 53), whereas URS2 is responsible for cell-cycle control (33). URS2 contains 10 copies of a repeated sequence (PuNNPyCACGAAAA, the cell-cycle box, CCB), which is sufficient to act as a UAS and confer cell-cycle-regulated transcription in a test plasmid (2, 1).

Six genes (SWI1-SWI6) that are required for transcription of HO and five genes (SIN1-SIN5) that may code for negative regulators have been identified (51, 53, 2). The SIN1 gene was identified because mutations in it relieve the requirement of SWI1 for HO transcription and thus render HO SWI-independent (53). We have shown that the sin1⁻ mutation allows the CCB elements in URS2 to function as a UAS (23; see 13 for review). We propose that in wild-type strains, SIN1 prevents the CCB elements from functioning as a

UAS until appropriate conditions are satisfied (i.e. mother cells in G1).

We show here that SIN1 is identical to SPT2, a gene identified because mutations in it restore expression to promoters inactivated by insertion of a Ty or δ element (58, 8). The predicted SIN1/SPT2 protein contains sequence similarity with the mammalian HMG1 protein, a non-histone component of chromatin. The SIN1 protein, like HMG1, is located in the nucleus and binds DNA in vitro with little or no sequence specificity. As an in vivo correlate, we demonstrate that promoters containing either URS2 sequences or random DNA sequences show similar SIN1-dependent regulation. We also show that sin1⁻ mutations restore HO transcription at the wild-type start site and cause an increased loss of chromosome III. Finally, we present genetic evidence that suggests that SIN1 may be part of a multi-protein complex. Based on these observations, we propose that SIN1 is a non-histone component of chromatin that creates the proper chromatin context for transcription.

Materials and Methods

Genetic analysis

Genetic methods were performed as described (51, and references therein). Mating tests and pheromone production assays were done as described (50, 51).

Strains

The strains used in this study are presented in Table 1. The swi1Δ and swi5Δ alleles were constructed in vitro by Michael Stern from cloned SWI1 and SWI5 genes (52). Both alleles are marked with LEU2. The sin1Δ allele is marked by TRP1. The HO-lacZ fusion allele (43) was used to score HO activity in crosses. Tester strains for **a**-factor and α-factor assays were: RC757 (α sst2) and XMB4-12B (**a** bar1). The mating testers were 1793 (α lys1) and 227 (**a** lys1). The chromosome loss tester was 333 (α thr4 lys2).

Cloning SIN1 and sin1-2

We failed in several attempts to find a plasmid able to complement a sin1⁻ mutant in existing libraries and thereby entertained the possiblity that a DNA segment containing SIN1 may be lethal in E. coli. This proved to be the case: SIN1 is adjacent to the RAD4 gene, which has been shown to cause lethality in E. coli (9). In order to circumvent this problem, we used a genomic library that was used to transform yeast directly after ligation in vitro. The genomic library was constructed by EcoRI partial digestion of total yeast DNA from strain XJJ10-8B (MATα ade2 his4 leu2 ura3-52 lys2 rna16-1 HOL1; a gift from Joe Couto) and ligated into the EcoRI site of pMR366 (obtained from Mark Rose), a vector similar to YCp50 but containing the pSC101 origin which is maintained at low copy in bacteria. The ligation mix was used directly to transform yeast strain WK9A-4b, and 5000 colonies were then screened by filter assay for white colonies. Two strains that formed white colonies were shown to form blue colonies after plasmid loss. DNA was isolated from these transformants and used to transform E. coli

(MH6). Only one colony was obtained upon transformation, from which DNA was isolated and used to retransform WK9A-4b. This DNA (pSIN1) was capable of complementing both WK9A-4b (swi5Δ sin1-2) and WK1-1c (swi1Δ sin1-2). Sequence analysis of a portion of pSIN1 showed that the RAD4 gene had been inactivated by insertion of a bacterial Tn10 element, while the plasmid was propagated in E. coli.

In order to confirm that pSIN1 actually contained the SIN1 locus, a 5 kb EcoRI fragment from pSIN1 was sub-cloned into YIp5, forming plasmid pYIpRI, linearized within the insert at a unique BglII site, and used to transform WK9A-4b. A cross was then performed between this strain and WK10-1a (swi5Δ SIN1+) to see if URA3 was linked to sin1-2. Upon dissection of ten tetrads, nine were parental ditype and one was a tetratype, indicating tight linkage between the cloned fragment and sin1-2.

The sin1-2 allele was cloned by gap repair (36) of a SIN1-containing plasmid in a sin1-2 strain. An SphI- EcoRI fragment containing SIN1 inserted in pMR366 (pΔSph) (see Figure 4c) was digested with XbaI and HindIII, releasing the 3' end of the SIN1 gene. This linear plasmid was then used to transform WK1-1c, and colonies were screened for β-galactosidase activity. 53 of 54 colonies were blue, indicating that the plasmid had been repaired by the sin1-2 locus. Plasmids from four independent transformants contained identical inserts as judged by restriction mapping. One representative was designated pSin1-2.

Construction of a sin1 deletion allele

A 3 kb SphI-EcoRI fragment containing SIN1 (see Figure 4c) was cloned into pUC18 (pUC-SIN1). This plasmid was then digested with XbaI and HindIII and the 5' overhangs were filled in by T4 DNA polymerase. An EcoRI fragment containing the TRP1 gene previously cloned into pUC18 (pUC18:TRP1, Bruce Patterson, unpublished) was digested with XbaI and HindIII, which released the TRP1 gene as well as surrounding polylinker sequences. The ends of the released fragment were filled in with T4 DNA polymerase and then inserted into the digested pUC-SIN1. This plasmid, pUC-SIN1-TRP1, was digested with PstI, which eliminated more of the SIN1 coding region, and was recircularized. The resulting construct, pUC-SIN1Δ-TRP1, lacks 292 of the 333 amino acids of SIN1 (see Figure 4c). The EcoRI-SphI fragment of this construction was isolated and used to introduce the sin1Δ allele into different strains. All gene replacements were done as described (36) and were confirmed by Southern hybridization.

Plasmids

For the experiments described in Table 3, pBA147 (gift of Brenda Andrews) was cut with BglII, treated with calf intestinal phosphatase, and subsequently ligated to total λ DNA cut with Sau3A. Ten plasmids were analyzed for insert size by cleavage with XhoI and EcoRI followed by PAGE (polyacrylamide gel electrophoresis). pBA147 was made by inserting the RPA39 UAS (-299 to -177) at position -178 upstream of CYC1-LacZ as described in (23).

The constructs used in Table 4 were created according to (1), except for P2-1, which was constructed by Joe Ogas. In this case, the CCB elements were first multimerized into the XhoI site of pUC18-Bgl[2], which contains the pUC polylinker flanked by two BglII sites (Brenda Andrews, unpublished). The BglII fragment was then subcloned into pΔSS-Bgl, which is pΔSS (10) with a BglII linker inserted into the SalI site (A. Mitchell, unpublished).

A plasmid which produced a truncated form of SIN1 missing its first 43 amino acids was produced as follows. Two primers (5'-GCATGCGTTGACAA-AGCGGAGGAAG; 5'-CTGCAGGCATAACTAAAATATTTCACT) were used to PCR amplify the promoter region and the start codon of SIN1. This fragment was then used to replace the PstI and SphI fragment from pΔSph. This plasmid is identical to pΔSph except that amino acids 2-51 of SIN1 are missing. This plasmid was tested for function by transformation into WK1-4d, followed by filter β-galactosidase assay.

Preparation of SIN1 antibodies

A PstI-EcoRI fragment from SIN1 was cloned into pATH21 (a gift of T.J. Koerner and A. Tzagaloff). Synthesis of the hybrid protein was induced with indoleacrylic acid and purified essentially as previously described (19). The protein was isolated by gel electrophoresis, and electroeluted protein was used to immunize rabbits (Babco, Inc., Berkeley, CA). Antibodies were affinity purified against bacterially produced TrpE-SIN1 according to (49).

Immunofluorescence

WK30-5c and WK30-1b were fixed, and immunofluorescence was performed essentially according to (12). Rhodamine-labelled goat anti-rabbit antibodies were obtained from Cappell Inc. (Trenton N.J.).

Immunoprecipitation assays

Cells producing either TrpE or TrpE-SIN1 were induced as above and then resuspended in 1/50 the initial volume in cold buffer A (100 mM Tris-HCl pH 8, 200 mM KCl, 1 mM EDTA, 0.1 mM DTT, 5% glycerol). Cells were then lysed by sonication, and the insoluble material was removed by centrifugation at 13,000 x g for 20 min. Extracts were then frozen at -70° C.

S. aureus cells were coupled to antibodies as in (18). Anti-TrpE antiserum was provided by Brenda Andrews. Anti-α2 antiserum was provided by Cynthia Keleher.

For binding reactions, 10 ul of antibody-coupled S. aureus cells were added to 25 ul of buffer B (25 mM Tris pH 7.0, 2 mM EGTA, 150 mM NaCl, 1% NP-40) and 5 ul of appropriate protein extract containing approximately 1ug of TrpE or TrpE-SIN1. After 30 min. on ice, S. aureus cells were collected by centrifugation and washed once with buffer A containing 0.5 M NaCl and once with buffer B. Cells were then resuspended in buffer C (20 mM Tris pH 7.5, 50 mM NaCl, 0.25 mM EDTA, 1 mM DTT, 10% glycerol) and approximately 5 ng of radiolabeled DNA was added, as well as any competitor DNA if indicated. After 30 min. on ice, S. aureus cells were pelleted and washed once with buffer C, and then any bound DNA was purified

away from the cells by phenol/chloroform extraction and ethanol precipitation. Products were visualized by electrophoresis through a 5% TBE gel followed by autoradiography. For α2 binding, the same procedure was used except instead of bacterial protein extracts, purified protein was added (a gift of Arkady Mak).

Assaying β-galactosidase

β-galactosidase assays of cells grown in culture were performed as described (51, 28). Colonies were assayed for β-galactosidase by replica plating to filters as described in (1).

Primer extensions

Primer extensions were performed using a modified version of the protocol described in (26). The primer used to assay the HO transcript was 5' GGGATCTAACCTACCAG-GTTCACC. The primer used to assay the URA3 transcript was 5'CGTGCATGATATTAAATAGC. Hybridization for HO was at 60°C for one hr; for URA3, at 55°C for one hr. AMV reverse transcriptase supplied by Boehringer Mannheim (Indianapolis, IN) was used.

Computer similarity search

A FASTP program (25) was utilized to search for sequences similar to SPT2/SIN1. The highest optimized similarity score was observed between SIN1 and bovine HMG1. The region of similarity contains 20.4% identity to porcine and bovine HMG1 over 191 amino acids of SIN1. In order to assess the statistical significance of the FASTP score, we ran the RDF2 program, which randomizes the region

of SIN1 similar to HMG1 and recalculates the FASTP similarity score. The original score was 4.81 SD away from the mean of the random scores obtained in two hundred randomizations.

Determining the percentile rank of charged residues in SIN1 relative to the rest of the proteins in the NBRF database was done by a search program developed by Robin Colgrove and Eric Fauman, which is available on request.

Results

Cloning and sequence examination of SIN1

The SIN1 gene was cloned by screening a low copy number yeast genomic library for plasmids able to complement a sin1-2 mutation. The starting strain, of genotype swi5Δ sin1-2 HO-lacZ, forms blue colonies on filters (see Materials and Methods), because the sin1-2 mutation allows transcription of HO in the absence of SWI5. In contrast, a strain that contains a complementing SIN1 plasmid should form white colonies. One plasmid, containing a 22 kb insert, was recovered which produced this phenotype. In order to ascertain whether the complementing plasmid actually contains SIN1, a 5 kb internal fragment was sub-cloned into YIp5 and integrated into the chromosome by homologous recombination. Tetrad analysis showed the plasmid to be integrated at the SIN1 locus (see Materials and Methods) and therefore confirmed that the insert contains SIN1. The same DNA fragment was used as a probe on a blot of separated yeast chromosomes (45), which indicated that SIN1 mapped to chromosome V (data not shown).

In the course of studying the sin1-2 mutation, we discovered an additional phenotype. In particular, we observed that sin1-2 was able to suppress the lys2-128δ mutation, an insertion of the 330 bp δ element in the 5' coding region of LYS2 (47, data not shown). The sin1-2 mutation thus exhibited the phenotype of spt mutations, which suppress transcription defects due to insertion of Ty and δ elements in and near promoters. Several observations indicate that SIN1 is identical to SPT2. First, both are located on chromosome V and have similar restriction maps (58, 40, data not shown). Second, sin1-2 and spt2-150 segregate as alleles: sporulation of a diploid formed by mating a spt2-150 strain (WK28-7a) to a sin1-2 strain (WK21-1d) yielded 20 tetrads in which all four of the spores were Sin$^-$. Third, limited nucleotide sequence analysis from the cloned SIN1 gene was identical to the published SPT2 sequence (data not shown). From these analyses, we conclude that SIN1 and SPT2 are identical.

SIN1/SPT2 contains an open reading frame of 333 codons encoding a polypeptide that is extremely hydrophilic and highly charged (40). The predicted sequence contains 25% basic residues and 20% acidic residues, with the acidic residues tending to be clustered in two long acidic stretches. An examination of the NBRF database (as of November 1990) revealed that SIN1/SPT2 was among the top 1% of all the entries in percentage of charged residues (data not shown; see Materials and Methods). This group of highly charged proteins was primarily composed of various protamines, histones, and HMG proteins. Of this group only the HMG proteins are rich in both basic and acidic residues.

We searched for sequences similar to SIN1/SPT2 in the protein database (25, see Materials and Methods) and found that SIN1/SPT2 has statistically significant sequence similarity to mammalian HMG1 proteins. This similarity extends over 191 amino acids in the C-terminal half of the predicted SIN1/SPT2 protein. 20% of the residues in SIN1/SPT2 are identical to porcine and bovine HMG1. Figure 1 shows the sequence alignment of this portion of SIN1 and an evolutionary cross-section of HMG1-like proteins from pig, trout, yeast, and Tetrahymena which reveals significant regions of similarity. 38% of the residues in this 191 amino-acid segment are similar to at least one of the other proteins. The unusual overall charge profile of SIN1, as well as the similarity between SIN1 and HMG1 and the indicate that SIN1 is an HMG1-like protein.

Nuclear concentration of SIN1

To determine whether SIN1 is concentrated in the nucleus, as expected for an HMG-like protein, we carried out immunofluorescence analysis using affinity-purified antibodies directed against SIN1 (see Materials and Methods). Cells were co-stained with anti-SIN1 antibodies to visualize SIN1 location and with DAPI to visualize the nucleus. As can be seen in Figure 2, nuclear staining with the antibody is visible in SIN1 cells and not in cells carrying a deletion of SIN1 (see Materials and Methods). Similar results were obtained using antibodies against β-galactosidase to localize a SIN1-LacZ hybrid that contains all but the C-terminal segment of SIN1 fused to LacZ (data not shown).

Although the antibody staining of SIN1 is somewhat diffuse, these observations show that SIN1 is concentrated in the nucleus.

TrpE-SIN1 binds non-specifically to DNA

We next examined the ability of SIN1 to bind to DNA. Our experiments were performed using a TrpE-SIN1 fusion protein containing the C-terminal 282 amino-acid segment of SIN1 fused to the C-terminus of the bacterial TrpE protein. We have shown that this segment of SIN1 is able to complement a sin1- mutation in vivo (data not shown; see Materials and Methods). Extracts were prepared from bacteria that produced either the TrpE-SIN1 hybrid or TrpE. TrpE-SIN1 and TrpE were immunoprecipitated from extracts using anti-TrpE antibodies coupled to S. aureus cells, and then radioactively labeled DNA fragments from a plasmid containing the URS2 region of HO were added. DNA fragments that associated with TrpE-SIN1 and TrpE were recovered and analyzed by non-denaturing PAGE. A typical experiment is shown in Figure 3A. Approximately 15 fragments were bound by the extract containing TrpE-SIN1 (lane 2); no fragments were bound by the extract containing TrpE (lane 3). In general the higher molecular weight fragments were precipitated by TrpE-SIN1 more efficiently than the lower molecular weight fragments. This behavior is expected for non-specific DNA binding because there are more non-specific sites on the larger fragments. These observations indicate that the SIN1 portion of the hybrid confers DNA binding in vitro.

To determine whether binding of TrpE-SIN1 exhibited any sequence specificity, we examined its binding behavior in the

presence of increasing amounts of non-specific competitor DNA. If any of the DNA fragments mixed with TrpE-SIN1 have strong specific sites for SIN1 binding, they should be more resistant to competition by non-specific DNA (see reference 5). As can be seen in Figure 3B, addition of calf thymus DNA competes equally well for all the plasmid fragments precipitated in the TrpE-SIN1 extracts. In a similar experiment, we found that a 10-fold molar excess of poly dI:dC entirely competed all the observed binding (Figure 3A, compare lanes 2 and 4). Experiments in which the ionic conditions and Mg^{++} concentrations were varied failed to reveal any indication of specific binding (data not shown). These experiments show that TrpE-SIN1 binds to random vector sequences and to URS2 sequences with similar affinities. Thus the binding activity we have detected is non-specific.

As a control for specific binding under the conditions used, we examined binding of the sequence-specific DNA-binding protein α2 (18) to a mixture of the same set of fragments to which an α2 binding site was added (See Figure 3c). As can be seen in lane 6, α2 clearly binds to its own operator with much higher affinity than to the non-specific plasmid sequences, whereas TrpE-SIN1 shows little or no specificity for the fragments containing URS2 (lane 2). The slight preference of α2 for one particular fragment of URS2 is probably due to the presence of two a1-α2 binding sites in URS2 (28) and has been observed previously (A. Johnson, personal communication). This analysis demonstrates that under conditions in which sequence-specific DNA binding can be observed for α2, the

TrpE-SIN1 protein exhibited little or no sequence-specific binding to DNA.

SIN1-dependent effects of random DNA fragments and URS2 on promoter activity in vivo

Because in vitro we observed SIN1 binding to both URS2 and random vector DNA fragments, we have determined whether random DNA and URS2 exert similar SIN1-dependent transcriptional effects in vivo. We have thus studied the effect of both random DNA segments and a 540 bp URS2 segment that are added to an otherwise-intact regulatory region, between the UAS of the RPA39 gene (59) and the TATA element of a CYC1-lacZ gene. A 540 bp URS2 fragment or Sau3A fragments from bacteriophage λ were inserted into a BglII site at position -178 with respect to the mRNA start site (see Figure 4A). Ten constructs were analyzed for insert size and introduced into isogenic SIN1$^+$ and sin1$^-$ strains by transformation. As a control, these strains were also transformed with an RPA39-CYC1-lacZ plasmid (RL0) containing no insert.

Insertion of the URS2 fragment in the SIN1$^+$ strain reduced transcription approximately 1200-fold, while the same insert in the sin1$^-$ strain reduced transcription only 35-fold (Table 2). Thus the URS2 segment exerted less inhibition of transcription in the absence of SIN1. The random DNA fragments showed similar behavior. In the SIN1$^+$ strain, insertion of the random DNA fragments reduced transcription between 1800-fold (for RL6) and 13-fold (for RL7). Transcription was reduced to a lesser extent in the sin1$^-$ strain: RL6 exhibited a 77-fold reduction, and RL7 exhibited a 2.6-fold

reduction. This experiment demonstrates that the random DNA fragments show SIN1-dependent regulation similar to that for the URS2 fragment. We have also discovered an unanticipated effect of SIN1 on the plasmid carrying no insert. Activity from plasmid RL0 was 9-fold higher in the SIN1$^+$ strain than in the sin1$^-$ strain. This observation suggests that SIN1 may also play a role in transcriptional activation. The implications of these findings are considered in the Discussion.

Other work (Kruger at al., in preparation) has indicated that the CCB (cell-cycle box) elements in URS2 are inhibited by the SWI1 and SIN1 gene products. The regions of URS2 responsible for this inhibition are not yet identified. Given the results above, we entertained the possibility that the addition of random DNA sequences to these CCB elements could confer regulation by SWI1 and SIN1. In order to test this hypothesis, we compared the activity of plasmids whose UAS contained either two CCB elements alone (P2), or two CCB elements and an adjacent a 43-basepair DNA segment derived from the polylinker of PUC18 (P2-1) in isogenic wild-type and swi1Δ sin1Δ cells (see Figure 4B). As controls, we examined the behavior of a plasmid (PU) whose UAS consists of a segment of URS2 and a plasmid (P3) whose UAS consists three CCB elements. The results of this experiment are shown in Table 3. In order to account for any non-specific effects between the two strains, we normalized the activity of the plasmids to P3 in each strain. The normalized results reveal that PU shows a 10-fold increase in activity in swi1-sin1- cells relative to its activation in wild-type cells. P2 shows less than a 2-fold increase in activity

between the two strains. Interestingly, P2-1 exhibits an 8-fold increase in activity. Thus the addition of the 43-basepairs of random DNA to two CCB elements causes the promoter to be regulated in a manner similar to URS2. The parallel behavior of PU and P2-1 suggests that the polylinker segment confers regulation by SWI1 and SIN1 and recreates a situation like that in the native URS2 region.

sin1⁻ mutations allow transcription at the wild-type start site

As noted above, inactivation of SIN1 restores HO transcription to mutants lacking SWI1, SWI2, SWI3, or SWI5 (53). We wished to determine whether this transcription utilized the wild-type start site or another site. We have thus examined the HO transcript by primer extension from congenic SWI⁺ SIN⁺, swi1⁻ SIN⁺, and swi1⁻ sin1⁻ strains. As can be seen in Figure 5a, the same predominant start site is used in both the wild-type (lane 1) and swi1⁻ sin1⁻ (lane 3) strains. This analysis also demonstrates that the efficiency of suppression due to the sin1-2 mutation is high: HO RNA is present in roughly equal amounts in wild-type and swi1⁻ sin1-2 cells (relative to the URA3 transcript controls). These experiments indicate that in the absence of SWI1, inactivation of SIN1 allows efficient transcription of HO from the normal start site.

