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Identification of the *Saccharomyces cerevisiae* genes *STB1–STB5* encoding Sin3p binding proteins

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Abstract The yeast *SIN3* gene functions as a transcriptional repressor, despite the fact that Sin3p does not bind DNA directly. We have conducted a two-hybrid screen to look for proteins that interact with Sin3p, using the PAH2 domain of Sin3p as bait. Five new genes, *STB1–STB5* were identified, as well as the *STB6* gene, which is similar to *STB2*. *STB1*, *STB2*, *STB3*, and *STB6* are novel genes, and *STB4* and *STB5* encode C6 zinc cluster DNA-binding proteins. None of these genes is essential for viability, and several of these genes may encode transcriptional activators. Several special problems were encountered in using a transcriptional repressor in a two-hybrid screen. For example, the *STB* genes will interact with a LexA-Sin3(PAH2) fusion protein containing a region of Sin3p, but a LexA-Sin3p fusion protein containing full-length Sin3p, along with a *STB* clone, does not produce two-hybrid activation of a transcriptional reporter. In addition, a *sin3* mutation reduces the transcriptional activation by two-hybrid partners, suggesting that a *sin3* mutation reduces the transcriptional efficiency of the Gal4p and VP16 activation domains. We have shown previously that Sin3p is part of a large multiprotein complex, and we show here that Stb1p and Stb2p are present in this complex.

Key words Yeast · Transcriptional repression *SIN3* · C6 zinc cluster

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Introduction

The *Saccharomyces cerevisiae SIN3* gene has been identified in several genetic screens for transcriptional regulators. Sin3p functions as a transcriptional repressor of a number of genes, including *HO*, a site-specific endonuclease that initiates mating type switching, *TRK2*, a potassium transporter, *IME2*, an inducer of meiotic genes, *INO1*, the inositol synthase gene, *SPO11* and *SPO13*, two sporulation genes, and *STA1*, a gene encoding an extracellular glucoamylase (Strich et al. 1989; Wang et al. 1990; Vidal et al. 1991; Yoshimoto et al. 1992; Bowdish and Mitchell 1993; Hudak et al. 1994). The Sin3p protein itself does not directly bind to DNA (Wang and Stillman 1990), and it has been suggested that Sin3p may interact with sequence-specific DNA-binding proteins in order to target Sin3p to specific promoters for repression (Wang and Stillman 1993). In support of this idea, a mouse *SIN3* homolog has been identified that interacts with the Mad family of DNA-binding proteins (Ayer et al. 1995), and we have recently shown that interaction between Mad1 and yeast Sin3p leads to transcriptional repression in yeast (Kasten et al. 1996). It is possible to target a LexA-Sin3p fusion protein to a promoter containing a LexA binding site, and such experiments further demonstrate that Sin3p functions as a transcriptional repressor (Wang and Stillman 1993).

SIN3 encodes a 175 kDa protein that contains four paired amphipathic helix (PAH) motifs, which have been proposed to mediate protein-protein interactions (Wang et al. 1990). These PAH motifs are highly conserved in the mouse (Ayer et al. 1995), in *S. pombe* (Accession Number Z54140) and *C. elegans* (Accession Number Z81494) homologs of Sin3p, suggesting that they play an important role in Sin3p function. The PAH2 region of mouse or yeast Sin3p mediates the interaction with the mouse Mad1 protein (Ayer et al. 1995; Kasten et al. 1996). Mouse Sin3p also interacts with the SMRT and N-CoR corepressors (Alland et al. 1997;

Heinzel et al. 1997; Nagy et al. 1997). The fact that Sin3p contains four proposed protein association domains implies that Sin3p may interact with multiple proteins. We have recently shown that Sin3p is part of a large multiprotein complex greater than 2 million daltons (> 2 MDa) in size (Kasten et al. 1997), and that Rpd3p, a histone deacetylase (Rundlett et al. 1996; Taunton et al. 1996), is part of the Sin3p complex (Kasten et al. 1997). Interactions between Sin3p and Rpd3 (or their mouse homologs) have also been demonstrated by others (Hassig et al. 1997; Kadosh and Struhl 1997; Laherty et al. 1997; Zhang et al. 1997).