A sin1 point mutation has a more severe phenotype than a sin1 deletion and is partially dominant

Cloning of SIN1 made it possible to construct a null allele of SIN1 and determine its phenotype (see Materials and Methods). We first noticed that expression of the HO-lacZ gene was lower in the swi1Δ sin1Δ strain than in the congenic swi1Δ sin1-2 strain (data not shown). Primer-extension analysis of HO RNA of congenic swi1Δ sin1-2 and swi1Δ sin1Δ strains showed that this difference is reflected at the level of transcription (see Figure 5b). In order to examine this result in isogenic strains, we introduced low copy (centromere-containing) plasmids with sin1-2 or no SIN allele into a swi1Δ sin1Δ HO-lacZ strain (WK44-9b). As shown in Table 4, the swi1Δ sin1Δ strain carrying a control (vector) plasmid exhibited 1.3 units of β-galactosidase activity. In contrast, introduction of the sin1-2 plasmid into this strain caused activity to increase to 3.2 units. It thus appears that the sin1-2 mutation creates a more severe Sin1$^-$ phenotype then the sin1 deletion mutation. Western analysis showed that the allele produces an apparently full-length protein, suggesting that sin1-2 is most likely a missense mutation (data not shown). These results indicate that the mutant sin1-2 protein has a more severe effect than elimination of SIN1 protein.

The two sin1 mutations also behave differently in dominance tests against SIN1. The same sin1-2 and control plasmids described above were introduced into a swi1Δ SIN1 HO-lacZ strain. As shown in Table 4, expression of sin1-2 in a SIN1 strain caused an increase in LacZ levels relative to the control plasmid (from 0.25 to 0.60 units). We have also observed that diploid lys2-128δ strains that carry both sin1-2 and SIN1 are Lys$^+$, which indicates that the sin1-2 mutation exhibits dominance with respect to the Spt$^-$ phenotype

(data not shown). These observations show that sin1-2 is a partially dominant allele.

SIN1 is required for proper mitotic stability of chromosome III

Mutants defective in SIN1 exhibit an additional phenotype, chromosome loss. Our first indication of this behavior came from the observation that colonies formed by α sin1⁻ cells secreted detectable amounts of a-factor (see Figure 6A). We also observed in mating assays that some of the cells in an α sin1⁻ patch mated with an α strain (Figure 6B). (Once again, the sin1-2 mutation caused a more severe phenotype than the sin1Δ allele.) These observations indicated that a-specific genes were being expressed at a low level in these α cells. One possible explanation for this behavior is that the sin1⁻ mutation affects repression of a-specific genes by α2. Another explanation is that some of the cells in the colony lose chromosome III and therefore lack MATα information. Such cells exhibit the mating behavior of a cells (54) and thus secrete a-factor and mate with α cells. Our observations favor the latter explanation.

To determine whether sin1⁻ mutants show increased loss of chromosome III, we performed rare mating assays (14) between genetically-marked α strains. In this analysis, two different α strains with complementary auxotrophic mutations are mixed, and prototrophs are selected. Prototrophic diploids can be formed in two ways. The first is by mating-type switching, a change from

MATα to MATa in either mating partner. Diploids can also be formed by loss of MATα, for example, due to loss of the entire chromosome. Chromosome loss can be monitored by scoring loss of an additional marker on chromosome III such as THR4. We have carried out two such rare matings, using SIN1$^+$ and sin1-2 strains respectively (see Table 5). In one mating (in which both partners are SIN1$^+$), 174 diploids were obtained after mixing 10^7 cells of each parent. Approximately 2/3 of these were Thr$^+$, indicating that they resulted from mating-type interconversion or from loss of MATα in the thr4$^-$ parent. The other mating (in which one partner is sin1$^-$) yielded 8-fold more diploids. Furthermore, the vast majority of these diploids were Thr$^-$, which indicated that they had acquired mating ability by loss of chromosome III in the sin1$^-$ parent.

A second test for chromosome loss involves the use of a/α diploids. Loss of the chromosome containing MATα will result in a cell that is phenotypically a, whereas losing the MATa chromosome will result in a phenotypically α cell. We observed that a/α cells homozygous for sin1-2 showed a ten-fold increase in mating behavior as a and α compared with isogenic cells heterozygous for sin1-2 (data not shown). These same diploids were also heterozygous for a recessive can1$^-$ mutation on chromosome V, which made it possible to measure loss of chromosome V simply by scoring canavanine-resistant colonies. We observed no difference in the frequency of canavanine-resistant colonies between these two strains. These observations taken together indicate that wild-type

SIN1 function is required for proper mitotic stability of chromosome III but apparently not for chromosome V.

Discussion

We have shown that SIN1 is identical to a previously described gene, SPT2, and that the predicted protein has similarity to mammalian HMG1, a non-histone component of chromatin. We have also observed that SIN1 is concentrated in the nucleus and binds to DNA non-specifically in vitro. These findings indicate that SIN1 is a non-histone component of chromatin. The SIN1 gene was discovered because sin1- mutations restore expression of HO in strains lacking SWI1 (53). We have shown here that this restored transcription utilizes the same start site as in wild-type strains. As discussed more fully elsewhere (13, 14), we believe that SIN1 prevents utilization of a specific UAS until the proper conditions have been achieved. We have not yet identified a consensus sequence for a SIN1 response element. Quite the contrary, we have found that a large fraction of random DNA segments can confer regulation by SIN1. We have also observed an additional phenotype of sin1 mutants, loss of chromosome III, which suggests that SIN1 may also be involved in maintaining chromosome stability.

SIN1 is HMG-like and is proposed to be a non-histone component of chromatin

SIN1 protein is highly charged (25% basic and 20% acidic) and is 20% identical and 33% similar to the mammalian HMG1 protein over 191 of the 214 amino acids found in both bovine and porcine HMG1. The HMG proteins were originally described as non-histone

components of chromatin which are extractable by 0.35 M NaCl or 5% perchloric acid (for review, see 56). There are four major mammalian HMG proteins (HMG1, 2, 14 and 17) as well as many minor uncharacterized species; their function is unknown. The mammalian HMG proteins, like SIN1, are highly charged (>45%), containing an overall basic charge but having some highly acidic stretches. HMG1 binds non-specifically to DNA (3, 44) and is found at a ratio of 1:10 relative to nucleosomes (24). Similarly, our studies show that SIN1 has little if any sequence specificity and is found at approximately 10,000 molecules per cell (a 1:10 ratio to nucleosomes; W.K., unpublished observation). The sequence similarity between SIN1 and HMG1, the location of SIN1 in the nucleus, and its ability to bind non-specifically to DNA are all consistent with the notion that SIN1 is a non-histone component of chromatin.

Other sequence-specific DNA binding proteins involved in transcriptional activation have recently been identified which have some sequence similarity to HMG1 and define a motif termed the "HMG box" (16). Though SIN1 has similarity to HMG1 over almost the entire length of HMG1, it does not contain any special similarity to the HMG box. Our experiments do not exclude the possiblity that SIN1 exhibits site-specific binding to a DNA sequence that we have not tested. It is also possible that SIN1 interacts with other proteins to bind to specific DNA sequences.

HMG-like proteins have been isolated from yeast based on the biochemical properties of mammalian HMG proteins (57, 22). These include the NHP6A and NHP6B proteins, which are very similar to each other and have approximately 40% amino-acid identity to

mammalian HMG1 (21). Another protein, ACP2, has been identified by cloning its gene using degenerate oligonucleotides based on a portion of HMG1-coding sequence as a probe (11). These HMG-like genes are essential for yeast: deletion of ACP2 or of both the NHP6A and NHP6B genes leads to inviability. The functions of these proteins are not known.

There are some differences between SIN1 and other HMG1-like proteins. SIN1 is somewhat larger than mammalian HMG1 (38 kd versus 26 kd) and much larger than the 10-kd yeast NHP6A and NHP6B proteins. The similarity between SIN1 and HMG1 extends only over the C-terminal half of SIN1. Unlike other yeast HMG proteins, SIN1 is not extractable from nuclei with 2% perchloric acid (W.K., unpublished). This behavior might reflect the addition of a segment to SIN1 that affects its properties. The highly basic N-terminal half of the protein has some weak similarity to mouse and nematode histone H1. These differences suggest that although SIN1 appears to be related to mammalian HMG1, it has diverged structurally and perhaps functionally as well.

SIN1 may be necessary for setting the proper chromatin context for gene regulation and other processes

The effect of a sin1 mutation can be seen in three different situations in which transcription is altered: (1) in transcription of the HO gene, when certain positive regulatory proteins (SWI1, SWI2, SWI3, or SWI5) are absent; (2) in transcription of LYS2 or HIS4 genes whose upstream region has been partially inactivated by insertion of a δ element; and (3) in transcription of the INO1 gene in strains

carrying a truncation of the carboxy-terminal domain of the largest subunit of RNA polymerase II.

Inactivation of SIN1 allows transcription of HO even in the absence of SWI1, SWI2, SWI3, or SWI5 products. It appears that the sin1 mutation allows utilization of the cell-cycle box sequences for transcriptional activation. The key observation is that sin1 mutations bypass the requirement for SWI1, SWI2, SWI3, and SWI5, but they are still dependent on SWI4 and SWI6 (53, 23). As described elsewhere (13, 23), we believe that SIN1 is involved in maintaining these cell-cycle boxes in a quiescent state until the appropriate conditions have been fulfilled. We imagine that the inhibition exerted by SIN1 is relieved by SWI1,2,3 in mother cells, which then frees the cell-cycle boxes for binding by the cell-cycle-box factor.

Insertions of a δ element in the upstream region of HIS4 or in the beginning of the coding region of LYS2 greatly reduces transcription of these genes. The precise reasons why these insertions reduce synthesis of the normal HIS4 or LYS2 transcripts are not clear and are likely to be complex. The his4-912δ insertion causes production of an abundant new HIS4 transcript initiated from the δ element and reduction of the normal HIS4 transcript (46). Apparently, removal of SPT2 (SIN1) allows more efficient transcription from both the δ element and the normal start site. (It should be noted that mutations in other SPT genes, for example in SPT15, lead to decreased transcription from the δ element; 7.)

We have recently identified another situation in which SIN1 plays a role (38). Truncation of the carboxy-terminal domain (CTD)

of the largest subunit of RNA polymerase II leads to a defect in transcription of the INO1 gene and to cold sensitivity for growth (34, 35). Deletion of SIN1 in these strains restores transcription of INO1 and reduces the cold-sensitive growth defect. We interpret these observations to indicate that the truncated CTD of RNA polymerase II is unable to initiate transcription of INO1 due to some action of SIN1. More specifically, we propose that SIN1 binding in the upstream region prevents this enfeebled RNA polymerase from functioning properly.

In the cases just described, inactivation of SIN1 allows enhanced or inappropriate transcription. We have also observed that SIN1 in some cases plays a stimulatory role. The intact RPA39 UAS carried on a test plasmid functions 10-fold better in a SIN1$^+$ host compared with a sin1$^-$ strain (Table 2). A UAS derived from the cell-cycle box elements is expressed approximately 2-fold better in SIN1$^+$ conditions (W.K., unpublished data). Similarly, an integrated GAL1-lacZ fusion gene is expressed 5- to 10-fold better in a SIN1$^+$ than in a sin1$^-$ strain (C. Peterson, personal communication). These observations lead us to propose that the normal role of SIN1 is to associate with DNA and provide a proper chromosomal context for other components of the transcription machinery to function. We have recently made another observation that supports the view that SIN1 affects chromatin structure (W. Kruger, unpublished): we have found that the SIN2 gene, which like SIN1 was identified as a bypass suppressor of swi1$^-$ mutations (53), is the HHT1 gene, one of the two genes coding for histone H3 (48).

SIN1 may also play a role in maintaining chromosome stability. We observed a 10- to 100-fold increase in loss of chromosome III by sin1 mutants. The lack of effect of a sin1⁻ mutation on behavior of chromosome V may reflect subtle differences between the centromere regions of these two chromosomes. Although we have not analyzed sin2⁻ mutants for their effects on chromosome stablity, imbalance of H3-H4 dimers are known to lead to chromosome loss (27).

We have observed that a wide variety of DNA segments can affect functioning of a UAS in a SIN1-dependent manner. In one experiment, we observed that inserts of 93 to 520 basepairs within a functional regulatory region (between the UAS and TATA) decreased the level of expression from that regulatory region. Such reductions due to insertions of this type have been observed previously (10). We observed two different effects of sin1 mutations on the behavior of the intact and modified regulatory regions: first of all, we observed that the intact regulatory region (carrying no insert) exhibited a nine-fold reduction in activity in the sin1⁻ strain in comparison with the SIN1⁺ strain. In contrast, the activity from the regulatory regions containing inserts was only decresed slightly or increased up to three-fold in the sin1 mutants. There are two ways to view these data. According to one view, SIN1 plays an essential *stimulatory* role in the regulatory region lacking an insert. According to this explanation, insertion of random DNA segments and the URS2 segment somehow eliminates this stimulatory action of SIN1. In the other view, the URS2 segment and the random DNA inserts contain a SIN1 binding site and thereby

inhibit activity of the UAS. The inhibition exerted by these segments thus can be viewed as an *in vivo* correlate of our observations on non-specific DNA binding by SIN1 *in vitro*. A second experiment, which examined the effect of a 43-basepair insert on expression promoted by cell-cycle-box elements, is most easily interpreted by proposing that the insert contains sites of SIN1 (and SWI1) action. In fact, addition of this DNA segment adjacent to a functional CCB element recreates the situation found in the native URS2 region of *HO*. We have suggested elsewhere that binding of SIN1 to DNA might limit communication between proteins bound at the UAS and the TATA element (38). If SIN1 indeed functions to set the chromatin context for transcription (and other processes), then we can expect to see complex phenotypes of *sin1* mutations.

Genetic behavior suggests that SIN1 may be part of a multi-protein complex

We have observed that the *sin1-2* mutation has a stronger Sin$^-$ phenotype than a *sin1* deletion mutation. This observation suggests that the mutant protein produced by *sin1-2* strains interferes with functioning of other SIN1-like proteins or inhibits function of a complex involving other proteins. A further suggestion that SIN1 may interact with other proteins comes from the observation that *sin1-2* is partially dominant, exhibiting both a Sin$^-$ and Spt$^-$ phenotype in *sin1-2/SIN1* strains. The dominant negative behavior of many *spt2* mutations had been previously noted (58, 40).

The SIN1 protein offers a great challenge: how to decipher the functional role of a protein that appears to be a non-specific DNA-binding component of chromatin. Why do mutations in *SIN1*

affect some genes and not others or affect some chromosomes and not others? We anticipate that further understanding of the role of SIN1 will require direct analysis of interacting proteins and chromatin structure.

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Table 1

Strain List

Strain	Genotype	source/comment
BDY12A-1c	MATα HO-lacZ leu2-a his ura3-52 met	Sternberg et al. (1987)
1368	MATa ho leu2- trp1 his ura3-52	Sternberg et al. (1987)
WK9A-4b	MATα swi5Δ sin1-2 HO-lacZ trp1 leu2- his ura3-52	this work; derived from crosses between derivatives of BDY12A-1c and 1368
WK21-1d	MATa swi5Δ sin1-2:URA3 HO-lacZ trp1 leu2- his ura3-52	" "
WK44-9b	MATα swi1Δ sin1Δ HO-lacZ trp1 leu2- his ura3-52	" "
WK1-9a	MATα swi1Δ HO-lacZ leu2- his ura3-52	" "
WK1-4d	MATα swi1Δ sin1-2 HO-lacZ leu2- his ura3-52	" "

WK1-1c	MATa swi1Δ sin1-2 HO-lacZ leu2- his ura3-52	" "
WK10-1a	MATα swi5Δ HO-lacZ trp1 leu2- his ura3-52	" "
WK10-1aΔ	MATα swi5Δ sin1Δ HO-lacZ trp1 leu2- his ura3-52	isogenic to WK10-1a
WK28-7a	MATα swi5Δ spt2-150 HO-lacZ trp1 leu2- his ura	this work; segregant from S573-7d x O95
S573-7d	MATa swi5Δ HO-lacZ ura4 leu2-	Sternberg et al. (1987)
WK24-7c	MATα swi5Δ HO-lacZ trp1 leu2- his lys2-128δ ura3-52	this work; segregant from WK21-1d x L206
WK24-20d	MATα swi5Δ sin1-2:URA3 HO-lacZ trp1 leu2- his lys2-128δ ura3-52	" "
WK30-5c	MATα ura3 his4 leu2 trp1 ho	Isogenic to EG123 from P. Siliciano
WK30-1b	MATα ura3 his4 leu2 trp1 ho sin1Δ	Isogenic to WK30-5c

WK36-4d	MATα ho ura3-52 lys2 ade2-101 his3Δ200 leu2-	Isogenic to YPH274 from P. Heiter
CY110	MATα ho ura3-52 lys2 ade2-101 his3Δ200 leu2- sin1Δ	Isogenic to WK36-4d
CY58	MATα ho ura3-52 lys2 ade2-101 his3Δ200 leu2- swi1Δ	Isogenic to WK36-4d
WK40-8c	MATα ho ura3-52 lys2 ade2-101 his3Δ200 leu2- sin1Δ swi1Δ	Isogenic to WK36-4d
O95	MATα spt2-150 ho lys2-128δ leu2-	F. Winston
L206	MATα lys2-128δ	M.A. Osley

[a] leu2- is a double point mutation in the LEU2 gene.

Table 2

Inserts of random DNA or a segment of URS2 cause SIN1-dependent alteration of promoter function

Plasmid (insert size)	β-galactosidase activity SIN1+	sin1-	Ratio (sin1-/SIN1+)
RL6 (440bp)	0.7 (0.05)	1.8 (1.3)	2.7 (26)
RL1 (380bp)	17 (1.3)	29 (21)	1.7 (16)
RL2 (140bp)	41 (3.1)	67 (48)	1.6 (15)
RL8 (240bp)	7.3 (0.56)	12 (8.3)	1.6 (15)
RL3 (100bp)	49 (3.8)	74 (53)	1.5 (14)
RL10 (93bp)	9.9 (0.93)	21 (13)	2.1 (14)
RL4 (110bp)	182 (14)	123 (88)	0.68 (6.3)
RL5 (160bp)	226 (17)	124 (89)	0.55 (5.2)
RL9 (260bp)	41 (3.1)	22 (16)	0.55 (5.2)
RL7 (115bp)	102 (7.7)	53 (38)	0.52 (5.0)
URS2 (540bp)	1.1 (0.08)	4.0 (2.9)	3.6 (36)
RL0 (no insert)	1311 (100)	139 (100)	0.11 (1.0)

Random DNA fragments of the indicated sizes were inserted between the RPA39 UAS and the TATA sequences of a CYC1-lacZ reporter gene (Figure 4A). These constructs were then introduced into isogenic wild-type (WK36-4d) and sin1 deletion (CY110) strains, and activity was measured. Activity was measured from three separate transformants for each construction and the average is given in

Miller units. Standard deviations in this experiment were generally <20%. Results in parentheses show the % of the activity exhibited by each plasmid in relation to RL0 in the same strain.

Table 3

A polylinker segment can mimic regulation in URS2

		β-galactosidase activity		
Plasmid	UAS	[1] SWI1+ SIN1+	[2] swi1- sin1-	[2]/[1]
P3	3xCCB	74 (100)	26 (100)	1
P2	2xCCB	1.6 (2.2)	1.0 (3.9)	1.8
P2-1	2xCCB + polylinker	0.3 (0.4)	0.9 (3.3)	8.3
PU	URS2	0.5 (0.6)	1.6 (6.2)	10.3

Fragments containing the indicated UAS were cloned upstream of CYC1-lacZ lacking a UAS (Figure 4B). The plasmids were used to transform an isogenic set of strains with the indicated genotypes, and β-galactosidase activity was measured. The strains used were WK36-4d, CY110, CY58, and WK40-8c, respectively. Activity was measured from three separate transformants and the average is given in Miller units. Standard deviations in this experiment were generally <20%. Results in parentheses are expressed as % of activity observed for plasmid P3 in the same strain. The right-hand column is the ratio of the normalized activities in columns 2 and 1.

Table 4

Functional Behavior of the sin1-2 Mutation

	Plasmid-borne locus		
Genotype	(-)	SIN1	sin1-2
swi1Δ sin1Δ	1.2	0.20	3.2
swi1Δ SIN1+	0.25	0.25	0.60

Yeast strains WK44-9b (swi1Δ sin1Δ HO-lacZ) and WK1-9a (swi1Δ sin1Δ HO-lacZ) were transformed with pMR366 containing either no insert, SIN1+, or sin1-2. Activity was measured from three separate transformants and the average is given in Miller units. Standard deviations in this experiment were < 16%.

Table 5

Chromosome loss in *sin1*⁻ mutants

Genotypes			Thr phenotype of diploid strains resulting from rare mating: Thr⁺	Thr⁻
MATα THR4 SIN1⁺ (WK10-1a)	X	MATα thr4⁻ SIN1⁺ (333)	105	69
MATα THR4 sin1-2 (WK9A-4b)	X	MATα thr4⁻ SIN1⁺ (333)	112	1340

Strains of indicated genotypes (and carrying complementary auxotrophic mutations; not shown) were mixed and plated on minimal medium sup-plemented with threonine to select for diploids. These colonies were then replica plated to minimal medium lacking threonine to determine the fraction that were Thr⁺. Because MAT and THR4 are both on chromosome III, Thr⁻ strains can result from loss of the chromosome III carrying THR4⁺ prior to mating. Thr⁺ strains can result from loss of the chromosome III carrying thr4⁻ or from switching of MATα to MATa prior to mating.

Figure 1. Similarity between the predicted amino-acid sequence of SIN1 and HMG1-related proteins.

HMG1-like proteins are shown aligned with SIN1; pig HMG1 (55), trout HMGT (37), yeast NHP6B (21), and Tetrahymena LG-1 (42). Numbers in parentheses indicate amino-acid positions. Identities between SIN1 and other HMG1-related proteins are indicated by bold type and shading. Identities and conservative changes between SIN1 and other members of the group are boxed. Conservative amino acid changes are grouped as follows: (F,Y), (K,R,H), (E,D), (Q,N), (I,L,V,A), (S,T). Gaps introduced to maximize alignment are indicated by -.