In this report, we present the results of a two-hybrid screen designed to identify yeast genes encoding proteins that interact with Sin3p. We have identified and characterized five yeast genes, *STB1–STB5*, that encode proteins that interact with Sin3p. We also identified a yeast gene which is paralogous to *STB2*, and we have named this new gene *STB6*. Genetic analysis demonstrates that none of these genes is essential for viability, and that several may be transcriptional activators. Finally, we show that the Stb1p and Stb2p proteins are present in the Sin3p complex.

Materials and methods

Plasmids and yeast strains

The plasmids used in this study are listed in Table 1. In many cases multiple steps were involved in plasmid construction, and details of plasmid construction are available on request. All strains used are isogenic in the W303 background (Thomas and Rothstein 1989), except those with an *HO*::*lacZ* reporter integrated at the *HO* locus, which are isogenic with K1107 (Nasmyth 1987). Standard genetic methods were used for strain construction and gene disruption (Rothstein 1991; Sherman and Hicks 1991). Gene disruptions were confirmed by Southern analysis. Strains DY1641 (*SIN3*) and DY2516 (*sin3*::*ADE2*) with an integrated *LexA-lacZ* reporter have been described (Kasten et al. 1996). Strains were transformed as

Table 1 Plasmids used

Name	Description	Type	Marker	Source
M1525	Gal4p DBD-SIN3(full length)	YEp	*HIS3*	This study
M1850	LexA-Sin3-Gal4AD	YEp	*HIS3*	This study
M1837	LexA-Sin3(PAH2)-VP16	YEp	*HIS3*	This study
pSH2-1	LexA fusion vector	YEp	*HIS3*	Hanes and Brent (1989)
M1965	LexA-lamin	YEp	*TRP1*	Stan Fields
M1776	LexA-Sin3(348–598)	YEp	*HIS3*	This study
M2629	LexA-Sin3(407–497)	YEp	*HIS3*	This study
M2630	LexA-Sin3(407–423,451–497)	YEp	*HIS3*	This study
M2344	*STB1* in pGAD1	YEp	*LEU2*	This study
M2346	GAD-Stb2 fusion in pGAD3	YEp	*LEU2*	This study
M2347	GAD-Stb3 fusion in pGAD1	YEp	*LEU2*	This study
M2354	*STB4* in pGAD1	YEp	*LEU2*	This study
M2359	*STB5* in pGAD1	YEp	*LEU2*	This study
YEplac181	YEp multicopy vector	YEp	*LEU2*	Gietz and Sugino (1988)
M2401	*STB1* in YEplac181	YEp	*LEU2*	This study
YEpFAT10	Very high-copy-number vector	YEp	*TRP1*, *LEU2-d*	Conrad et al. (1990)
M2467	*STB1* in YEpFAT10	YEp	*TRP1*, *LEU2-d*	This study
M2613	*stb1*::*TRP1* disruptor	YIp	*TRP1*	This study
M2687	*stb2*::*TRP1* gamma disruptor	YIp	*TRP1*	This study
M2679	*stb3*::*TRP1* gamma disruptor	YIp	*TRP1*	This study
M2942	*stb4*::*HIS3* gamma disruptor	YIp	*HIS3*	This study
M2804	*stb5*::*TRP1* gamma disruptor	YIp	*TRP1*	This study
M2805	*stb6*::*LEU2* gamma disruptor	YIp	*LEU2*	This study
M2806	*stb6*::*HIS3* gamma disruptor	YIp	*HIS3*	This study
pPL5925	*ume6*::*LEU2* disruptor	YIp	*LEU2*	Strich et al. (1994)
M1398	*IME2*::*lacZ* reporter	YEp	*URA3*	Wang and Stillman (1993)
pJH330	*INO1*::*lacZ* reporter	YEp	*URA3*	Lopes and Henry (1991)
p(spo13)28	*SPO13-lacZ* reporter	YEp	*URA3*	Strich et al. (1989)
M2948	LexA-Ras	YEp	TRP1	Vojtek et al. (1993)
M2949	Vp16-Raf	YEp	LEU2	Vojtek et al. (1993)
M3255	LexA-A65	YEp	TRP1	McCright and Virshup (1995)
M3256	GAD-B72	YEp	LEU2	McCright and Virshup (1995)
pRS313	Control plasmid	YCp	*HIS3*	Sikorski and Hieter (1989)
M1635	Wild type Sin3p	YCp	*HIS3*	Wang and Stillman (1993)
M1636	Sin3pΔPAH1	YCp	*HIS3*	Wang and Stillman (1993)
M1637	Sin3pΔPAH2	YCp	*HIS3*	Wang and Stillman (1993)
M1638	Sin3pΔPAH3	YCp	*HIS3*	Wang and Stillman (1993)
M1639	Sin3pΔPAH4	YCp	*HIS3*	Wang and Stillman (1993)
pRD56	p*GAL1*:GST vector	YCp	*URA3*	Park et al. (1993)
M2950	p*GAL1*:GST -Sin3p	YCp	*URA3*	Kasten et al. (1997)
M2708	LexA-Stb1p	YEp	*HIS3*	This study
M2727	LexA-Stb2p	YEp	*HIS3*	This study

described (Jiang and Stillman 1995), and strains were grown on selective complete media (Sherman 1991) containing 2% glucose (except where indicated) supplemented with adenine, uracil and amino acids as appropriate, but lacking the essential components to select for plasmids.

β-Galactosidase assays

Blue/white assays for colony color were performed using the chromogenic substrate 5-bromo-4-chloro-3-β-D-galactopyranoside (X-Gal) on nitrocellulose (Breeden and Nasmyth 1985) or Whatman (Dohrmann et al. 1996) filters. Extracts were prepared and quantitative assays for β-galactosidase activity were performed using the chromogenic reagent *o*-nitrophenyl-β-D-galactopyranoside (ONPG) as described (Breeden and Nasmyth 1987).

Two-hybrid screen

The two-hybrid screen was conducted essentially as described by Chien et al. (1991). Yeast strain DY1641 containing plasmid M1776 (LexA-Sin3(PAH2)) was transformed with the YL-1, YL-2, and YL-3 libraries, generously provided by Stan Fields. Approximately 50 000 transformants were screened for blue colony color on X-Gal. Sixteen positive plasmids were isolated which still conferred blue colony color on the strain with the LexA-Sin3(PAH2) bait upon rescreening. Based on a combination of restriction digests and DNA sequence analysis, the sixteen plasmids were placed into five different groups.

Other methods

Physical mapping of the *STB* genes was performed by Southern hybridization to yeast chromosomal blots (a gift of Jeff Singer and Tim Formosa) and a yeast genomic library of ordered prime clones (Riles et al. 1993) provided by Linda Riles. *STB3* and *STB6* were cloned from lambda clones using plasmid pMB2240 as described (Erickson and Johnston 1993). The sequences of the *STB1*, *STB2* and *STB3* genes were determined from sets of nested deletions prepared with ExoIII (Henikoff 1984), and deposited with GenBank (Accession Numbers U33439, U33438, and U33440). Chromatography of yeast protein fractions on Sephacryl S-400 and glutathione-agarose resins, and immunoblot analyses with antibodies to LexA (generously provided by Erica Golemis) and to GST, were performed as described (Kasten et al. 1996).

Results

Two-hybrid screen for proteins that interact with the PAH2 region of Sin3p

The Gal4p DNA-binding domain (Gal4-DBD) is often used to tether a protein to the promoter to function as the "bait" in a two-hybrid screen. However, we found that when full-length Sin3p is fused to the Gal4-DBD, this plasmid construct (M1525) is toxic in yeast. It was very difficult to recover transformants, and examination of the few transformants obtained showed cells with a very abnormal morphology. The cells were very large in size, but did not appear to be arrested in any particular stage of the cell cycle. This toxicity is not due to overproduction of Sin3p, as previous work has demonstrated that Sin3p overproduction is tolerated well (Wang et al. 1990). We attribute this effect to inappropriate repression of certain promoters by Gal4-DBD-Sin3p. These promoters are probably not normally regulated by Gal4p, but contain weak Gal4p binding sites and are only affected by Gal4-DBD-Sin3p because of the high expression level of the fusion protein. Since the Gal4-DBD-Sin3p fusion is toxic in yeast, we switched to a LexA-Sin3p fusion protein, which is not toxic (Wang and Stillman 1993).