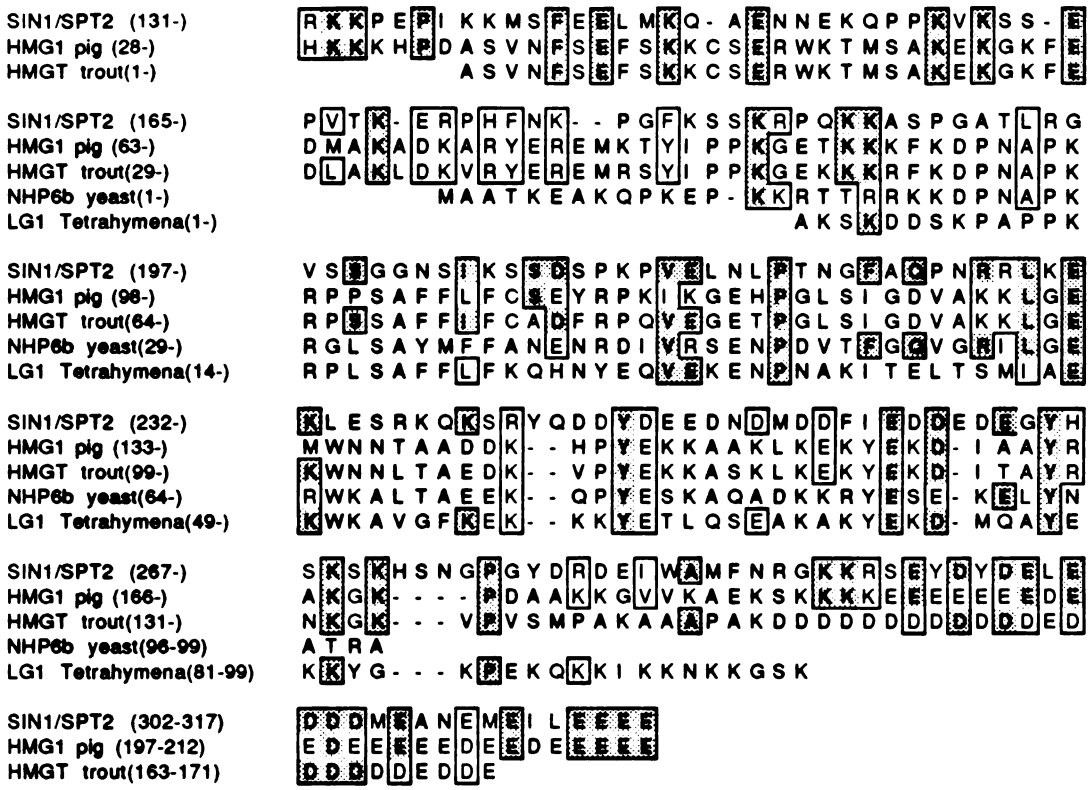

SIN1/SPT2 (131-)
HMG1 pig (28-)
HMGT trout(1-)
SIN1/SPT2 (165-)
HMG1 pig (63-)
HMGT trout(29-)
NHP6b yeast(1-)
LG1 Tetrahymena(1-)
SIN1/SPT2 (197-)
HMG1 pig (98-)
HMGT trout(64-)
NHP6b yeast(29-)
LG1 Tetrahymena(14-)
SIN1/SPT2 (232-)
HMG1 pig (133-)
HMGT trout(99-)
NHP6b yeast(64-)
LG1 Tetrahymena(49-)
SIN1/SPT2 (267-)
HMG1 pig (166-)
HMGT trout(131-)
NHP6b yeast(96-99)
LG1 Tetrahymena(81-99)
SIN1/SPT2 (302-317)
HMG1 pig (197-212)
HMGT trout(163-171)

Figure 1

Figure 2 SIN1 localization

Isogenic SIN1 and sin1Δ strains were fixed and stained with affinity-purified anti-SIN1 antibodies from rabbit. Afterwards they were stained with rhodamine-conjugated goat anti-rabbit IgG and stained with DAPI. (A) SIN1$^+$ cells visualized for DAPI. (B) is identical to (A) except visualized on the rhodamine channel. (C) and (D) correspond to (A) and (B) except that sin1Δ cells were used. Strains used were WK30-5c and WK30-1b respectiviely.

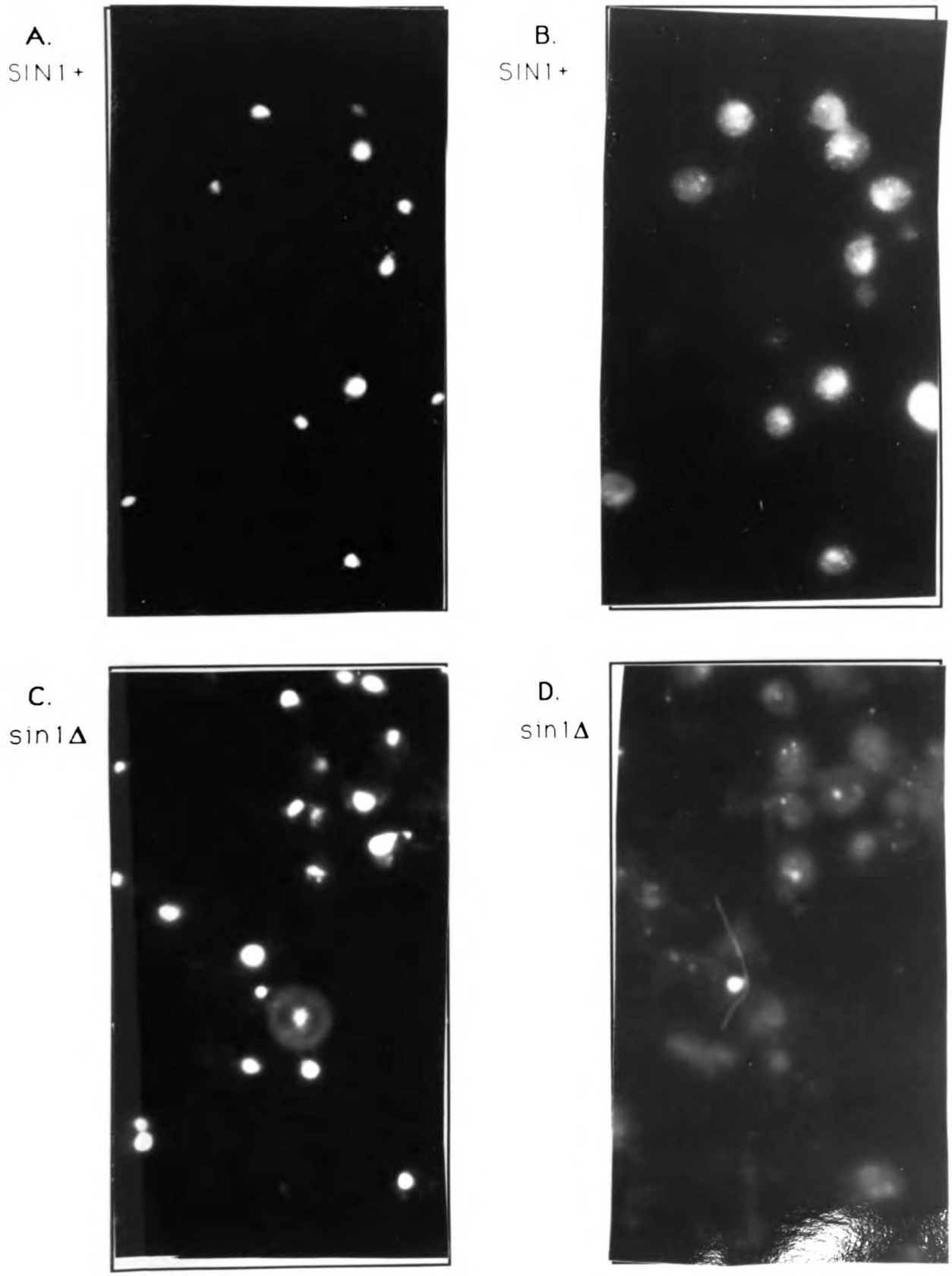


Figure 2

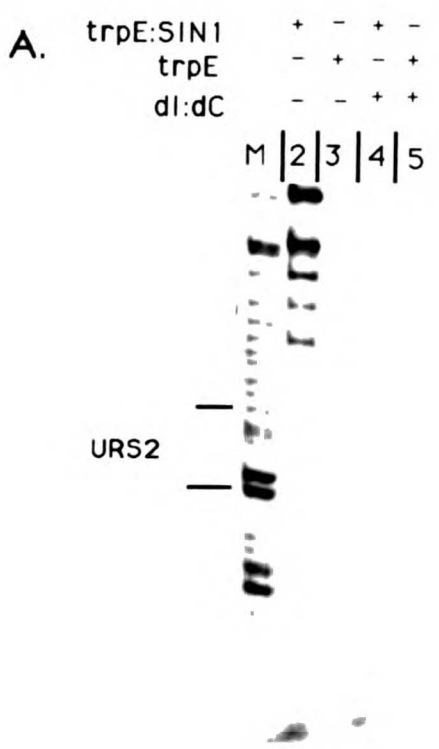
Figure 3 DNA binding properties of TrpE-SIN1

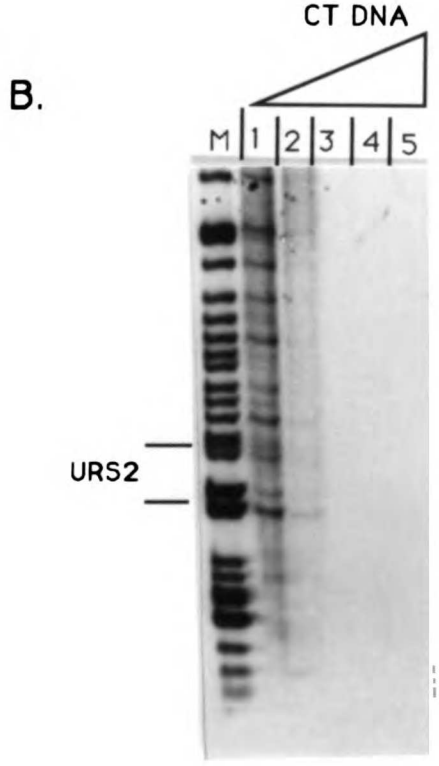
S. aureus cells coupled to anti-TrpE antibodies were used to immunoprecipitate either TrpE-SIN1 or TrpE from bacterial extracts. Cells were then incubated with labeled DNA fragments obtained from Sau3A digestion of plasmid pBA144 (See Materials and Methods). DNA fragments bound to the cells were recovered and visualized by PAGE and autoradiographed.

(A) Lane M shows 20% of the input DNA used in lanes 2-5. Lane 2 shows recovered fragments using extracts containing TrpE-SIN1. Lane 3 shows recovered fragments using TrpE extract. Lanes 4 and 5 are identical to lanes 2 and 3 except that 10-fold molar excess of poly dI:dC was added.

(B) Lane M shows 20% of input DNA used in lanes 1-5. Lane 1 contains TrpE-SIN1 extract with no competitor DNA. Lanes 2-5 show binding reactions in the presence of increasing amounts of non-specific calf thymus DNA competitor (3-, 9-, 27-, 72-fold molar excess).

(C) Lane M shows the same input DNA fragments as in (A) and (B) to which has been added a 93 bp fragment containing an $\alpha 2$ binding site. Lane 1 shows recovered fragments using TrpE-SIN1 extracts. Lane 2 shows recovered fragments using TrpE extracts. Lane 3 shows recovered fragments when no extract is added. Lanes 4-6 show immuno-precipitations using anti-$\alpha 2$ antibodies and decreasing amounts of purified $\alpha 2$ protein (60, 30, 15 ng).


A.
trpE:SIN1 + − + −
trpE − + − +
dI:dC − − + +
M 2 3 4 5
URS2


B.
CT DNA
M 1 2 3 4 5
URS2

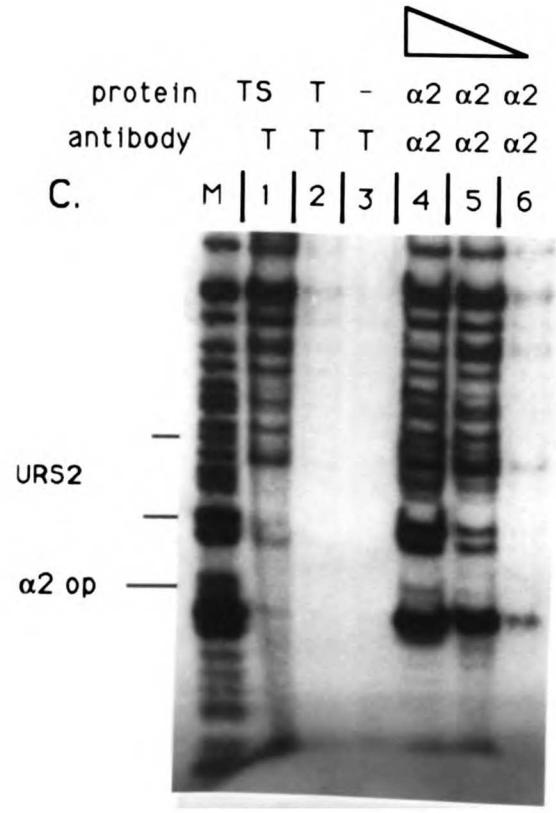

protein TS T − α2 α2 α2
antibody T T T α2 α2 α2
C.
M 1 2 3 4 5 6
URS2
α2 op

Figure 3

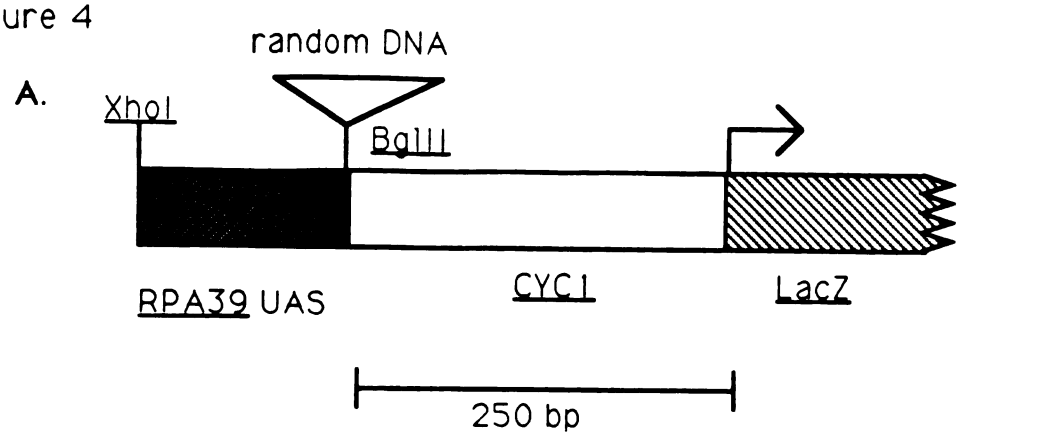
Figure 4 Structures of various DNA constructs used in this study.

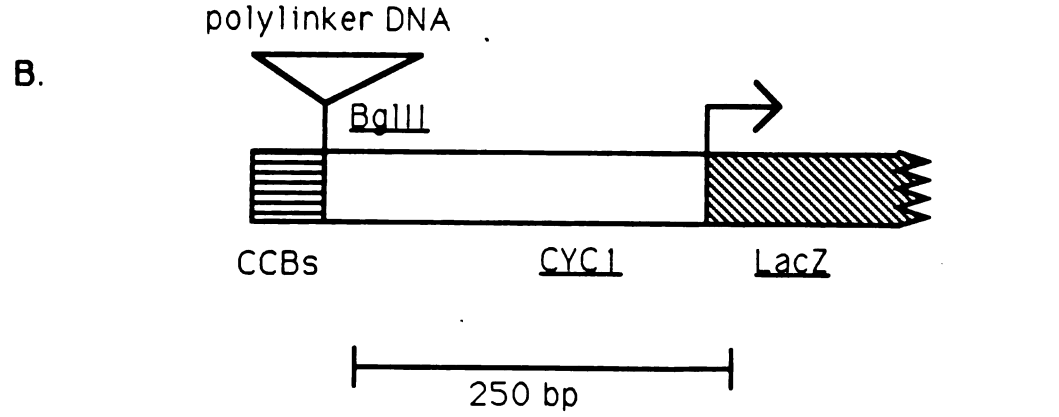
(A) Structure of the RPA39-CYC1-lacZ promoter segment of pBA147 and derivatives used in Table 2.

(B) Structure of CCB-CYC1-lacZ promoter segment of plasmid P2 and derivatives used in Table 3.

(C) Structure of the SIN1 region of plasmid pΔSPH and the structure of the sin1Δ allele of plasmid pSIN1Δ:TRP1. Grey region indicates SIN1 coding region. Black-striped region indicates TRP1 coding region.

Figure 4


A.
random DNA
XhoI
BglII
RPA39 UAS
CYC1
LacZ
250 bp


B.
polylinker DNA
BglII
CCBs
CYC1
LacZ
250 bp

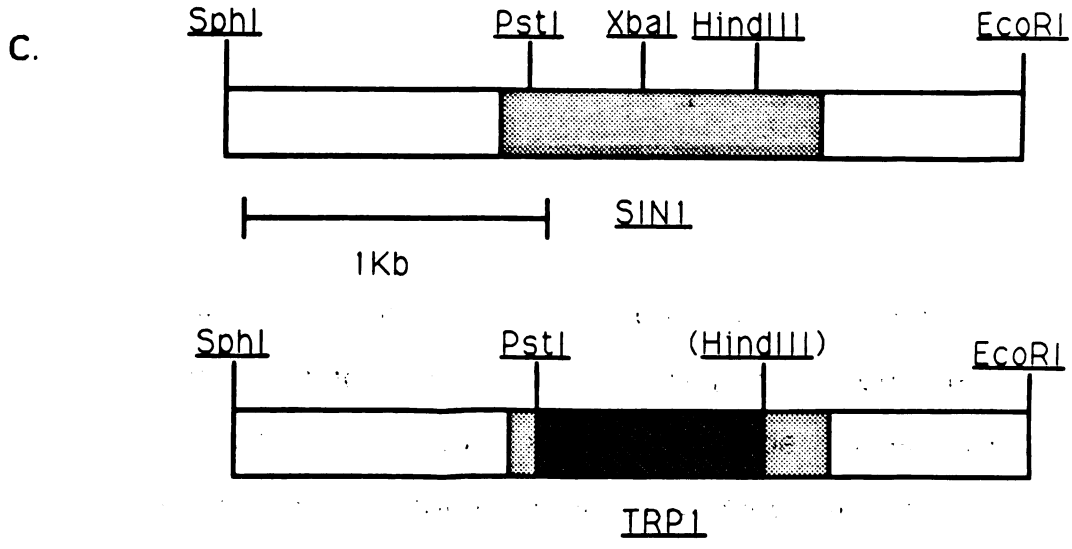

C.
SphI
PstI
XbaI
HindIII
EcoRI
SIN1
1Kb
SphI
PstI
(HindIII)
EcoRI
TRP1

Figure 5. Sin1⁻ restores expression of HO-lacZ utilizing the normal start site

A.) Primer extension analysis (see Materials and Methods) of HO and URA3 transcripts. Lane 1: wild-type yeast strain BDY12A-1c. Lane 2: swi1Δ strain WK1-9a. Lane 3: swi1Δ sin1-2 strain WK1-4d.

B) Primer extension analysis comparing suppression of swi1Δ by sin1-2 and sin1Δ. Lane 1: swi1Δ sin1-2 strain WK1-4d. Lane 2: swi1Δ sin1Δ strain WK44-9b.

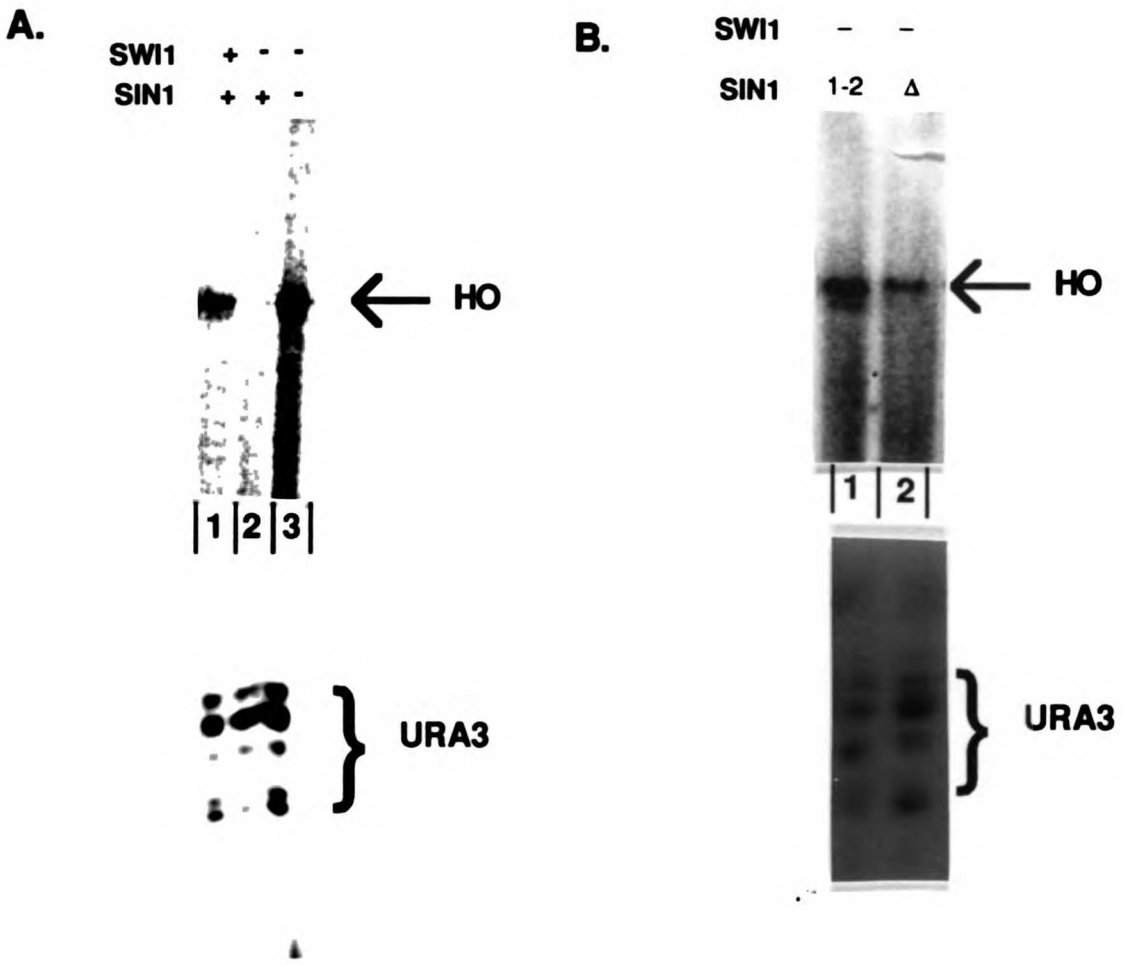


Figure 5

Figure 6 Unusual mating behavior of α sin1⁻ mutants.

A) Patches of cells of the indicated genotype were replica plated to a lawn of α sst2 cells, which are supersensitive to **a**-factor. Production of **a**-factor is seen by a zone of inhibition surrounding the patch. The strains used (from top to bottom) are: WK10-1a, WK10-1aΔ, and WK9A-4b.

B) Patches of cells of the indicated genotype were replica plated to a lawn of α cells containing a lys2⁻ mutation on minimal SD media. Diploids formed by mating are able to grow and are seen as papillae.

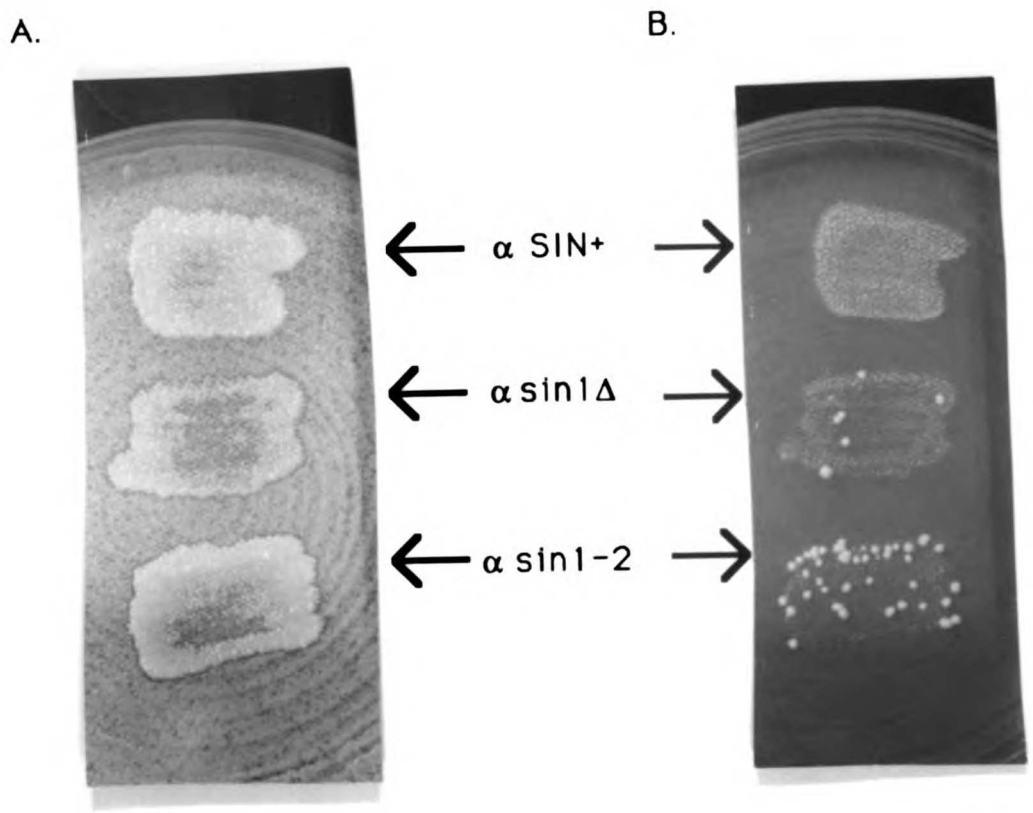


Figure 6

CHAPTER 3

DOMINANT INTERFERING MUTATIONS AFFECTING HISTONE H3 ALLOW EXPRESSION FROM ENFEEBLED PROMOTERS IN YEAST

by Warren Kruger, Craig Peterson, Anita Sil and Ira Herskowitz

To be submitted in revised form to Nature.

Abstract

The sin2-1 mutation was identified because it restores HO expression to a strain that lacks the positive regulator SWI1. We show here that sin2-1 is a point mutation in HHT1, one of the two yeast genes coding for histone H3; it results in an arginine to histidine change at amino acid 117 of histone H3. The sin2-1 mutation is partially complemented by low copy plasmids carrying either HHT1 or the other histone gene, HHT2. In addition to its effect on HO, the sin2-1 mutation also suppresses transcriptional defects due to truncation of the carboxy-terminal domain of RNA polymerase II and to insertion of a δ element into a promoter. These results indicate that alterations in chromatin structure can affect gene expression in discrete ways in vivo. The analogous mutational change introduced into the HHT2 gene (hht2-1) also confers a Sin$^-$ phenotype. Sin2-1 and hht2-1 are partially dominant to wild-type HHT1 and HHT2; additionally, the hht2-1 mutation gives a more severe phenotype then deletion of HHT2. These observations demonstrate the phenotypic consequences of dominant interfering mutations in redundant gene families. Random mutagenesis of the HHT2 gene has revealed two additional mutations which confer a Sin$^-$ phenotype. This result suggests a general approach of using yeast to identify dominant interfering mutations.

Introduction

The SIN2 gene of Saccharomyces cerevisiae was initially identified as a regulator of HO transcription. Mutations in SIN2 (sin2-1) or in SIN1 allow HO transcription in the absence of the SWI1, SWI2, SWI3, or SWI5 gene products (Sternberg et al.,1987). In addition to affecting HO expression, mutations in SIN1 are able to reverse the transcription defects due to truncation of the carboxy-terminal domain (CTD) of the largest subunit of RNA polymerase II (Peterson et al., 1991) and δ element insertions in the 5' region of certain yeast genes (Kruger and Herskowitz, 1991). The SIN1 gene codes for a protein with sequence similarity to HMG1, a non-histone component of chromatin, and it has been proposed that SIN1 may also to be a component of chromatin (Kruger and Herskowitz, 1991).

In this paper we show that SIN2 is identical to HHT1, one of the two structural genes for histone H3. The sin2-1 allele is a point mutation which results in an amino acid substitution at position 117. We have constructed the analogous mutation in the other histone gene, HHT2, and show that this mutation also creates a Sin$^-$ phenotype. Characterization of these mutations indicates that they are semi-dominant and have transcriptional effects identical to sin1$^-$ mutations. Random mutagenesis of HHT2 has identified two additional mutations which cause a Sin$^-$ phenotype. These results indicate it is possible to obtain informative mutations in functionally redundant genes, and the feasibility of using random mutagenesis of a cloned gene followed by phenotypic screening in yeast to identify dominant interfering mutations. Additionally,

these studies indicate an important role for chromatin in the regulation of transcription.

Materials and Methods

Strains and general methodologies

Growth of yeast and general yeast genetic methods are described in Hicks and Herskowitz 1976, and references therein. Yeast were transformed as described (Ito et al., 1983). β-galactosidase activity was quantitatively measured in cells grown in liquid media as described (Miller, 1972). β-galactosidase activity of cells grown on plates was examined qualitatively by filters as described (Andrews and Herskowitz, 1989).

Isolation and analysis of sin2-1 complementing clones

A genomic low-copy library (see Kruger and Herskowitz, 1991) was used to transform yeast strain WK3-7c (relevant genotype: α swi1Δ sin2-1 HO-lacZ ura3-52) and approximately 5000 colonies were screened for reduced β-galactosidase activity as described (Kruger and Herskowitz, 1991). Forty-three candidates were tested for plasmid dependence of the phenotype by selecting for loss of the plasmid on 5-FOA (Boeke et al., 1984) and subsequent rescreening. Only one of the candidates was plasmid dependant. This plasmid was recovered, restriction mapped, and the indicated fragments in Figure 1 were subcloned into YCp50. pHHT1 was constructed by subcloning a 7 kb HindIII fragment containing HHT1 derived from pMS191 (Smith, 1983) into the HindIII site of YCp50. The sin1-2 containing plasmid used in Table 1 is pSin1-2 described in Kruger and

Herskowitz, 1991. All of the plasmids described above were tested for complementation in WK3-7c.

The HindIII fragment contained in pH3-2 was further subcloned into two BamHI-HindIII fragments which were inserted into both mp18 and mp19 for subsequent sequencing using Sequenase^{tm}. The partial sequence was analyzed and found to be already in the database as part of the HHT2 gene (Smith, 1983).

In order to see if pSIN2c actually contained SIN2, a HindIII fragment indicated by subclone pH3 was inserted into Yip5 and the subsequent plasmid, pYipH3, was linearized with BglII and used to transform WK3-7c. A resultant transformant was mated with WK10-8d, sporulated, and 17 tetrads dissected. Of the 12 swi1Δ sin2-1 spores, only 7 were URA+ indicating that HHT2 and SIN2 were not tightly linked and therefore not the same gene.

Recovery and analysis of sin2-1

To recover the HHT1 loci from a sin2-1 strain we employed the following procedure. First, A YCp50 plasmid was digested with EcoRI, filled in with Klenow, and subsequently recircularized which resulted in YCp50ΔR1. Into the HindIII site of this plasmid was inserted a 6 kb HindIII fragment isolated from pMS351 (a gift from M. Smith), which contains the HHT1 loci in which the HHT1 coding sequences have been deleted and replaced with an EcoRI site (see Smith and Stirling, 1988). This resultant plasmid, designated pHHT1Δ, was then digested with EcoRI and used to transform WK3-7c. Four of the resultant colonies were isolated and their plasmids recovered. Two of plasmids' restriction maps indicated that they

had been repaired by the resident HHT1 loci. Subsequently these two plasmids were used to transform WK1-9a. Both plasmids behaved identically as the representative one shown in Table 2. The recovered plasmid was designated phht1-1.

The HindIII-SmaI fragment containing hht1-1 (sin2-1) was subcloned into mp18 and mp19 and the entire H3 coding region was sequenced using the primers 5'CTAAAACTGATGACAATCAA and 5'GAAAAACATCTAACATAAT.

Construction of hht2-1 and hht2Δ

Site directed mutagenesis of HHT2 was performed essentially as described (Climie et al., 1990). The oligo used for the mutagenesis was 5'GATAGTAACATGCTTAGCGTG. An EcoRI-BglII fragment containing either HHT2 or hht2-1 was cloned into pRS314 (Sikorski and Hieter, 1989) which is a centromere based vector containing the TRP1 gene. A The hht2Δ allele was constructed by first subcloning the HindIII fragment in pH3-2 into pUC18, followed by digestion of the resultant plasmid with AccI and BamHI. A BamHI-SmaI fragment containing URA3 derived from CY243 (S. Michaelis, unpublished) was subsequently ligated into the above vector. The HindIII fragment from the resultant plasmid, pUC:hht2Δ, was used for gene replacement as described (Orr-Weaver et al., 1983) and was confirmed by Southern hybridization.

In vitro mutagenesis of HHT2 and screening for additional Sin- alleles

pH3-2 was mutagenized by treatment with hydroxlamine by the procedure of Mendenhall et al., 1988, with the following modifications. Ten micrograms of plasmid DNA in 100 microliters of 1M potassium phosphate (pH 6.0) was treated with 100 microliters of fresh 1M hydroxylamine at 70°C for 1.5 hours. Reactions were desalted over G-25 spin columns, ethanol precipitated, and resuspended in 10 microliters of TE. Half of the resultant pool of mutagenized plasmid DNA was used to transform Cy240. Approximately 1500 transformants were obtained, which were subsequently patched and tested for β-galactosidase activity by filter assay. A total of six suppressors were obtained. Growth on 5-FOA plates resulted in loss of the suppressor phenotype, confirming that the phenotype was plasmid-dependent.

Five of the plasmids were recovered and the HindIII insert fragments were subcloned into mp18 and the HHT2 gene of each insert was sequenced using the primers 5'GGATGTTTGTATGATGTCCC and 5'TTCCCGCTTTATATTCATGA. The recovered plasmids were subsequently retested by transformation into WK1-9a.

INO1 primer extension analysis

For analysis of INO1 transcripts, cells were grown and RNA was isolated as described (Peterson et al., 1991). Primer extension analysis was performed on 20 micrograms of RNA as described (Kruger and Herskowitz, 1991). The primer used to measure INO1 transcripts was 5'GCTGTCTTCGTAACTACAGAC.

Results

SIN2 is HHT1

The SIN2 gene was cloned by screening for low copy plasmids able to complement a sin2-1 mutation. We expected only partial complementation because we had observed that sin2-1 was partially dominant to SIN2$^+$ (data not shown; see Table 2). One plasmid was obtained with this behavior and we designated it pSIN2c. The structure of the insert of this plasmid is presented in Figure 1. Subcloning experiments revealed that the complementing portion of this plasmid lies on a 2.7 kb HindIII fragment, and that a BamH1 site lies in or very near the complementing region (Figure 1). Sequencing of the DNA adjacent to the BamH1 site revealed that the complementing region contained the HHT2 gene, one of the two genes in yeast which code for histone H3 (Smith, 1983).

We next determined whether HHT2 was identical to SIN2. We used a portion of the pSIN2c insert to direct integration of a URA3 gene to the HHT2 locus and then performed an appropriate cross (see Materials and Methods) to examine whether URA3 was now tightly linked to SIN2. The results of this cross showed that that URA3 was unlinked to SIN2, indicating that SIN2 was not HHT2.

Since there were two genes in yeast which coded for histone H3, we reasoned that sin2-1 might be an alteration in the HHT1 gene. Indeed, we observed complementation of a sin2-1 mutation by both HHT1 and HHT2 (Table 1). Additionally, we noted that expression of a semi-dominant allele of sin1-2 exacerbated the Sin- phenotype of sin2-1. In order to determine whether SIN2 was actually HHT1, we

recovered the HHT1 locus from a sin2-1 strain by gap repair (see Materials and Methods). Since sin2-1 is semi-dominant, we expected that a plasmid containing sin2-1 would confer a Sin$^-$ phenotype to a SIN2$^+$ strain. As shown in Table 2, the HHT1 locus from a sin2-1 strain (designated hht1-1) has this behavior. These experiments indicate that sin2-1 is a mutation in the HHT1 gene.

To determine the exact nature of the sin2-1 mutation, we sequenced the recovered hht1-1 locus. The sequence revealed three differences from the previously published sequence (Smith, 1983), only one of which resulted in an amino acid change. This change substitutes a histidine for an arginine at amino acid position 117.

sin2-1 and sin1$^-$ mutations show the same set of transcriptional alterations

Because sin2-1 and sin1$^-$ mutations show similar phenotypes with respect to HO transcription (Sternberg et al., 1987), we have examined the effect of sin2-1 on other genes known to be affected by sin1$^-$. Transcription of the INO1 gene is very sensitive to mutations in the carboxy-terminal domain (CTD) of the large subunit of RNA polymerase II (RPB1) (Nonet et al., 1987; Nonet and Young, 1989). Mutations in SIN1 partially reverse the 100-fold effect of these CTD mutations on INO1 transcription (Peterson et al., 1991). We therefore examined whether sin2-1 had a similar effect.

We measured INO1 RNA levels by primer extension analysis in isogenic strains expressing wild-type and mutant alleles of RPB1 in combination with plasmids expressing either nothing, sin2-1, or sin1-2 (a semi-dominant allele of SIN1). The results of this

analysis are presented in Figure 2. In the strain carrying the RPB1 truncation (rpb1-103) no INO1 transcript was detected (lane 2). When sin2-1 is introduced, INO1 expression levels increased to approximately 15% of wild-type levels (lane 3). A similar level of suppression was exhibited by the strain carrying sin1-2 (lane 4). This experiment shows that sin2-1 is able to partially reverse the phenotype of a CTD truncation of RNA polymerase II.

We also examined whether sin2-1, like sin1$^-$, could suppress the transcriptional defect caused by insertion of a yeast δ element in the 5' region of the LYS2 gene. This suppression ability is called the Spt$^-$ phenotype (Winston et al., 1984). A strain containing a lys2-128δ allele (WK51-5d) normally behaves as a lysine auxotroph because insufficient LYS2 transcript is produced (Simchen et al., 1984). However, transformation of this strain with a plasmid containing sin2-1 converts the strain into a lysine prototroph (data not shown). Thus sin2-1, like sin1$^-$, causes cells to exhibit an Spt$^-$ phenotype.

These results show that sin2-1 and sin1$^-$ cells exhibit a similar set of transcriptional defects.

Genetic analysis of a small multi-gene family

In yeast there are two genes which encode identical histone H3 proteins, HHT1 and HHT2. Thus these two genes can be considered to be part of a small multi-gene family. We were interested in understanding the phenotypic consequences of mutations at either of the genes in this small gene family. Specifically, we wished to determine whether a mutation that was analogous to sin2-1 in the

other histone gene (HHT2) would result in a Sin⁻ phenotype. In addition, we wanted to examine the phenotypes of isogenic cells carrying mutant, wild-type, and null alleles of HHT2. The HHT2 alleles used in this experiment are shown in Figure 3a. Using site-directed mutagenesis, we introduced a single base pair change into HHT2 that should cause production of a histone H3 molecule with an arginine to histidine substitution at position 117. We introduced this allele (designated hht2-1) on a low copy number plasmid into a yeast strain (WK50-2a) which contained a deletion of HHT2 and appropriate markers to score both the Sin and Spt phenotypes. As controls, we also transformed strain WK50-2a with the vector plasmid carrying no insert or the wild-type HHT2 gene.

Both Sin⁻ and Spt⁻ phenotypes were observed in cells carrying hht2-1 (Figure 3b). Quantitation of the levels of HO-lacZ levels is shown in Table 4. In cells carrying either the plasmid with no insert or the plasmid with the wild-type HHT2 gene, no Sin⁻ or Spt⁻ phenotypes were observed. From this experiment we can draw two conclusions: (1) production of mutant histone H3-sin protein from either histone locus is sufficient to create both Sin⁻ and Spt⁻ phenotypes, and (2) deletion of one of the two histone genes is not sufficient to give these phenotypes.

In order to determine the phenotype of a strain that produces only histone H3-sin protein we attempted to generate a double mutant strain containing both sin2-1 and hht2Δ. We constructed a diploid (WK48-20a/WK48-6c) of the following genotype: swi1Δ:LEU2 /SWI1 hht1-1/HHT1 hht2Δ:URA3/HHT2 HO-lacZ/HO-lacZ and dissected 45 tetrads from which we obtained 95 viable spores. In

this cross we were only able to follow hht1-1 in swi1Δ spores. None of the 43 swi1Δ spores contained both hht2Δ:URA3 and hht1-1, indicating that the triple mutant combination is lethal. Additionally, only 18 of the 52 SWI+ spores were URA+, which is consistent with hht1-1 and hht2Δ:URA3 being a lethal combination even in the presence of SWI1. The failure to recover hht1-1 hht2Δ:URA3 containing spores suggests a cell is not viable which produces only histone H3-sin protein.

Identification of additional Sin- alleles

We wished identify additional changes in histone H3 which, like sin2-1, could give a dominant Sin- phenotype. We mutagenized plasmids containing the HHT2 gene in vitro (see Materials and Methods) and then used the mutagenized plasmids to transform a swi1Δ HO-lacZ strain. We subsequently screened the transformants for plasmids which conferred a Sin- phenotype. We recovered five plasmids that were sufficient to confer the Sin- phenotype. The strength of the Sin- phenotype conferred by these plasmids fell into two classes, strong and weak (See Table 4). Sequence analysis revealed all three of the plasmids which conferred strong Sin- phenotype contained the same mutation, designated hht2-3, which results in a threonine to isoleucine change at amino acid 119. Likewise, both of the plasmids which gave a weak Sin- phenotype contained the same mutation, designated hht2-2, which results in an aspartate to lysine change at amino acid 109. A summary of the changes in the histone H3 protein which can cause a Sin- phenotype is shown in Figure 4. All three of the changes which create a

thought to be a component of chromatin (Kruger and Herskowitz, 1991). In wild-type cells, SIN1 and SIN2 are thought to control utilization of the cell-cycle box elements in the URS2 region of the HO promoter (Herskowitz, 1989; Kruger et al., in preparation). These cell-cycle-box elements are required for the proper cell-cycle regulation of HO transcription, and bind a site-specific DNA-binding factor called CCBF which contains the SWI4 and SWI6 proteins (Nasmyth, 1985; Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). Certain mutations in SIN1 and SIN2 allow inappropriate activation of these cell-cycle-box elements (Kruger et al., in preparation), and bypass the need for SWI1, SWI2, SWI3, and SWI5. The observation that both SIN1 and SIN2 are components of chromatin suggest that chromatin structure may some how interfere with CCBF formation or function. These observations also indicate that the role of the SWI1, SWI2, SWI3, and SWI5 gene products may be to antagonize the negative influence of chromatin on cell-cycle-box activation.

Alterations in histone H3 and SIN1 also derepress genes whose promoters have been inactivated by insertion of δ elements. Insertion of a δ element in the upstream region of HIS4 or in the beginning of the coding region of LYS2 greatly reduces transcription of these genes (Silverman and Fink, 1984; Simchen et al., 1984). The precise reason why these insertions reduce transcription is not clear and is likely to be complex. The SPT genes were identified because mutations in these genes increased transcription from these inactivated promoters (Winston et al., 1984; Fassler and Winston, 1988). SPT11 and SPT12 code for yeast histones H2A and H2B

respectively (Clark-Adams et al., 1988). Experiments have shown that altering the balance between H2A-H2B dimers and H3-H4 dimers inside the cell can create a Spt⁻ phenotype. These observations, as well as our own, indicate that promoters crippled by insertion of a δ element are very sensitive to alterations in chromatin structure.