A two-hybrid screen requires a specific interaction between two proteins, one a DNA-binding domain fusion and the second a fusion protein with an activation domain. A LexA-Sin3p protein represses transcription of a heterologous reporter (Wang and Stillman 1993), raising the question of whether LexA-Sin3p would block the activation domain present on the second hybrid protein. We constructed a plasmid (M1850) expressing a LexA-Sin3-Gal4-AD triple fusion, consisting of the LexA DNA-binding domain fused to Sin3p fused to the Gal4p activation domain. This LexA-Sin3-Gal4-AD triple fusion was unable to activate transcription from an integrated *lexA-lacZ* two-hybrid reporter, suggesting that repression by Sin3p was dominant over the Gal4p activation domain. We therefore examined smaller regions of Sin3p (1539 amino acids) for the ability to activate transcription when they formed part of a triple fusion with the VP16 activation domain. A LexA-Sin3(PAH2)-VP16 triple fusion with the PAH2 region of Sin3p (residues 347–598) is able to activate transcription of a *lexA-lacZ* reporter. Additionally, a LexA-Sin3-(PAH2) fusion does not activate or repress transcription. Finally, the LexA-Sin3(PAH2)-VP16 triple fusion partially activates transcription of an *HO*::*lacZ* reporter in a *swi5* mutant; the ability of the triple fusion to activate *HO*::*lacZ* in the absence of Swi5p suggests that this region of Sin3p may interact with *HO* DNA-binding proteins. Based on these results, we proceeded with a two-hybrid screen using LexA-Sin3(PAH2) as bait.

A two-hybrid screen was performed to identify proteins that interact with the PAH2 region of Sin3p. A yeast strain with an integrated *lexA-lacZ* reporter was transformed with the LexA-Sin3(PAH2) bait (M1776) and the YL-1, YL-2, and YL-3 yeast genomic libraries in plasmids pGAD1, pGAD2, and pGAD3, respectively (Chien et al. 1991). Activation of the *lacZ* reporter was monitored by screening double transformants for blue color on filters soaked in a solution containing X-Gal. From these screens sixteen prey plasmids were identified and found to represent five new yeast genes which are referred to as the *STB* genes for *S*in *T*hree *B*inding proteins. None of the five *STB* candidates activate the *lacZ* reporter with the LexA vector or LexA-lamin fusion (Fig. 1, lines 1 and 2), indicating that the interactions between the *STB* candidates and Sin3p are specific.

A *sin3* mutation reduces two-hybrid activation

During the course of analyzing the interaction of the LexA-Sin3(PAH2) bait with the Stb proteins, we examined two-hybrid activation in a *sin3* mutant strain.

A

Bait: Control or Sin3p Derivative	*STB* gene	*STB1*	GAD-*STB2*	GAD-*STB3*	*STB4*	*STB5*
	GAD Dependent	no	yes	yes	no	no
	Number of Independent Isolates	4	1	2	8	1
	Fold Activation	580	3600	3500	68	410
1. M1132 LexA vector		white (1)	white (1)	white (1)	white (1)	white (1)
2. M1965 LexA-Lamin		white (3)	white (2)	white (3)	white (2)	white (1)
3. M1776 Library Screen Bait	PAH2 348–598	blue (580)	blue (3600)	blue (3500)	blue (68)	blue (410)
4. M2629 PAH2 only	407–497	blue (53)	blue (110)	blue (34)	white (2)	white (5)
5. M2630 PAH2 deleted	407 497	white (1)	white (3)	white (2)	white (1)	white (1)

B

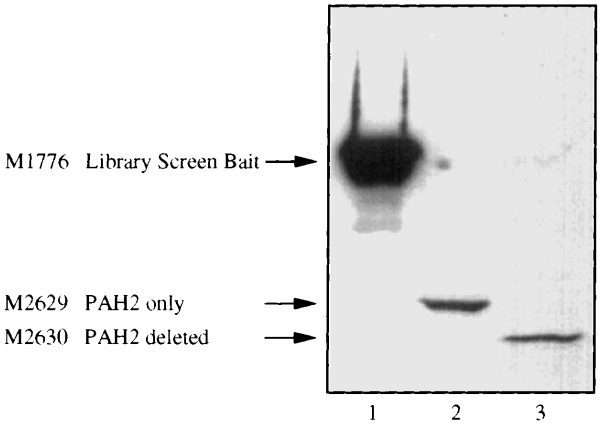
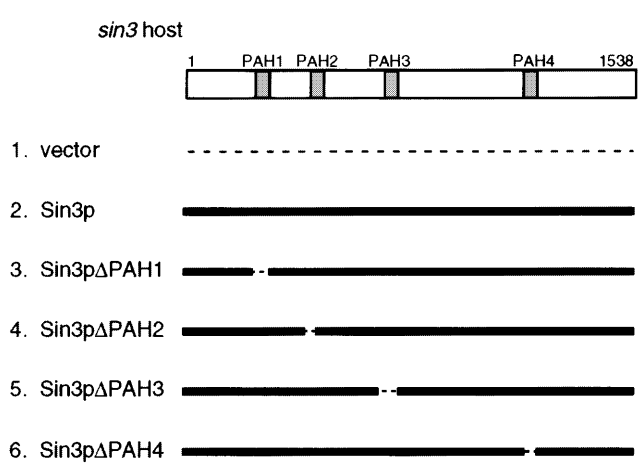