Alterations in SIN1 and histone H3 are also capable of partially suppressing the transcriptional effects of truncation of the carboxy-terminal domain (CTD) of RNA polymerase II at the INO1 promoter. The CTD consists of a conserved heptapeptide that is present in 26 or 27 copies in yeast and in greater numbers in other eukaryotes (Nonet et al., 1987). Deletion of this domain is lethal in yeast, while truncation to 10 copies results in a temperature sensitive phenotype and greatly reduced expression of the INO1 gene (Nonet and Young, 1989). Mutations in SIN1 have been shown to alleviate some of the effects of the CTD truncation (Peterson et al., 1991). The observation that sin1⁻ mutations alleviate the effects of a shortened CTD has led to the proposal that function of the CTD may be to antagonize the effect of chromatin structure at the promoter (Peterson et al., 1991). We have shown here that the sin2-1 mutation has a similar effect. We observe a 10- to 20-fold increase in the levels of INO1 transcript when histone H3-sin is produced.

How does production of histone H3-sin effect transcription? One possibility is that production of histone H3-sin results in nucleosome depletion. It is possible that a nucleosome which contains a histone H3-sin molecule is not functional, so that the amount of nucleosomes in a cell producing both wild-type histone H3

and histone H3-sin is reduced. Studies have demonstrated that massive nucleosome depletion can result in gene activation for a variety of genes and raises the possibility that some positive regulators may function by mimicking this process (Han and Grunstein, 1988; Han et al., 1988). Alternatively, it is possible that histone H3-sin is incorporated into nucleosomes, but these nucleosomes have altered properties. They may bind to DNA in some abnormal way or fail to interact properly with other proteins. This kind of explanation could also be used to explain the behavior of cells which contain mutations in the N-terminus of H4. These cells have partially derepressed transcription of HMLα and HMRa, but do not appear to have substantially reduced numbers of nucleosomes (Kayne et al., 1988). The fact that all three histone H3-sin alterations map to the same region of the histone H3 protein may define a region of histone H3 important for protein-protein interactions. Given the phenotypic similarity between cells containing either sin2-1 or sin1$^-$, an intriguing possibility is that nucleosomes that contain histone H3-sin fail to interact properly with SIN1, an HMG1-like protein. Interestingly, calf thymus histone H3 has been shown to cross-link in vivo to HMG1 (Stos, 1987). Biochemical studies will be necessary to address these possibilities.

Genetics of redundant genes

The genetic interactions between the various alleles of the two different histone loci are summarized in Table 5. There are several notable points. First, the arginine to histidine mutation at

position 117 in either of the two loci is sufficient to create a Sin$^-$ phenotype. Thus, this mutant form of histone H3 is dominant in combination with the wild-type form. Second, deletion of one of the two histone loci does not create a Sin$^-$ phenotype. This indicates that the Sin$^-$ phenotype requires production of a mutant histone H3 protein, and not simply a reduction in the amount of wild-type histone H3. Third, the ratio of wild-type to mutant genes affects the strength of the Sin$^-$ phenotype. Sin2-1 strains containing an extra wild-type histone H3 gene (either HHT1 or HHT2) on a low copy plasmid exhibit a weakened Sin$^-$ phenotype (see Table 1). Together, these observations indicate the histone H3-sin protein is interfering with wild-type histone H3. For this reason, we refer to sin2-1 as a dominant interfering mutation.

The ability to create dominant interfering mutations is extremely useful in genetic analysis of redundant gene families. In the case described here, a null mutation in HHT2 causes no phenotype because the HHT1 gene is still intact and able to provide sufficient amounts of histone H3. Deletion of both HHT1 and HHT2 is lethal and therefore provides little information about the transcriptional role of histone H3. In this situation, only the production of a mutant histone H3 protein that partially interferes with wild-type histone H3 function creates an informative phenotype. In multicellular organisms dominant interfering mutations have been identified in various gene families. In the nematode, dominant interfering alleles in the genes which code for myosin and actin have been identified which affect movement (Waterston et al., 1984; Epstein, 1990). In Drosophila, dominant interfering mutations in one of the genes

coding for tubulin has been identified which affect sperm (Fuller, 1986). Thus when a gene is redundant, dominant interfering mutations are an extremely useful way to get in vivo information about gene function.

Use of yeast to identify dominant interfering mutations

It has been previously suggested that the use of dominant interfering or dominant negative mutations could be useful in providing information on the in vivo function of cloned genes from higher cells (Herskowitz, 1987). Since the histone H3 protein is among the most highly conserved of all genes, 93% identical between yeast and man, the mutations identified here may be useful in analyzing the functions of histones in multicellular organisms. For example, it would be possible to express histone H3-sin under the control of a regulated promoter in Drosophila in order to study its effects on development and differentiation.

In this study we were able to identify additional dominant interfering alleles of histone H3 by random in vitro mutagenesis of the cloned histone H3 gene followed by phenotypic screening in yeast. It should be possible to use a general version of this strategy to identify dominant interfering mutations in other cloned genes. The starting point in such a screen is a yeast strain which expresses a cloned gene of interest in such a way that its function is required for some assayable phenotype in yeast. Into this strain randomly mutagenized versions of this cloned gene could be transformed and then subsequently the yeast could be screened for plasmids which conferred a dominant interfering phenotype. Recently, a wide variety of mammalian genes have been shown to function in yeast

including steroid hormone receptors (Schena et al., 1989), Neurotransmitter receptors (King et al., 1990), and G-proteins (Kang et al., 1990). Thus the strategy described above may be generally useful in identifying dominant interfering mutations in many different kinds of genes.

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Table 1

HHT1 and HHT2 both can complement sin2-1

Plasmid Insert	HO-lacZ Activity (Miller units)
none	1.1
HHT1	0.4
HHT2	0.4
sin1-2	2.1

Low copy-number plasmids (YCp50-based) containing the indicated inserts were introduced by transformation into a swi1Δ sin2-1 HO-lacZ strain (WK3-7c). Three transformants were picked and β-galactosidase assays were performed. Averages are presented and varied less then 15%. A swi1Δ SIN$^+$ HO-lacZ strain gave < 0.1 units.

Table 2

The HHT1 locus from a sin2-1 strain confers a Sin⁻ phenotype

Strain	Genotype	Plasmid insert	HO-lacZ expression
WK1-9a	swi1Δ SIN2	none	0.1
WK1-9a	swi1Δ SIN2	HHT1	0.1
WK1-9a	swi1Δ SIN2	hht1-1	0.7
WK3-7c	swi1Δ sin2-1	none	1.1

Low copy-number plasmids (YCp50-based) containing the indicated inserts were used to transform the indicated strains and β-galactosidase activity from three separate transformants was measured. Averages are presented and varied less than 15%. The insert designated hht1-1 contains the HHT1 locus recovered from the sin2-1 strain WK3-7c by repair of a gapped plasmid (see Materials and Methods).

Table 3

Suppression of *swi1Δ* by *hht2-1*

Insert	Activity
none	<0.1
hht2-1	1.0
HHT2	<0.1

The indicated inserts, carried on a low copy vector (pRS314), were introduced by transformation into strain WK50-5c (*swi1Δ HO-lacZ lys2-128δ hht2Δ*). Three colonies were isolated and liquid β-galactosidase assays were performed. Averages are presented and varied less then 15%.

Table 5

Summary of genetic interactions at HHT1 and HHT2

HHT1 allele	HHT2 allele	Phenotype
1. HHT1+	HHT2+	Sin+
2. hht1-1	HHT2+	Sin-
3. HHT1+	hht2-1	Sin-
4. HHT1+	hht2Δ	Sin+
5. hht1-1	hht2Δ	inviable[a]
6. hht1Δ	hht2Δ	inviable[b]

a Unable to recover viable spores of this genotype

b Smith and Stirling, 1988

Table 4

Strength of the Sin⁻ phenotype of the different histone H3-sin alleles

Strain	SWI Genotype	plasmid borne allele	HO-lacZ activity
WK1-9a	swi1Δ	hht1-1	0.8
WK1-9a	swi1Δ	hht2-2	0.5
WK1-9a	swi1Δ	hht2-3	2.0
WK50-4c	SWI1	HHT2	5.0
WK1-9a	swi1Δ	HHT2	<0.2

Low copy-number plasmids (YCp50-based) containing the indicated histone H3 producing alleles were used to transform the indicated strains and β–galactosidase activity from three separate transformants was measured. Averages are presented and varied less than 15%.

Table 6

Yeast Strain List

Strain	Genotype	Scource
WK1-9a	α swi1Δ:LEU2 HO-lacZ ura3-52 leu2-	Kruger and Herskowitz, 1991
WK3-7c	α swi1Δ:LEU2 HO-lacZ ura3-52 leu2-	This study
WK10-8d	α HO-lacZ trp1-289 ura3-52 leu2-	This study
WK50-2a	a hht2Δ:URA3 lys2-128δ ura3-52 leu2- HO-lacZ	This study
WK48-20a	α hht2Δ:URA3 ura3-52 leu2- HO-lacZ	This study
WK48-6c	a swi1Δ:LEU2 sin2-1 HO-lacZ leu2- ura3-52	This study
CY240	a swi1Δ:LEU2 HO-lacZ, ura3-52 leu2-	This study
JAY47	a leu2- trp1-1 ura3-1 ade2-1 his3-11,15 can1-100 ho	Archambault et al., 1991

Figure 1

Structure of the plasmid inserts that partially complement sin2-1 in low-copy. The restriction sites shown are: R1= EcoRI, H3=HindIII, BH1= BamHI, Bgl= BglII, and SmaI= SmaI.

Structure of plasmid inserts that complement sin2-1

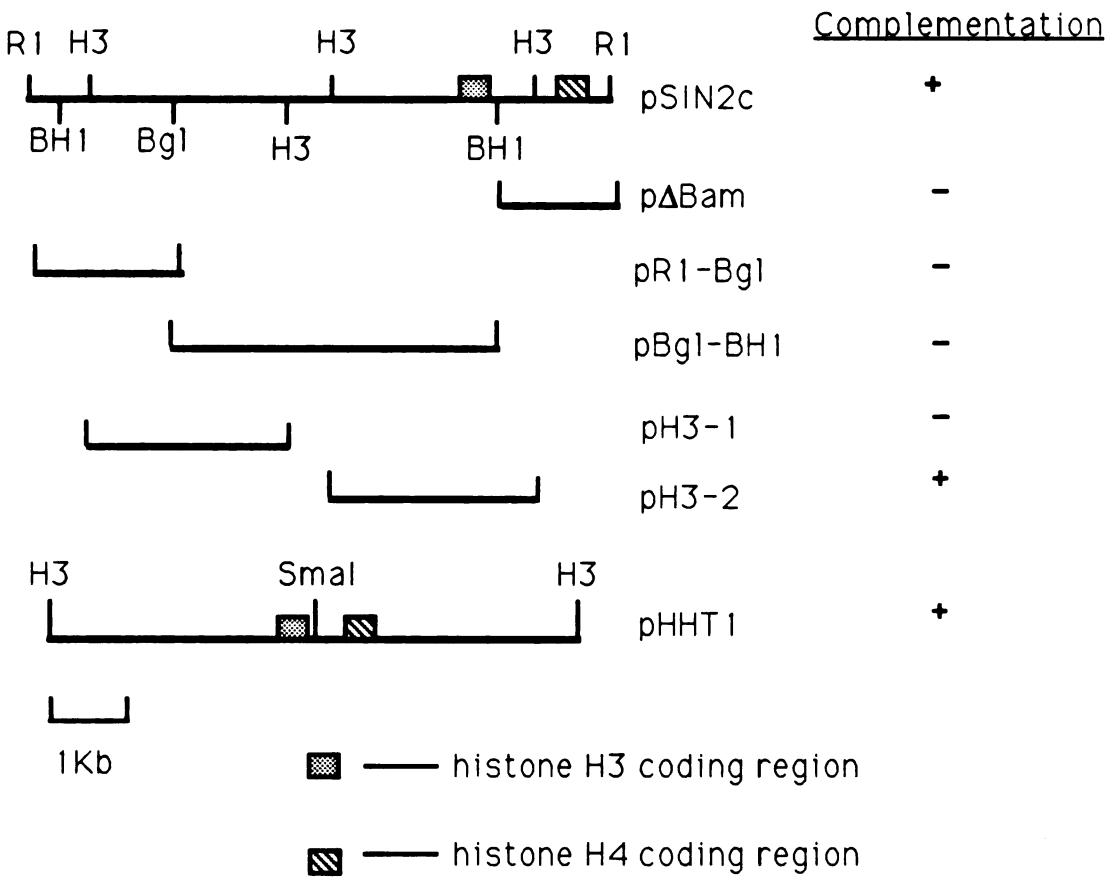


Figure 1

Figure 2

Partial suppression of CTDΔ transcription defect of INO1 by sin2-1. Plasmids expressing either a wild-type RPB1 gene (RP112) or rpb1-103 (Ry2203) which has a truncated CTD, were introduced into JAY47. Into these strains were also introduced plasmids which expressed either sin2-1, sin1-2, or nothing (YCp50 alone). These strains were then grown in glucose under inducing conditions for INO1 transcription. RNA was isolated and transcription of INO1 was measured by primer extension analysis as described in Materials and Methods. Lane 1 contains RP112 and YCp50. Lane 2 contains Ry2203 plus YCp50. Lane 3 contains Ry2203 plus phht1-1. Lane 4 contains Ry2203 plus pSin1-2.

hht1-1 (sin2-1) can partially suppress transcription defects at INO1 due to CTD truncation of Pol II.

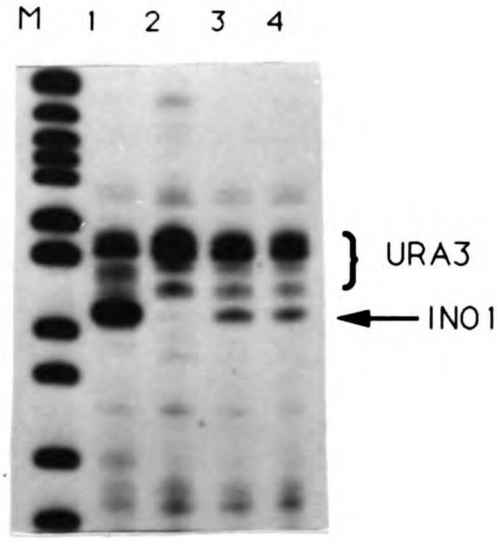


Figure 2

Figure 3a.

HHT2 alleles used in this study. The structure and locations of the various HHT2 alleles used in Figure 3b are shown. The HindIII fragment shown is identical to that of pH3-2 shown in Figure 1. The amino acid located at position 117 is shown in a different font.

Alleles of HHT2

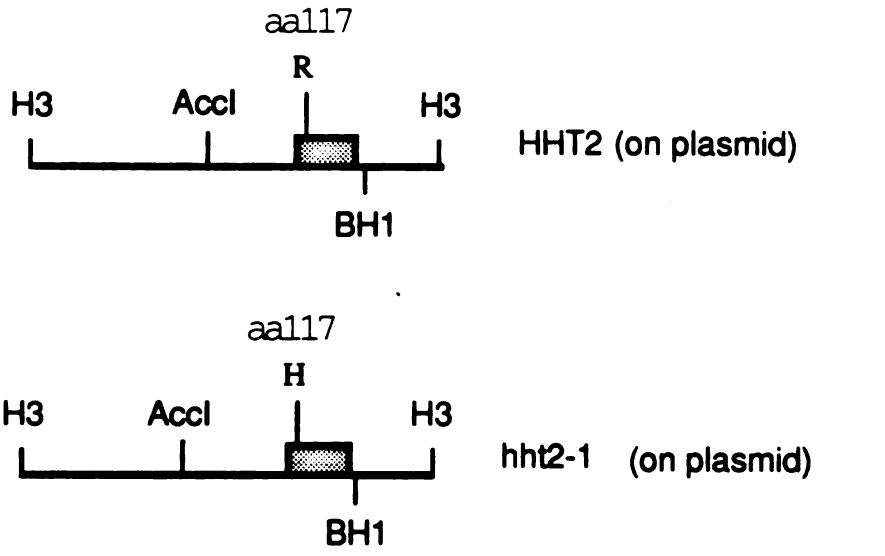
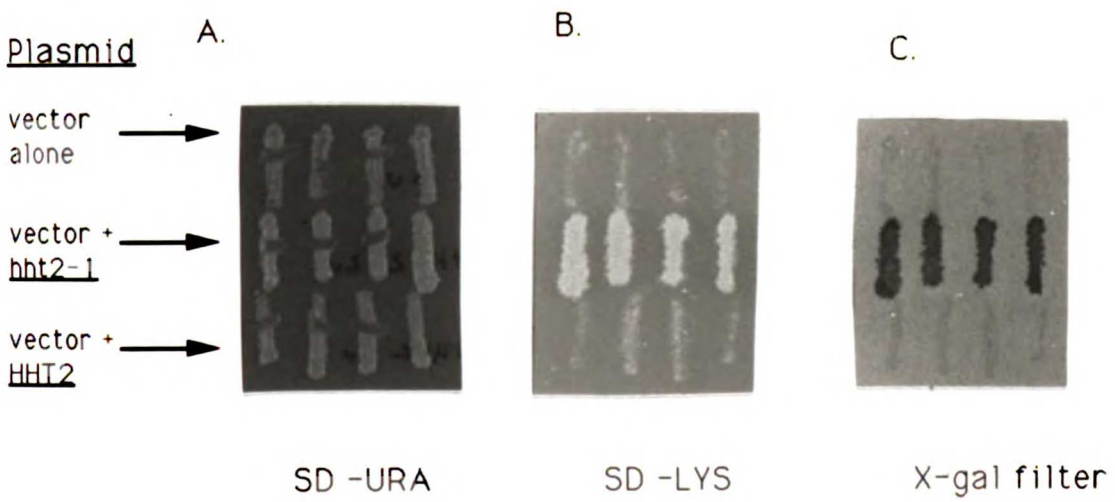


Figure 3a

Figure 3b.

hht2-1 causes both Sin$^-$ and Spt$^-$ phenotypes. The alleles described above were introduced into WK51-5d (genotype shown above), and four colonies from each transformation were patched on SD-URA (panel A), subsequently tested for growth on SD-Lys media (Panel B), and β–galactosidase activity using a filter assay (Panel C).

Substitution of Arginine to Histidine at amino acid 117 of HHT2 causes both Sin- and Spt- phenotypes.



Strain genotype: swi1Δ HHT1 hht2Δ HO-lacZ lys2-128δ

Figure 3b.

Figure 4

Analysis of the Sin$^-$ mutations in histone H3. The various changes in the histone H3 protein which give a dominant Sin$^-$ phenotype are shown above. The shaded area of the histone H3 protein (below) shows the region in which the three described changes occur.

Sin mutations in histone H3

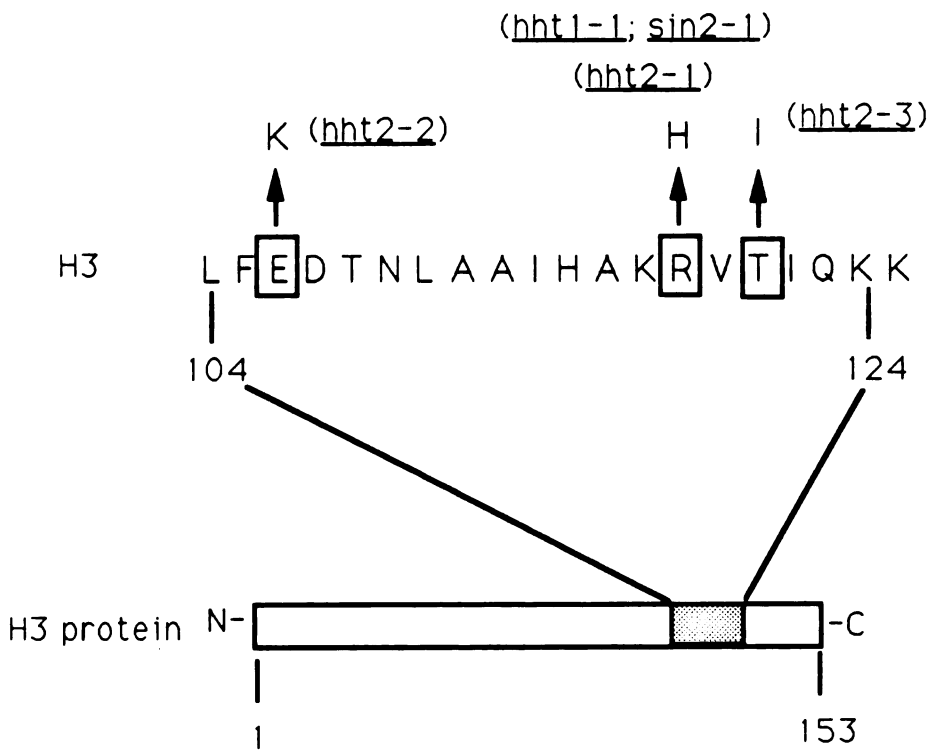


Figure 4

Chapter 4

THE URS2 REGION OF THE YEAST HO GENE CONTAINS A REGULATED UAS UNDER CONTROL OF SWI1 AND SIN1.

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Abstract

The yeast HO gene is transcribed in mother cells only during the late G1 phase of the cell cycle. This transcriptional control is exerted by the interplay of genes coding for both positive (SWI) and negative regulators (SIN) of HO. These regulators exert their effects through a large regulatory region which can be functionally subdivided into two parts, URS1 and URS2. URS1 contains UAS activity by two criteria: it is required for HO expression, and it is sufficient to drive expression from a heterologous promoter lacking a UAS. In this study we show that URS2 contains a regulated UAS that can also drive expression from a heterologous promoter. This UAS is inhibited by the SIN1 and SWI1 genes and requires SWI4 and SWI6. The UAS activity of URS2 is sufficient to drive HO expression in the absence of URS1, but this expression is no longer properly regulated. These findings lead to a model in which URS1 and URS2 act sequentially to control HO transcription.

Introduction

The HO gene codes for an endonuclease that initiates the process of mating-type interconversion in Saccharomyces cerevisiae (Kostriken and Heffron, 1984). HO is transcribed only in haploid mother cells during the late G1 phase of the cell cycle (Nasmyth, 1983). The regulated expression of HO is sufficient to account for the precise pattern of mating type interconversion observed in vivo (Hicks and Herskowitz, 1976; Strathern and Herskowitz, 1979; Nasmyth, 1983).

Transcriptional regulation of HO is mediated by a 1.4 kb segment upstream of the transcription start site. Previous studies (Nasmyth, 1985a,b) have subdivided this region into two functionally distinct segments, URS1 and URS2 (see Figure 1). URS1 has UAS activity: deletion of URS1 inactivates the HO promoter, and insertion of URS1 upstream of a heterologous promoter lacking its endogenous UAS allows expression. URS1 is responsible for the mother/daughter control of HO (Nasmyth, 1987). URS2 is responsible for the cell cycle control of HO but is not essential for expression: deletion of URS2 causes HO to lose cell cycle control (Nasmyth, 1985b). This region of HO contains a repeated sequence, PuPyCACGAAAA (known as the cell cycle box; CCB) that governs cell-cycle regulation (see Figure 1). When this sequence is multimerized and placed upstream of a test promoter, it exhibits cell-cycle-regulated UAS activity (Breeden and Nasmyth, 1987). This expression occurs at the same time in the cell cycle when HO is normally expressed. Studies have detected a DNA binding activity,

cell-cycle-box-factor (CCBF), which binds specifically to these regions (Andrews and Herskowitz, 1989). However, these sequences in the context of URS2 are not sufficient for UAS activity: a chromosomal deletion of URS1 which leaves URS2 intact results in almost undetectable levels of HO transcript (Nasmyth, 1985a). This observation suggests that other sequences in URS2 inhibit the activity of the CCB elements.

At least eleven genes which control HO expression have been identified. The SWI genes (SWI1- SWI6) were identified as activators of HO (Stern and Herskowitz, 1984; Breeden and Nasmyth, 1987). Mutation in any of these genes causes HO to be unexpressed. SWI4 and SWI6 are required for expression from the multimerized cell cycle-box sequences, as well as for formation of CCBF (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). SWI5 is necessary for UAS activity of URS1; its product binds to sites within this region (Stillman et al., 1988). Mutations in SWI1, SWI2, and SWI3 decrease the UAS activity of URS1 and share the same set of pleiotropies; therefore, the SWI1, SWI2, and SWI3 products are thought to act together (Stern and Herskowitz, 1984; Sternberg et al., 1987; C. Peterson, unpublished).

The SIN genes (SIN1- SIN5) were identified as mutations that eliminate the need for various SWI genes for HO transcription. These genes can be divided into two groups based on the ability to suppress different swi mutations (Sternberg et al., 1987). Mutations in SIN1 and SIN2 bypass the need for SWI1, SWI2, SWI3, and partially alleviate the requirement for SWI5; they do not bypass the requirement for SWI4 (SWI6 was not tested at this time).

Mutations in SIN3, SIN4 and SIN5 suppress mutations only in SWI5 (Sternberg et al.,1987). No mutations that genuinely relieve the need for SWI4 have been identified (B. Andrews and J. Ogas, unpublished).

Here we present a hypothesis that explains the paradoxical behavior of the CCB element in functioning as a UAS by exploiting our observations on the functional interactions between SWI and SIN gene products. This hypothesis relies on the observation that inactivation of SIN1 alleviates the need for all the other SWI genes except for SWI4 (and presumably SWI6). Since SWI4 and SWI6 are the only two SWI genes required for UAS activity of the isolated CCB elements, this observation leads to the view that the SIN1 gene product is responsible for inhibiting the activity of the CCB elements when they are located within URS2. A corollary of this hypothesis is that the other SWI genes (SWI1, SWI2, SWI3, and SWI5) are normally required for overcoming inhibition by SIN1. These SWI genes are thus responsible for controlling the ability of URS2 to function as a UAS only under the proper conditions, i.e. in mother cells.

In this paper we test the hypothesis that URS2 contains regulated UAS activity. We show that SIN1 inhibits this UAS activity and that SWI4 and SWI6 are required for UAS activity. We find, unexpectedly, that full inhibition of URS2 also requires SWI1. In addition, we show that mutations in SIN1 do not bypass the need for SWI6. Consistent with the above hypothesis, we show that inappropriate activation of the UAS activity in URS2 (in swi1- sin1- strains) leads to mating-type switching of daughter cells. From

these results, we present a unified model for regulation of HO expression in which the role of URS1 is to allow activation of URS2.

Materials and Methods

Strains

The strains are described in Table 1. The swi1Δ and swi5Δ alleles are gene replacements marked by LEU2, constructed in vitro by Michael Stern from the cloned SWI1 and SWI5 genes (Stern, 1985). The sin1Δ allele was constructed as described and marked by TRP1 (Kruger and Herskowitz, submitted). The swi4Δ and the swi6Δ alleles are gene replacements marked with HIS3 or LEU2 respectively and were constructed from the cloned SWI4 and SWI6 genes by Joe Ogas (unpublished). The HO-lacZ fusion allele is described by Russell et al. (1986). Genetic methods were performed as described by Hicks and Herskowitz (1976), Stern et al. (1984), and references therein.

Identification of swi6 sin1 HO-lacZ segregants

Swi6Δ segregants from a diploid formed by mating JO223-2d and WK10-3c were identified by leucine prototrophy. Six of these segregants containing MATα were mated to strain D262-1a (see Table 1), which contains a cry1 mutation linked to MATa, which allows for subsequent selection of a/a diploids. These diploids were then used to score for HO-lacZ as described (Sternberg et al., 1987). The a/α diploids were sporulated and approximately ten tetrads were dissected for each cross. The presence of sin1-2 was

inferred from the recovery of leucine prototrophs which contain β-galactosidase activity.

Plasmids

All plasmids used in Tables 2 and 3 are derivatives of pΔSS (Hoar and Guarente, 1984). pCYC1-lacZ is p ΔSS with a BglII linker inserted into the SalI site (A. Mitchell, unpublished). pURS2inv-CYC1-lacZ was created by inserting a BamHI fragment from pHO-Lac-c12 (Russell et al., 1986), containing a portion of HO and its upstream region (-762 to +275), into the BglII site of pCYC1-lacZ. pURS2-CYC1-lacZ was created identically as above except that the BamHI insert was in the opposite orientation. This construct, pURS2, was non-functional because the HO transcription start-site was present between URS2 and CYC1-lacZ. This region was eliminated by digestion with KpnI and XhoI and recircularization. The resulting plasmid, pURS2-CYC1-lacZ, contains URS2 sequences from -762 to -131. pURS1-CYC1-lacZ is described in Sternberg et al. (1987), and contains sequences -1516 to -901 from URS1. pRPA39-CYC1-lacZ was created by digestion of pLG699-Z (Guarente and Hoar, 1984) with XhoI and BglII, and insertion of a BglII and XhoI segment from PMR10-299 (Rotenberg and Woolford, 1986). This construct contains promoter sequences (-299 to -171) from RPA39 fused to CYC1-lacZ. pRP:URS2-CYC1-lacZ was created by insertion of a BglII-KpnI fragment from the HO promoter (-710 to -131) in which a BglII site has been added to the KpnI end, into pRPA39-CYC1-lacZ digested with BglII.

Assays of plasmid encoded activities

In the experiments presented in Tables 2 and 3, the indicated strains were transformed using lithium acetate as described in Hicks et al. (1978). At least three individual colonies were isolated and β-galactosidase assays of cells grown in culture were performed as described in Stern et al. (1984) and Miller et al. (1972).

Pedigree analysis

Pedigree analysis was performed as described by Hicks and Herskowitz (1976). Spores of the indicated strains were allowed to germinate in the presence of α-factor provided by a streak of α cells (strain 1793). Pedigrees of α spores were followed for 3 to 8 generations, using micromanipulation to separate mothers from daughters. Cells from the strains heterozygous for the swi1Δ were subsequently tested for leucine auxotrophy to determine which pedigrees were derived from swi1⁻ and SWI1 spores.

Results

sin1⁻ does not alleviate the requirement of SWI6 for HO expression

Previous work had shown that mutations in SIN1 bypass the requirement for SWI1, SWI2, SWI3, and SWI5, but not SWI4 for HO expression. In order to determine whether sin1⁻ bypasses the need for SWI6, we crossed a strain (JO223-2d) containing a swi6 deletion

(marked with LEU2) to a strain (WK10-3c) containing a sin1-2 mutation (a very strong Sin⁻ allele; Kruger and Herskowitz, submitted) and an HO-lacZ gene to assess HO expression. If sin1-2 is able to suppress swi6, we would expect (assuming no linkage between SIN1 and SWI6) one quarter of the swi6 segregants to express HO-lacZ. Nineteen tetrads were dissected from which 59 viable spores were obtained. None of the 25 swi6 segregants from the cross expressed HO-lacZ, suggesting that sin1-2 was unable to suppress the swi6 deletion.

To confirm that a swi6⁻ sin1⁻ HO-lacZ strain had actually been generated, we examined six of the slower-growing swi6 segregants for the presence of HO-lacZ (see Materials and Methods), and found that three of these segregants did contain HO-lacZ. We then crossed these segregants to determine whether they contained sin1-2 (see Materials and Methods). All three segregants contained the sin1-2 allele. These experiments show that sin1⁻ is not able to suppress a swi6 deletion for HO expression.

SIN1 and SWI1 inhibit UAS activity in URS2

The experiment above and previous work (Sternberg et al., 1987) show that sin1 mutations bypass the need for all of the SWI genes except for SWI4 and SWI6. These are the only SWI genes required for UAS activity exhibited by the multimerized CCB elements when placed upstream of a test promoter (Andrews and Herskowitz, 1989; Breeden and Nasmyth, 1987). It had been previously observed that the URS2 region of HO, in the absence of URS1, did not contain appreciable UAS activity even though it contains ten CCB elements (Nasmyth, 1985a). These observations

imply that the CCB elements in URS2 are somehow inhibited by the flanking URS2 sequences. We have examined whether the SIN1 gene product inhibits the UAS activity of the CCB elements located in URS2. If so, a sin1⁻ mutation would allow URS2 to act as a UAS and thus bypass the need for URS1.

To test this hypothesis, we constructed plasmids which contain a portion of the URS2 region inserted in two different orientations upstream of a CYC1-lacZ fusion gene lacking its own UAS (see Figure 2). We then used these plasmids to transform wild-type, swi1⁻ SIN1, SWI1 sin1⁻, and swi1⁻ sin1⁻ strains and measured promoter activity. (In these experiments the sin1 mutation used was the sin1-2 allele.) For controls, we included plasmids with no UAS, the RPA39 UAS, or the URS1 region of HO all inserted at the same location. As can be seen in Table 2, the URS2 fragment in both orientations shows a substantial increase in UAS activity in swi1⁻ sin1⁻ cells relative to that exhibited in wild-type strains. In the normal orientation there is a five-fold increase in activity (6.3 versus 1.2), while in the inverted orientation there is an eighteen-fold increase (18 versus 1.0). Surprisingly, the UAS activity exhibited by SWI1 sin1⁻ cells, although 3- to 5-fold greater than in wild-type cells, is still 2- to 4-fold lower then in swi1⁻ sin1⁻ cells. This result was unexpected because SWI1 behaves as a positive regulator for the entire HO promoter and for URS1 (see line 3). These experiments show that both SIN1 and SWI1 inhibit UAS activity of URS2.

The inhibitory effects of SWI1 and SIN1 are specific to the URS2 segment. Neither swi1⁻ or sin1⁻ mutations have a dramatic

effect on the plasmids containing either the RPA39 UAS or no UAS. Both SWI1 and SIN1 are required for maximal activity in URS1, indicating that in URS1 they act as activators. These results suggest that effects of SWI1 and SIN1 are context dependent.

SWI4 and SWI6 are activators of URS2

We next wished to determine whether the UAS activity of URS2 was dependent on SWI4 and SWI6. For these experiments, we tested pURS2-CYC1-lacZ (see Figure 2A) in a set of isogenic swi4$^-$, swi4$^-$ sin1$^-$, swi6$^-$, swi6$^-$ sin1$^-$, wild-type, and sin1$^-$ strains, which contain deletion alleles of the indicated genes. We detected only background levels (<0.1u) of activity in swi4$^-$, swi4$^-$ sin1$^-$, swi6$^-$ and swi6$^-$ sin1$^-$ cells, but measurable activity for wild-type (0.39u) and sin1$^-$ (0.6u) strains (Table 2, line 1). As a control for specificity we included the CYC1-lacZ promoter containing its endogenous UAS, which showed less than three-fold differences in the different strains. These results show that SWI4 and SWI6 are required for UAS activity from URS2. Given that maximal UAS activity from URS2 is observed when both SIN1 and SWI1 were mutated (Table 1), we would have liked to examine UAS activity in a swi1$^-$ sin1$^-$ strain also defective in SWI4 or SWI6. However this proved to be not possible, as swi4$^-$ and swi6$^-$ mutations are lethal in combination with swi1$^-$ (Stern and Herskowitz, 1984; this paper).

Because the UAS activity of the URS2 segment is so low (Table 3, line 1), we have analyzed this segment of URS2 in another context to ascertain whether it confers regulation by SWI4 and SWI6. In this case, we have not assayed the ability of this segment to function as a UAS but rather its effect on an intact, functional UAS (from the

RPA39 gene). We have inserted the URS2 segment between the RPA39 UAS and the CYC1-lacZ gene and assayed activity in different strains. The RPA39 UAS functions well in swi4⁻ and swi6⁻ strains, exhibiting 104% and 37% of the activity seen in wild-type strains (Table 3, line 4). Addition of the URS2 segment (Table 3. line 3) causes a great drop in UAS activity in wild-type strains -- from 597 units to 1.9 units. This residual activity is dependent on SWI4 and SWI6: activity is reduced to 0.40 and 0.24 units respectively in swi4⁻ and swi6⁻ mutants. Although the nature of the inhibition of the RPA39 UAS due to the URS2 segment is unknown, it appears that the behavior of this segment is once again governed by the state of the SWI4 and SWI6 genes.

Inhibitory roles of SIN1 and SWI1 on chromosomal HO-lacZ

We noted earlier the unexpected finding that the UAS activity of a segment of URS2 is greatest in the absence of both SIN1 and SWI1. Although SWI1 is ordinarily a positive regulator, needed for transcription from the intact HO regulatory region, it appears that it functions as an inhibitor in the absence of URS1. We wished to examine the UAS activity of URS2 in another situation in which URS1 is not functional. This situation can be created by inactivating the SWI5 gene, which is necessary for function of the URS1 region (Sternberg et al., 1987; Nasmyth, 1987). As shown in Table 4 (lines 1 and 2), inactivation of SIN1 increases expression of HO 4.5- fold in a swi5⁻ mutant, as measured by the β-galactosidase activity of an HO-lacZ fusion gene (Russell et al., 1986). A striking finding is that inactivation of the SWI1 gene in the swi5⁻ sin1⁻ strain leads to a further 3-fold increase in expression of HO (Table 4, line 3).

Expression of HO in a swi1⁻ sin1⁻ strain is independent of SWI5 (Table 4, lines 3 and 4). Examination of the levels of URA3 and HO-lacZ mRNA in these strains indicate that these effects are specific to the HO containing transcripts (data not shown). These findings are fully consistent with our earlier observations and indicate that the UAS activity in URS2 is independent of URS1 and is inhibited by SWI1.

Effect of sin1⁻ and swi1⁻ sin1⁻ mutations on the pattern of mating type switching

Previous work has shown that URS1 is responsible for mother/daughter regulation of HO (Nasmyth, 1987). If activation of URS2 UAS activity is entirely responsible for HO expression in swi1⁻ sin1⁻ cells, we expect that HO should be expressed in daughter cells as well as mother cells and thus that daughter cells should be able to switch their mating types. To determine if sin1⁻ daughter cells can switch mating types, we performed pedigree analysis on wild-type, sin1⁻, and swi1⁻ sin1⁻ cells (see Materials and Methods). In swi1⁻ sin1⁻ cells, URS1 is largely inactive, but URS2 is derepressed, and this should result in mostly deregulated switching. For these experiments, we analyzed two different mutations, a deletion allele of SIN1 and the sin1-2 point mutation, the latter which exhibits a stronger Sin⁻ phenotype (Kruger and Herskowitz, submitted).

As seen in Table 5 (line 1), wild-type daughter cells never undergo mating type switching, whereas a few daughters switch (~4%) in the sin1Δ mutant (line 2). In contrast, mother and daughter cells that are swi1⁻ sin1Δ exhibit almost the same rate of

switching (16% versus 9%) (line 3). Similar results are observed in the strain carrying the sin1-2 allele (Table 5, lines 4-6). In this strain, wild-type daughter cells again exhibit no switching (line 4), whereas sin1-2 daughters show low levels of switching (9%; see line 5). In swi1⁻ sin1⁻ cells, daughters again exhibit rates of switching similar to mothers (41% vs. 47%; see line 6). These observations support the view in swi1⁻ sin1⁻ cells, URS2 is functioning as a UAS independently of URS1. Once again, we observe that SWI1 exhibits an inhibitory effect on the UAS activity of URS2 seen in sin1⁻ mutants.

Discussion

The HO gene of Saccharomyces cerevisiae is transcribed only in mother cells during the G1 phase of the cell cycle. This precise transcriptional control is exerted by a large regulatory region extending 1400 base pairs upstream of the transcriptional start site. Previous studies have demonstrated that this control region can be functionally dissected into two parts, URS1 and URS2. URS1 contains sequences necessary and sufficient for mother/daughter control of HO expression, while URS2 contains sequences necessary for cell cycle regulation (Nasmyth, 1985a,b). URS1, in the absence of URS2, exhibits UAS activity as defined by its ability to promote transcription from a heterologous promoter lacking an endogenous UAS. We have shown here that the URS2 region of HO also contains UAS activity that is controlled by the SWI1, SWI4, SWI6, and SIN1 gene products. The observation that both URS1 and URS2 contain UAS activity leads to the following question: Which UAS is actually responsible for driving HO transcription? We propose that URS2

contains the UAS used to promote transcription, and that the URS1 UAS activity is used to regulate URS2 activity. The evidence leading to this sequential activation model is summarized below.

Both URS1 and URS2 have regulated UAS activity

Previous work has demonstrated that URS1 has UAS activity. When URS1 is placed upstream of a CYC1-lacZ fusion gene lacking its endogenous UAS significant activation of transcription is observed (Sternberg et al., 1987; also Table 2). This UAS activity is normally under mother/daughter control. Deletion of URS2 results in HO transcription in mother cells only, but it is no longer properly cell-cycle regulated (Nasmyth, 1985a,b). UAS activity of URS1 normally requires the SWI1, SWI2, SWI3, and SWI5 gene products (Sternberg et al., 1987; C. Peterson, unpublished information).

In this paper we showed that a segment containing URS2 when placed in either orientation upstream of the CYC1-lacZ fusion gene carried on a plasmid could activate transcription. This activation was minimal in wild-type cells and maximal in swi1⁻ sin1⁻ cells. Interestingly, it is necessary to have both SWI1 and SIN1 inactivated for maximal UAS activity. We initially thought that the inhibition by SWI1 of URS2 may have been an artifact of the plasmid assay, because SWI1 acts as an activator for the intact HO promoter and for URS1 in isolation, but this does not appear to be the case. A chromosomal HO-lacZ fusion gene in which URS1 function has been eliminated by a deletion of SWI5 is expressed better when both SWI1 and SIN1 are deleted. Additionally, mating type switching experiments indicate that both SWI1 and SIN1 inhibits HO expression

in daughter cells. These results indicate that URS2 UAS activity is negatively regulated by both SWI1 and SIN1.

The CCB elements appear to be the source of the UAS activity in URS2. These sequences when multimerized and placed upstream of a reporter gene exhibit cell-cycle-regulated UAS activity (Breeden and Nasmyth, 1987; Andrews and Herskowitz, 1989). This UAS activity is dependent on only two of the SWI genes, SWI4 and SWI6. These two genes are the only SWI genes which are still required for HO expression when SIN1 is altered or deleted. Thus SWI4 and SWI6 are required for UAS function even in the absence of SIN1. Our results here demonstrate that SWI4 and SWI6 are activators of URS2, both in the presence and absence of SIN1 (see Table 3). Taken together, these observations imply that the Cell-Cycle Boxes are the source of UAS activity exhibited by URS2.

UAS activity of URS2, but not URS1, is essential for HO transcription.

The observation that both URS1 and URS2 can exhibit UAS activity as judged by their ability to stimulate transcription from a minimal promoter (i.e. TATA and initiation sequences) does not necessarily imply that these two elements are both acting as UAS elements in the intact HO promoter. Since the molecular mechanisms of UAS activity are not understood, it is difficult to know how the observed UAS activity of URS1 and URS2 sequences in isolation relates to the activation of HO transcription in the intact promoter. Some sequence elements exhibit UAS activity when placed upstream of a heterologous promoter, but do not have UAS

activity in their native contexts. For example, the RAP1 binding site has UAS activity when placed in front of a test promoter, but is essential for transcriptional repression as part of the HMR-e silencer (Brand et al., 1987; Shore et al., 1987). A similar relationship may exist at HO. It is possible that the UAS activity exhibited by either URS1 or URS2 in isolation is not actually used to promote transcription directly. One way to address this question is to examine the effects of mutations in the regulators of URS1 and URS2 UAS activity, in order to see which UAS is absolutely required for transcription from the intact HO promoter. In cells containing swi1, URS1 is inactive and intact HO is not expressed. However, in cells containing both swi1 and sin1 URS1 is still inactive (see Table 2), but HO is expressed. This result indicates that URS1 UAS activity is not absolutely required for transcription. In contrast, URS2 UAS activity is absolutely required for HO transcription. In swi4 cells, URS2 UAS activity is inactivated and HO is unexpressed, even though URS1 UAS activity is intact (W.K. unpublished data). Additionally, no mutations which can bypass the need for SWI4 in HO expression have been identified, even though they have been screened for extensively (B. Andrews and J. Ogas, unpublished data). These observations suggest that SWI4 dependent UAS activity from URS2 is absolutely required for HO transcription. Thus it appears that URS2 is the actual UAS for the HO promoter.

How URS1 and URS2 act together to regulate HO

If URS2 UAS activity is normally critical for HO expression, what is the role of URS1? Deletion analysis has shown that in wild-type cells URS1 is essential for HO expression (Nasmyth, 1985a).