Fig. 1A, B *STB* gene two-hybrid activation with LexA-Sin3p fusion proteins. **A** The LexA vector, the LexA-Lamin control, and the various LexA-Sin3p baits were tested for two-hybrid activation with various *STB* and GAD-*STB* plasmids. The white color indicates a failure to activate the *lacZ* reporter while blue color indicates activation of the *lacZ* reporter. Quantitative measurements of LacZ activity are given in *parentheses*; standard deviations were less than 10%. GAD-dependent clones require the Gal4p activation domain for two-hybrid activation; GAD-independent *STB* genes presumably contain their own activation domains. The five *STB* genes were isolated between one and eight times in the library screen with M1776. Fold activation refers to two-hybrid activation of an *STB* plasmid with the M1776 LexA-Sin3p bait, normalized to the two-hybrid activation with the LexA vector control. **B** Protein extracts were prepared from strains expressing the LexA-Sin3p fusion proteins and analyzed by immunoblotting with anti-LexA antibody. The positions of the three LexA-Sin3p fusion proteins are indicated

Although there was no significant difference in the relative levels of activation in a wild type versus a *sin3* strain, we did detect a reduction in the level of *lacZ* expression in a *sin3* mutant. A decrease in two-hybrid activation in a *sin3* mutant has been previously noted for the LexA-Mad1 and VP16-Max interaction (Kasten et al. 1996). Although the Stb proteins and Mad1 interact with Sin3p, the following experiments demonstrate that this decrease in two-hybrid activation in a *sin3* mutant is not seen only with Sin3p-interacting proteins.

To determine whether the *sin3* mutation affected only Sin3p-interacting proteins or all two-hybrid activation, we examined other examples of two-hybrid activation in a *sin3* mutant strain. LexA-Ras and VP16-Raf are commonly used as positive controls for two-hybrid analysis, and neither Ras nor Raf are believed to interact with Sin3p. The two-hybrid activation by LexA-Ras and VP16-Raf was decreased 13-fold in a *sin3* mutant compared to a *SIN3* strain (Fig. 2, compare lines 1 and 2). Additionally, a *sin3* mutation caused an eight-fold decrease in two-hybrid activation by LexA-A65 and GAD-B72, two subunits of a mammalian protein phosphatase (McCright and Virshup 1995). These results suggest that the reduction in two-hybrid activation is not specific to Sin3p-interacting proteins. Moreover, a *sin3* mutation reduces two-hybrid activation by both VP16 and Gal4p-AD, and thus the effect is not specific to a particular activation domain.

We used *SIN3* deletion alleles to determine which of the PAH domains is required for this increased two-hybrid activation. As shown in Fig. 2, deletion of the



	LexA-Ras and VP16-Raf		LexA-A65 and GAD-B72	
	lacZ Activity	Relative Activation	lacZ Activity	Relative Activation
1. vector	260 ± 50	8%	280 ± 140	12%
2. Sin3p	3300 ± 410	100%	2300 ± 130	100%
3. Sin3pΔPAH1	2600 ± 450	79%	1600 ± 360	70%
4. Sin3pΔPAH2	1700 ± 180	25%	1400 ± 590	61%
5. Sin3pΔPAH3	330 ± 90	10%	130 ± 45	6%
6. Sin3pΔPAH4	1400 ± 120	42%	1600 ± 580	70%