URS2 UAS activity is normally repressed by the action of the SWI1 and SIN1. A logical hypothesis is that URS1 activity normally controls the utilization of the UAS activity of URS2. According to this hypothesis, the UAS activity observed by URS1 in isolation, is actually an activity involved in controlling the "real" UAS located in URS2. We call this hypothesis the sequential activation model for HO regulation (see Figure 3).

This model can be utilized to explain the precise transcriptional control of HO. According to this model, URS1 would be inactive (Nasmyth, 1985a,b) in daughter cells, and therefore URS2 UAS activity would also be inactive primarily because of inhibition by SIN1. This results in no HO transcription in daughter cells. In mother cells, URS1 is active which then causes SWI1 to inhibit SIN1, thus allowing activation of the cell cycle boxes in URS2 by SWI4 and SWI6. Alternatively, activation of URS1 could increase SWI4 and SWI6 activity, enabling them to overcome inhibition by SIN1. In either case, this results in HO expression only in the correct cells at the proper time during the cell cycle. Thus by allowing URS1 to control URS2 activity, the HO gene is able to be under both mother/daughter and cell cycle control at the same time. The idea of using one regulatory module to control the activity of another module may be of general use in the control of complex gene expression.

How does URS1 regulate activity of URS2? The SWI1 gene may play an important role in this process. SWI1 has sites of action in both URS1 and URS2. In UAS assays SWI1 acts as an activator of URS1, but as an inhibitor of URS2. This suggests that the function of

SWI1 is context dependent. The fact that SWI1 has a site of action in both URS1 and URS2 suggests it could be involved in communication between the two. One possibility is that in the intact promoter activation of URS1 changes the SWI1 function observed in URS2 from inhibitor to activator (as shown in Figure 3). This activating function may then work to inhibit SIN1 function, and allow utilization of the cell cycle box elements as a UAS.

The model described above is oversimplified in that it does not take into account the roles of any of the other SWI and SIN genes. Given the similarity in phenotypes exhibited by cells containing swi1, swi2, or swi3, as well as the ability of sin1 to suppress many of these phenotypes, it is likely that all three of these genes have the same role in regulating HO expression. Similarly, sin2 mutations, like sin1 mutations, are able to bypass the requirements for SWI1, SWI2, SWI3, and SWI5, but not SWI4. Thus, it is likely that these other genes contribute to the functions attributed to SWI1 and SIN1 in the model. The other described SIN genes (SIN3, SIN4, and SIN5) only suppress mutations in SWI5, and are probably involved in regulating activity of URS1.

It should be noted that the model described above does not attempt to explain the molecular mechanisms by which these genetic interactions occur. For this we need to understand the biochemical relationships between the various proteins interacting at these sequences. We have some preliminary information. SWI5 encodes a site-specific DNA binding protein which binds to sequences in URS1 (Stillman et al., 1988). SWI4 and SWI6 are both part of a site-specific DNA binding complex which binds to the CCB

elements in URS2 (Andrews and Herskowitz, 1990; B. Andrews, personal communication). The SIN1 gene product is a non-specific DNA binding protein with sequence similarity to mammalian HMG1, and is thought to be a non-histone component of chromatin (Kruger and Herskowitz, submitted). The SWI1 gene has been cloned, and recently has been shown to be identical to ADR6, a regulator of the ADH1 and ADH2 genes (C. Peterson, personal communication). The SWI1/ADR6 gene product is nuclear localized, but its predicted protein sequence does not reveal similarity with any proteins of known biological function (O'Hara et al., 1988). Understanding the complex biochemical relationships between these various proteins will be required in order to understand the molecular basis for the interactions between URS1 and URS2.

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Table 1

Strain	Relavent Genotype	Source/Comment
1369	α ho leu2-a ura3-52 trp1-	Sternberg et al. (1987)
S172-8a	α ho swi1Δ leu2- ura3-52	"
IC134-3a	α ho swi1Δ sin1-2 leu2- ura3-52	"
IC104-11c	α ho sin1-2 leu2- ura3-52	"
WK36-4d	α ho ura3-52 his3Δ200 leu2- trp1	Isogenic to YPH274 from P. Hieter
CY110	α ho ura3-52 his3Δ200 leu2- trp1 sin1Δ	"
WK36-9d	α ho ura3-52 his3Δ200 leu2- trp1 swi4Δ	"
WK38-4a	α ho ura3-52 his3Δ200 leu2- trp1 swi4Δ sin1Δ	"
WK37-5d	α ho ura3-52 his3Δ200 leu2- trp1 swi6Δ	"
WK39-1b	α ho ura3-52 his3Δ200 leu2- trp1 swi6Δ sin1Δ	"
X10-1b (1879)	a/α HO/HO ura4/ura4 leu2-/leu2-	Hicks and Herskowitz (1976)

1875	**a**/α HO/HO ura4/ura4 leu2-/leu2- sin1-2/sin1-2	Sternberg et al. (1987)
1875sw	**a**/α HO/HO ura4/ura4 leu2-/leu2- sin1-2/sin1-2 swi1Δ/SWI1	This study
WK31-1b	**a**/α HO/HO leu2-/leu2- trp1/trp1	"
WK31-1bs	**a**/α HO/HO leu2-/leu2- trp1/trp1 sin1Δ/sin1Δ	"
WK31-1bssw	**a**/α HO/HO leu2-/leu2- trp1/trp1 sin1Δ/sin1Δ swi1Δ/SWI1	"
WK44-1a	α swi5Δ swi1Δ HO-lacZ trp1 leu2- ura3-52	This study
WK44-9a	α swi5Δ HO-lacZ leu2- ura3-52	"
WK44-4a	**a** swi5Δ sin1Δ HO-lacZ leu2- ura3-52	"
WK44-5b	**a** swi5Δ swi1Δ sin1Δ HO-lacZ leu2- ura3-52	"
WK44-2a	α swi1Δ sin1Δ HO-lacZ leu2- ura3-52	"
WK10-3c	α sin1-2 HO-lacZ leu2- ura3-52	This study

D262-1a	**a** cry1 swi1Δ HO-lacZ ura4	Sternberg et al. (1987)
JO223-2d	**a** swi6Δ:LEU2 ho leu2- ura3	J. Ogas

[a] A double point mutation in the LEU2 gene.

Table 2

UAS activity of URS2

	β-galactosidase activity			
Insert	SWI1 SIN1	swi1⁻ SIN1	SWI1 sin1⁻	swi1⁻ sin1⁻
URS2	1.2	0.4	3.2	6.3
URS2^{i}	1.0	0.7	5.3	18
URS1	23	1.4	7.3	3.4
none	0.2	0.3	0.2	0.3
RPA39 UAS	313	280	150	200

Fragments containing the indicated inserts were cloned upstream of a CYC1-lacZ promoter lacking its endogenous UAS (see Figure 2). The plasmids were used to transform congenic strains with the indicated genotypes, and activity was measured in four separate transformants. The strains used were 1369, S172-8a, IC134-3a, and IC104-11c. Results shown are averages expressed in Miller units. Variation between transformants was less than 20%.

Table 3

Effect of SWI4, SWI6 and SIN1 on URS2

UAS segment	wt	swi4⁻	swi6⁻	sin1⁻	swi4⁻ sin1⁻	swi6⁻ sin1⁻
1) URS2	0.39 (0.06)	<0.1 (<0.02)	<0.1 (<0.05)	0.60 (0.18)	<0.1 (<0.02)	<0.1 (<0.04)
2) CYC1	155 (26)	335 (54)	286 (58)	109 (34)	150 (67)	135 (47)
3) RPA39 &URS2	1.9 (0.32)	0.40 (0.06)	0.24 (0.1)	3.7 (1.2)	0.90 (0.4)	0.63 (0.2)
4) RPA39 alone	597 (100)	620 (100)	222 (100)	231 (100)	485 (100)	281 (100)

Isogenic strains of the indicated genotypes were transformed with plasmids containing the indicated segments inserted upstream of a CYC1-lacZ reporter gene (see Figure 2). Three separate colonies were picked and β-galactosidase assays were performed, and the results were averaged. The data in parentheses are normalized to the RPA39 insert in the same strain. Variation between transformants was less than 30%. The strains used in this experiment were WK36-4d, CY110, WK36-9d, WK38-4a, WK37-5d, and WK39-1b.

Table 4

Relief of *swi5*⁻ defect by mutations in *SWI1* and *SIN1*

Strain	Genotype	Expression of HO-lacZ
1) WK44-9a	*SWI1 swi5Δ SIN1*	0.21
2) WK44-4a	*SWI1 swi5Δ sin1Δ*	0.90
3) WK44-5b	*swi1Δ swi5Δ sin1Δ*	2.6
4) WK44-2a	*swi1Δ SWI5 sin1Δ*	3.0
5) WK44-1a	*swi1Δ swi5Δ SIN1*	0.18
6) WK44-1b	*SWI1 SWI5 SIN1*	12.0

β-galactosidase activity of each of the above strains was measured in triplicate and the average is presented above. Standard deviations were less than 20% of the averages.

Figure 1. Diagram of the regulatory sequences of HO.

URS1 is shaded, while URS2 is unshaded with the Cell-Cycle Boxes indicated by thick black vertical lines.

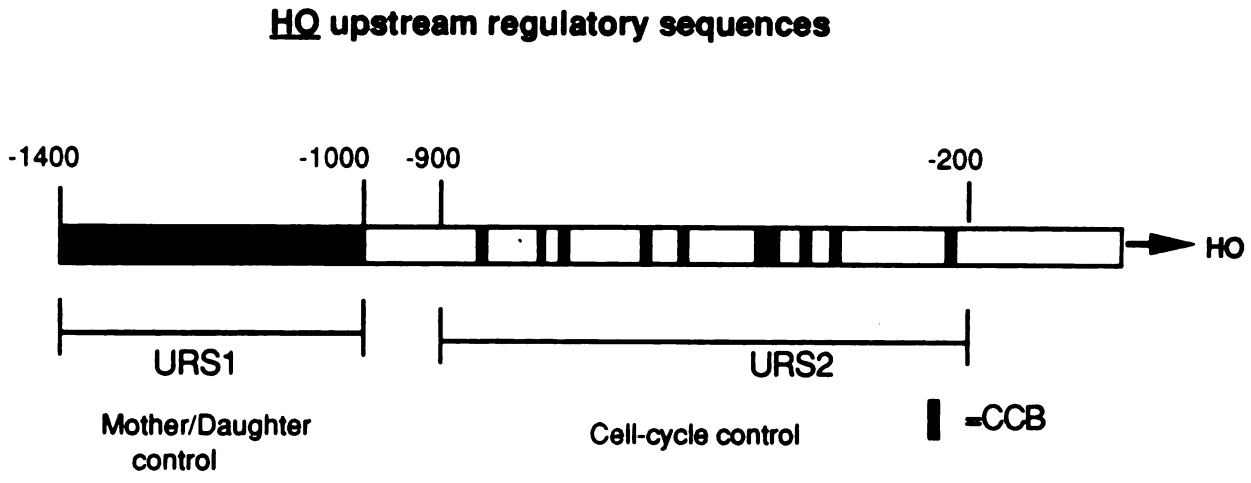

HO upstream regulatory sequences
-1400
-1000
-900
-200
HO
URS1
URS2
Mother/Daughter control
Cell-cycle control
=CCB

Figure 1

Figure 2. Structures of the upstream regulatory regions of *CYC1-lacZ* used in Tables 2 and 3.

The DNA segments used are drawn to scale and the same shading pattern is used as in Figure 1. The diagonal stripped region represents the *RPA39* UAS sequences.

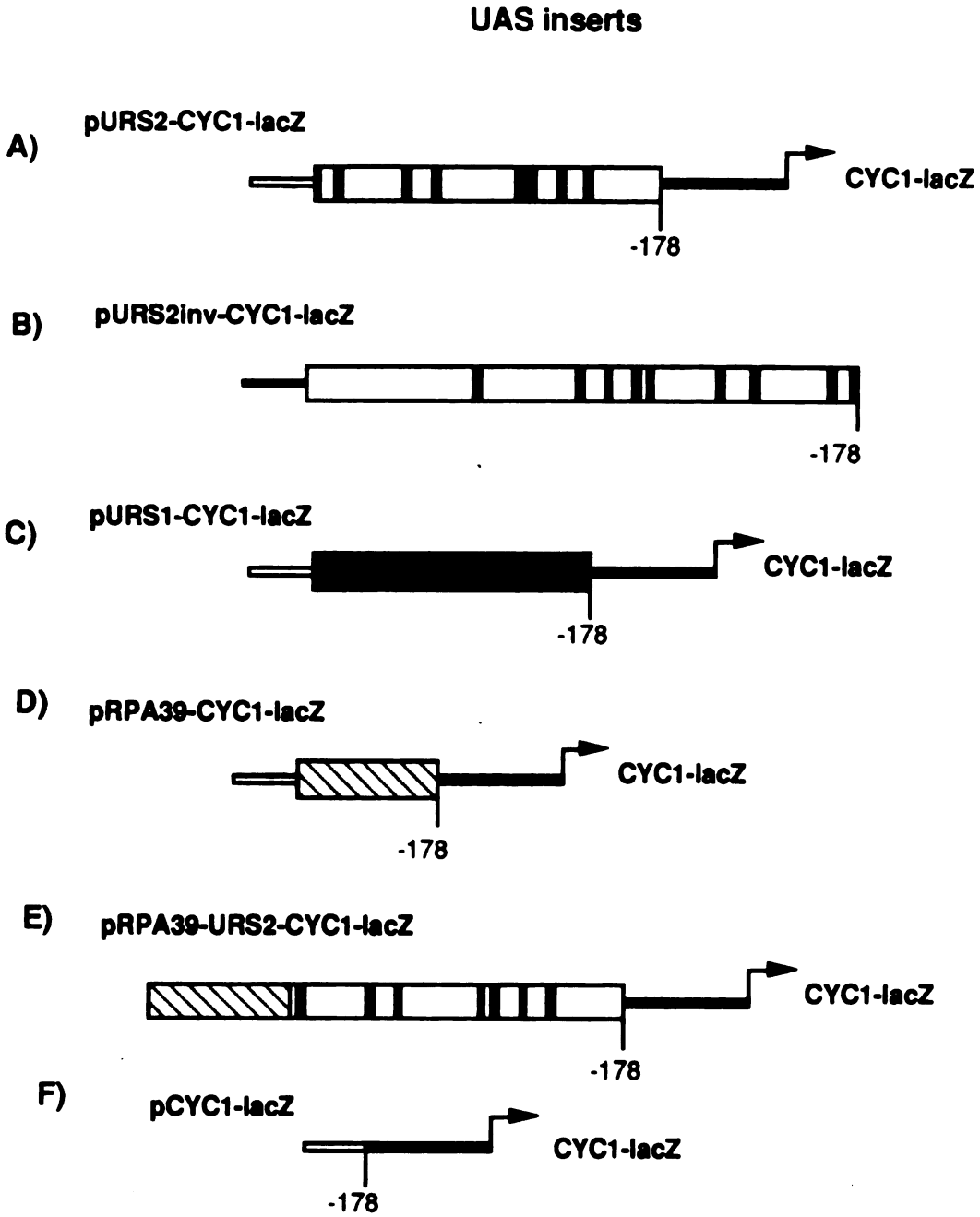
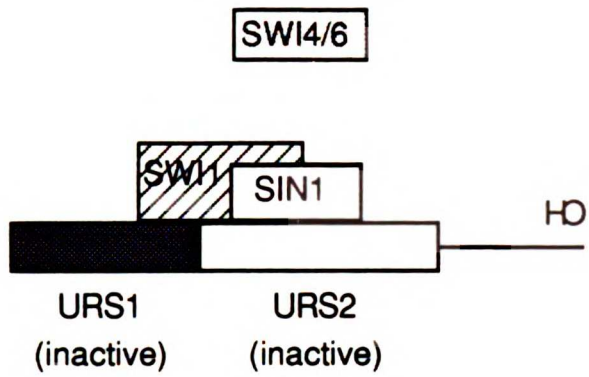

UAS inserts
A)
pURS2-CYC1-lacZ
CYC1-lacZ
-178
B)
pURS2inv-CYC1-lacZ
-178
C)
pURS1-CYC1-lacZ
CYC1-lacZ
-178
D)
pRPA39-CYC1-lacZ
CYC1-lacZ
-178
E)
pRPA39-URS2-CYC1-lacZ
CYC1-lacZ
-178
F)
pCYC1-lacZ
CYC1-lacZ
-178

Figure 2

Figure 3. Sequential activation model for control of HO transcription.

The situation in mother cells is shown above, while the situation in daughters is shown below. In the daughters, URS1 is inactive and URS2 is also inactive due to inhibition of SWI4 and SWI6 activity by the combined actions of SIN1 and SWI1. In mothers, URS1 becomes activitated by SWI5, and this activation of URS1 leads to an alteration in the function of SWI1 in URS2. This alteration in SWI1 results in its inhibiting SIN1, such that there is no longer anything inhibiting of SWI4 and SWI6 from activating URS2. The arrows eminating from both URS1 and URS2 represents the "UAS" activity measured by the plasmid assay. The numbers next to the arrows indicate the sequential nature of the actions taking place. The role of SWI1 in URS1 activation has been left out for simplification.

Daughters



Mothers

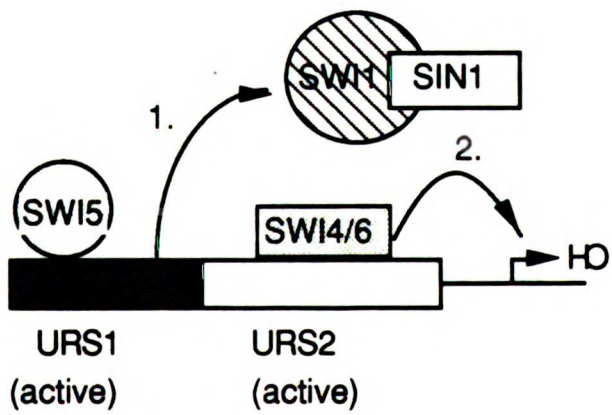


Figure 3

CHAPTER 5

CONCLUSION

Conclusion

The SIN1 and SIN2 genes were originally identified in a screen designed to discover mutations in genes which encoded negative regulatory proteins involved in HO transcription (Sternberg et al., 1987). It was found that mutations in either SIN1 or SIN2 can alleviate the need for the SWI1, SWI2, SWI3, and SWI5 activators. In addition to HO, SIN1 or SIN2 can also affect transcription of other yeast genes. Specific mutations in either SIN1 or SIN2 allow expression of HIS4 or LYS2 genes inactivated by δ insertions in their 5' regulatory sequences and also allow expression from the INO1 gene in cells lacking SWI1, SWI2, SWI3, or the C-terminal domain of RNA polymerase II (Peterson et al., 1991). In this dissertation I have characterized SIN1 and SIN2 and discovered that they encode for proteins which are components of chromatin. SIN1 codes for a protein with similarity to HMG1, while SIN2 codes for histone H3. These findings imply that chromatin is intimately involved in transcriptional regulation.

Specific point mutations in either SIN1 or SIN2 have stronger transcriptional affects than deletion of these genes. In the case of SIN2 the reason for this is apparent: since there are two genes in yeast which code for identical histone H3 proteins, simply deleting one gene has no effect because the other gene produces sufficient quantities of histone H3 for the cell to function properly. Given the similar genetic behavior of SIN1 and the existence of multiple HMG1-like proteins in higher cells (Walker, 1982), I think it is likely that there exist other SIN1-like proteins in yeast. The simplest

explanation for the genetic behavior of SIN1 and SIN2 is to suggest that slightly mutated SIN1 and histone H3 protein is incorporated into the chromatin complex but fail to make all the proper contacts with other chromatin proteins or DNA. Thus they may be viewed as altering chromatin structure. Since a major disruption of chromatin structure appears lethal (Han et al., 1988), it is probable that mutations in SIN1 and SIN2 are altering chromatin structure only slightly.

The idea that mutations in SIN1 and SIN2 cause only slight alterations in chromatin structure is consistent with their very discreet effects on transcription. The genes which are derepressed by mutations in SIN1 and SIN2 have all been enfeebled in some way, either by loss of a transactivator protein or by a cis-acting promoter insertion. It should be noted that not all enfeebled genes are derepressed by mutations in SIN1 and SIN2. For example, sin1 and sin2 mutations derepress the HO promoter enfeebled by loss of SWI1 but not by loss of SWI4. A similar observation is observed at the INO1 promoter: sin1 suppresses the loss of SWI1 but not of INO4 (Peterson et al., 1991). In these cases, I believe that SWI4 and INO4 are the "essential" activators and that mutations in SIN1 and SIN2 allow these activators to activate in the absence of help from other gene products. This idea suggests that the other activator gene products' (e.g. SWI1, SWI2, SWI3, SWI5, and the CTD of PolII) function is to help an essential activator in some fashion, perhaps by aiding it in binding to DNA or in interacting with the general transcription machinery. The fact that mutations in SIN1 and SIN2 are able to partially alleviate the need for these gene products

suggests that these "helper" products' function is to help overcome the effects of chromatin at the promoter.

The observation that at some intact promoters mutations in SIN1 cause decreased transcription is also explainable in this light. It seems likely that all promoters must have a mechanism to antagonize the effects of chromatin. In cells which contain a slightly altered chromatin structure due to alterations in SIN1 or histone H3 this "chromatin neutralization" machinery might not function quite as well. Such a machinery might be designed for optimal "neutralization" of normal chromatin, and may not interact quite properly with altered chromatin. This weakened interaction with altered chromatin could result in reduced transcription of these genes.

At the time I began these studies the role of chromatin in transcriptional regulation had largely been ignored. Essentially, even though chromatin proteins make up the bulk of DNA-binding proteins in the cell, they were treated as invisible entities in the process of transcriptional regulation. This study, and work by others, now clearly show that the role of chromatin cannot simply be ignored and must be factored into the process of transcriptional regulation. Future studies, hopefully, will focus on the molecular details of the interactions between chromatin proteins, site-specific DNA binding complexes, and the general transcription machinery.

References

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APPENDIX

ANALYSIS OF SIN1 PROTEIN

Summary

We have used anti-SIN1 antibodies to examine whether the levels or modification of SIN1 protein change in several mutant cell types. We have determined that there are approximately 10^5 molecules of SIN1 protein per cell and that these levels are unaffected by mutations in SWI1, SWI2, SWI3, SWI5, SIN2 and SIN3. We also show that the sin1-2 allele produces an apparently full-length protein, while the sin1-1 and sin1Δ alleles produce no detectable SIN1 protein. We demonstrate that full-length SIN1 protein produced in bacteria and native SIN1 migrate similarly in SDS-polyacrylamide gels. This observation suggests that there are no gross modifications of the SIN1 protein in yeast.

Introduction

Mutations in SIN1 are able to bypass the requirement for the SWI1, SWI2, SWI3, and SWI5 gene products for expression of HO (Sternberg et al., 1987). Thus in formal genetic terms, the role of these SWI gene products is to inhibit the function of the SIN1 gene product. Because of these observations, we were interested in determining whether these SWI genes had an influence on the state or levels of SIN1 protein. Additionally, the hypothesis that SIN1 encodes an HMG1-like protein (see Chapter 2) predicts that SIN1 protein should be found at moderately abundant levels. In order to address these and other questions, we generated anti-SIN1 protein antibodies and performed immunoblot analyses shown in this appendix.

Materials and Methods

Strains and extract preparation

Yeast and bacterial strains used in this study are described in Table 1. Yeast extracts were prepared from logarithmically growing cells by beadbeating in SDS sample buffer as described (Current Protocols in Molecular Biology, 1989). E. coli extracts were made by resuspending cells in cracking buffer as described (Klied et al., 1981).

Preparation of antibodies and glutathione transferase-SIN1

Preparation of SIN1 antiserum and affinity purification was performed as described (Kruger and Herskowitz, 1991). Preparation

of glutathione transferase-SIN1 was done as described (Peterson et al., 1991).

Gels and immunoblotting

Samples were run on 8% SDS protein gels and then transferred to nitrocellulose as described (Andrews and Herskowitz, 1990). The blots were probed with either a 1/1000 dilution of the crude anti-serum, or a 1/500 dilution of the affinity-purified serum, followed by incubation with goat anti-rabbit antibody conjugated to alkaline phophatase.

Results

Antiserum raised against TrpE-SIN1 recognizes SIN1 produced in yeast.

Antiserum which recognized SIN1 was produced by injection of TrpE-SIN1 fusion protein into rabbits (Kruger and Herskowitz, 1991). This TrpE-SIN1 fusion protein contains all of SIN1 except the N-terminal 51 amino acids. After several boosts we examined the serum to see if it was capable of recognizing native SIN1 produced in yeast. We used the serum to probe immunoblots which contained total yeast extracts run on gels from wild-type and sin1Δ cells. As can be seen in Figure 1a, there is one distinct band present in the SIN1 lane but absent in the sin1Δ lane, although the serum also recognizes several other bands present in both lanes. The SIN1-specific band migrates as a 43 kDa protein, which is close to the 37 Kda predicted molecular weight of SIN1. In order to confirm that

this band was actually SIN1, we affinity purified the antiserum against a glutathione transferase-SIN1 fusion protein (g-SIN1) purified from bacteria (Peterson et al., 1991). As can be seen in Figure 1b, the affinity purified serum still recognizes the 43 kDa protein, but not most of the other cross-reacting bands. Interestingly, a 70 kDa band is also recognized by the affinity-purified antiserum that is present in both SIN1 and sin1Δ extracts. The nature of this cross-reacting band is not known. These experiments identify a 43 kDa band as SIN1.

The effect of sin⁻ and swi⁻ mutations on SIN1.

Given the genetic interactions between SIN1 and SWI1, SWI2, SWI3, and SWI5 (Sternberg et al., 1987), it was possible that mutations in these genes could affect the amount or state of SIN1. Therefore, we examined SIN1 in cells containing mutations in these genes. Since mutations in SIN2 give the same phenotype as mutations in SIN1, we also examined SIN1 in sin2⁻ cells. In Figure 2, we show immunoblot analyses of SIN1 in cells containing swi1⁻, swi2⁻, swi3⁻, swi5⁻, sin2⁻ and sin3⁻ mutations. None of the swi⁻ or sin⁻ mutations tested have a noticeable effect on the amount or mobility of SIN1. These experiments suggest that the mutations tested do not exert their phenotypes by affecting SIN1.

We also have examined the behavior of a full-length SIN1 protein produced in bacteria. As can be seen in Figure 2, a full length SIN1 protein produced from the bacterial T7 promoter produces a SIN1 protein which migrates with the same mobility as yeast SIN1. There is also a major 25 kDa SIN1 degradation fragment

not observed in yeast. This observation suggests that the SIN1 protein is not grossly modified in yeast.

To determine how the various characterized SIN1 alleles affected SIN1 protein, we examined the state of SIN1 in strains containing different SIN1 alleles. We were especially interested in the state of SIN1 in strains containing the sin1-2 allele, because swi1Δ cells containing this allele actually transcribe HO better than swi1Δ cells containing a null allele of SIN1 or the sin1-1 allele (Kruger et al., 1991; Sternberg et al., 1987). In Figure 3, cells containing either sin1Δ or sin1-1 do not produce any detectable SIN1 protein, but the sin1-2 allele produces full-length SIN1 at a slightly reduced level from wild-type or sin2⁻. This analysis, along with the semi-dominant genetic behavior exhibited by sin1-2, suggests that the sin1-2 allele contains a missense mutation (or a non-sense mutation very near the C-terminus of the coding region).

Quantitation of SIN1 in yeast

Given the hypothesis that SIN1 encodes a yeast HMG1-like protein, we would expect SIN1 to be moderately abundant. In mammalian cells HMG1 is found at approximately a 1/10 ratio to nucleosomes. We were interested in determining whether SIN1 was in roughly the same abundance in yeast. In order to quantitate the amount of SIN1 in yeast, we performed an antibody titration experiment, comparing the signals attained from a known amount of purified glutathione S-transferase-SIN1 fusion protein to the signal in extracts made from a determined number of yeast cells. Data are presented in Figure 4. First, we determined the concentration of our

glutathione transferase-SIN1 fusion protein (g-SIN1) by comparison to a BSA standard (Figure 4a). This gel shows that some of the glutathione-SIN1 is partially degraded, with a major degradation product running at 26 kDa. This degradation product cross-reacts with the SIN1 antiserum (see Figure 4b), and it is the same size as the degradation product observed for full-length SIN1 expressed in bacteria. Therefore, we believe that it is composed entirely of SIN1 and refer to this fragment as proteolyzed SIN1 (p-SIN1). These experiments indicate that our glutathione-SIN1 solution contained approximately 5ng/ul of both g-SIN1 and p-SIN1.

We next compared the signals obtained on an immunoblot between SIN1 protein contained in total cell extract from a known amount of yeast cells and SIN1 present in the glutathione transferase-SIN1 preparation. The signal obtained from 10ul of yeast extract (10^7 cells) is about the same as that obtained for approximately 10ng of g-SIN1 or p-SIN1. In order to rule out the possibility that the yeast extract was somehow affecting the signal, we performed a mixing experiment shown in Figure 4c. This experiment confirms that the signal obtained from total yeast extract from 10^7 cells is approximately equal to that obtained with either 10ng of g-SIN1 or p-SIN1. Because of the size differences between these two molecules, the amount of calculated SIN1 present varies between 8,500 per cell (using gSIN1) and 20,000 per cell (using pSIN1). This estimate suggests that there is approximately one molecule of SIN1 per five or ten nucleosomes (assuming one nucleosome per 200 basepairs). This ratio of one molecule of SIN1

for every five to ten nucleosomes is comparable to that of HMG1 found in mammalian cells.

Discussion

We have analyzed the gross state and levels of SIN1 protein in various swi⁻ and sin⁻ cells and have found no detectable changes in cells containing mutations in SWI1, SWI2, SWI3, SWI5, SIN2 and SIN3 within the sensitivity of our assay. It is possible that there are slight modifications of SIN1 that are not detected by one-dimensional electrophoresis in SDS. However, our results argue that mutations in the above genes do not grossly affect SIN1 protein. These results suggest that the formal genetic antagonism exhibited by SWI1, SWI2, SWI3 and SWI5 of SIN1 does not occur by gross changes in the SIN1 protein. We also found that cells containing either the a sin1Δ or a sin1-1 allele do not produce any detectable SIN1, indicating that these are true null alleles. Cells containing the sin1-2 allele produce slightly lower amounts of an apparently full-length SIN1 protein. This observation is consistent with the dominant genetic behavior of sin1-2 (Kruger and Herskowitz, 1991).

A full-length SIN1 protein produced in bacteria has the same mobility on a one-dimensional SDS-gel as SIN1 produced in yeast. This observation indicates that SIN1 does not undergo any gross modifications in yeast, such as glycosylation or extensive phosphorylation. Again, however, we cannot rule out less extensive modifications which were not detected by our assay.

Using antibodies and a bacterially produced glutathione transferase-SIN1 fusion protein, we have estimated the amount of

SIN1 to be between 8,500 and 20,000 molecules per cell . This amount represents about one SIN1 molecule per 1000 bp of DNA, or one for every 5 nucleosomes. This ratio is very similar to the ratio for HMG1 protein calculated in mammalian cells (Kuehl et al., 1984). This finding is consistent with SIN1 encoding an HMG1-like protein.

References

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

Table 1

Strain designation	Genotype	Source
WK44-9b	α swi1Δ sin1Δ HO-lacZ trp1 leu2- his ura3-52	Kruger and Herskowitz, 1991.
WK30-5c	α ura3 his4 leu2- trp1 ho	"
WK30-1b	α ura3 his4 leu2- trp1 ho sin1Δ	"
1368	a ho leu2- trp1 his ura3-52	"
WK1-4d	α swi1Δ sin1-2 HO-lacZ leu2- his ura3-52	"
WK2-11a	a ho leu2- his ura3-52 sin2-1	This study
WK3-7c	α swi1Δ HO-lacZ leu2- ura3-52 sin2-1	Chapter 3
D271-5a	a cpr1 swi1Δ his ura4 sin1-1	Sternberg et al., 1987.
CY70	α ura3 lys2 ade2 trp1 his3 leu2	C. Peterson
CY55	α swi5Δ sin3Δ ura3 lys2 trp1 his3 leu2 ade2	C. Peterson

CY93	α swi1Δ swi2Δ swi3Δ ura3 lys2 trp1 his3 leu2 ade2	C. Peterson

Figure 1. Immunoblot analysis of SIN1.

Antiserum raised against TrpE-SIN1 fusion protein produced in bacteria was used to probe immunoblots containing total yeast extract made from either SIN1 (WK30-5c) or sin1Δ (WK30-1b) cells. Figure 1a shows this analysis using the crude serum. Figure 1b is identical to 1a except the antiserum has been affinity purified using a purified glutathione-SIN1 fusion protein.

Figure 1

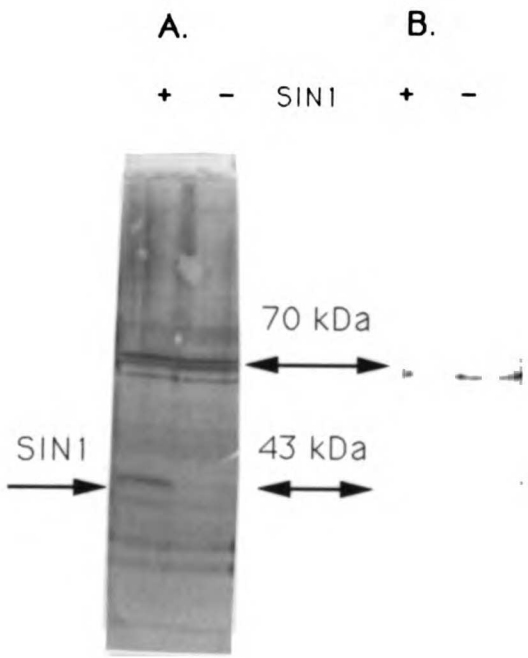


Figure 2. Analysis of SIN1 produced in various yeast strains and bacteria. Immunoblot analysis of total cell extracts made from either yeast (left-side) or bacteria (right-side) using affinity purified anti-TrpE-SIN1 antiserum. On the left side the relevant genotype of the strain is indicated above each lane. The strains used from left to right were: 1368, WK2-11a, CY93, CY70, CY55. The right side of the figure shows E. coli containing either the expression vector alone (lanes marked pT7), or the expression vector with SIN1 inserted (lanes marked pT7:SIN1).

Figure 2

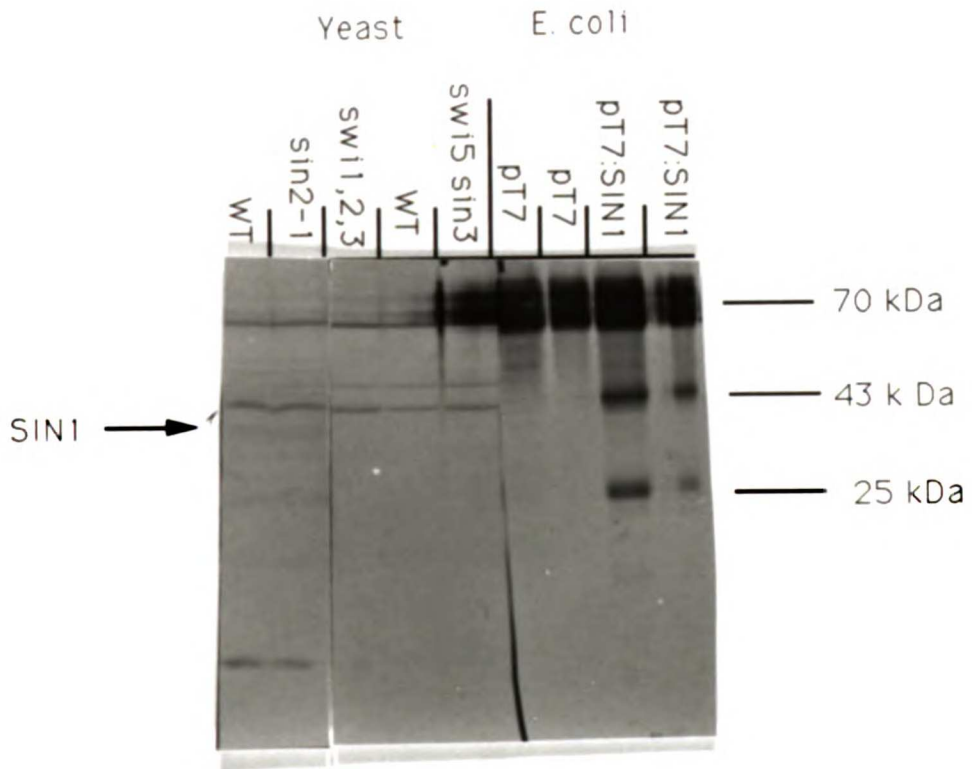

Yeast
E. coli
WT
sin2-1
swi1,2,3
WT
swi5 sin3
pT7
pT7
pT7:SIN1
pT7:SIN1
SIN1
70 kDa
43 k Da
25 kDa

Figure 3. Analysis of SIN1 in sin⁻ cells.

The figure shows immunoblot analysis using crude serum of total yeast extracts made from strains carrying the indicated sin⁻ alleles. The strains used from left to right are: 1368, WK3-7c, WK1-4d, D271-5a, and WK44-9b.

Figure 3

Analysis of SIN1 protein produced by various SIN1 alleles

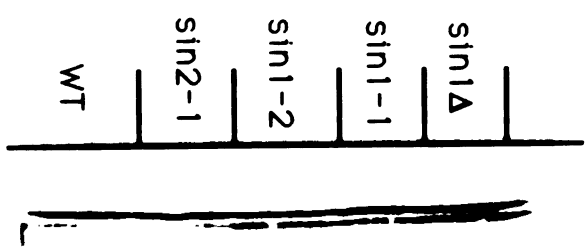


Figure 4. Quantitation of SIN1 in yeast.

Panel A. shows a coomasie stained gel comparing 40ul of a glutathione transferase-SIN1 preparation with known amounts of BSA. The intact glutathione transferase-SIN1 (g-SIN1) and a proteolyzed SIN1 fragment (p-SIN1) are indicated.

Panel B shows an immunoblot using affinity-purified anti-SIN1 antibodies to examine the relative signals produced by the indicated amounts of the same preparation of glutathione transferase-SIN1 used in A and the signal produced by total yeast extract produced from 10^7 cells.

Panel C also shows immunoblot analysis except in the left hand lanes the indicated amounts of the glutathione transferase-SIN1 preparation has been mixed with total yeast extract from 10^7 cells. The right hand portion of the blot shows the strength of the SIN1 signal using different amounts of yeast extract.

Figure 4A.

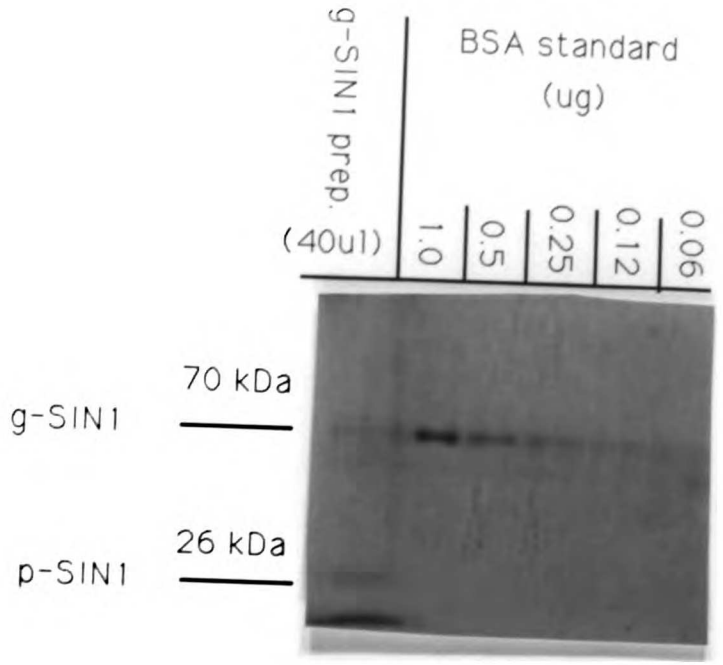


Figure 4B.

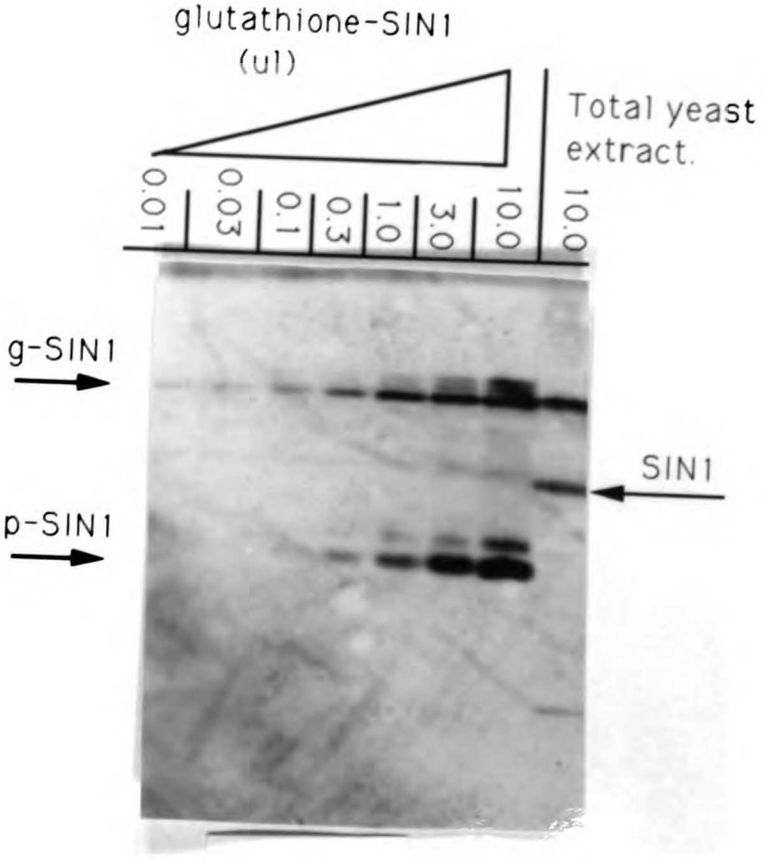
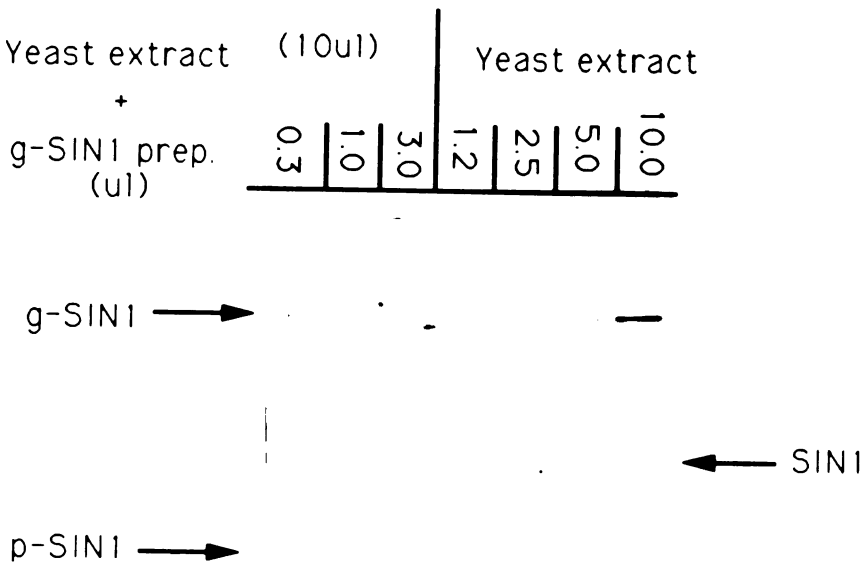


Figure 4C.


Yeast extract
+
g-SIN1 prep.
(ul)
(10ul)
0.3
1.0
3.0
Yeast extract
1.2
2.5
5.0
10.0
g-SIN1
SIN1
p-SIN1