Fig. 2 A *sin3* mutation decreases two-hybrid activation by the VP16 and Gal4p activation domains. The various *SIN3* deletion constructs on YCp plasmids were transformed into strain DY2516 (*sin3 LexA-lacZ*) with either LexA-Ras and VP16-Raf or LexA-A65 and GAD-B72. β-Galactosidase activity (units ± s.d.) was determined as a measure of promoter activity from three independent transformants. The following plasmids were used: M1093, vector plasmid; M1635, wild type Sin3p; M1636, Sin3ΔPAH1p; M1637, Sin3ΔPAH2p; M1638, Sin3ΔPAH3p; and M1639, Sin3ΔPAH4p

PAH1, PAH2 and PAH4 regions of Sin3p leads to two-hybrid activation at levels close to that seen in strains expressing the native Sin3p protein. In contrast, deletion of the PAH3 region results in two-hybrid activation at the levels seen in *sin3* mutants, consistent with previous observations that PAH3 is important for *SIN3* function (Kasten et al. 1996; Wang and Stillman 1993).

Interaction requires the PAH2 region of Sin3p

Two-hybrid assays were performed to characterize further the interaction between Sin3p and the five *STB* candidate proteins. All five *STB* prey plasmids interact and activate with the original LexA-Sin3p(PAH2) fusion spanning residues 347–598 (M1776) (Fig. 1A, line 3). They also interact with several other LexA-Sin3p baits that contain PAH2, but do not respond to LexA-Sin3p baits that lack PAH2 (data not shown). To determine whether the two-hybrid interaction requires PAH2, plasmids M2629 and M2630 were constructed. Plasmid M2629 expresses a LexA-Sin3p fusion containing little more than just PAH2 (residues 407–497), and M2630 expresses a similar fusion protein in which part of PAH2 has been deleted. The *STB1*, *STB2*, and *STB3* prey candidates all interact with the M2629 bait, producing blue colonies (Fig. 1A, line 4), but not with the M2630 bait (Fig. 1A, line 5), demonstrating interaction with the PAH2 region of Sin3p. The *STB4* and *STB5* clones do not show any two-hybrid interaction with the M2629 LexA-Sin3p(PAH2) bait, suggesting that additional regions of Sin3p are required for interaction.

To address the question of whether the PAH2 domain is sufficient for the two-hybrid interaction, we used quantitative assays to measure *lacZ* expression. The *lacZ* assays show that the *STB1*, *STB2*, and *STB3* prey candidates provide much higher levels of *lacZ* expression with the M1776 bait than with the M2629 bait containing PAH2 only (Fig. 1A). This difference could mean that additional domains present in M1776, in addition to PAH2, contribute to protein-protein interaction. Alternatively, it is possible that there is a difference in stability between the LexA fusion proteins expressed by M1776 and M2630. To address this question, an immunoblot experiment was performed using antibody to LexA (generously provided by Erica Golemis), to determine protein levels of the LexA-Sin3p proteins. The results in Fig. 1B show that the M2629 LexA-Sin3p bait protein accumulates to much lower levels than the M1776 bait protein. This result suggests that the lower level of two-hybrid activation by M2629, compared to M1776, could be due to the decreased levels of the M2629 bait protein.

Stb1p, Stb4p, and Stb5p are GAD-independent activators in a two-hybrid assay

The prey construct isolated from a two-hybrid library is typically a fusion protein to the Gal4p activation domain (GAD) or to the VP16 activation domain (VP16). Three of the *STB* genes, however, do not require the GAD to activate transcription. We refer to these candidates as being "GAD independent." *STB1*, *STB4* and *STB5* activate the two-hybrid reporter in a GAD-independent manner. Although several pieces of data suggested the GAD-independent nature of these candidates, GAD independence was clearly demonstrated by expressing these genes from their native promoters on standard yeast multicopy plasmids and examining activation in a two-hybrid assay. *STB1*, *STB4*, and *STB5* on a multicopy YEp plasmid were each able to activate in a two-hybrid assay in the presence of the LexA-Sin3 (PAH2) bait. Typically these multicopy YEp plasmids are present at 20–40 copies per cell (Rose and Broach 1991). We also examined the ability of *STB1*, when present on a YEpFAT vector with the LEU2-d allele

(plasmid copy number around 200 copies/cell), to activate a reporter via the LexA-Sin3(PAH2) bait. *STB1* on the YEpFAT vector produced three-fold higher activation than the YEp-*STB1* plasmid, consistent with the multicopy nature of the GAD-independent activation. Our results thus suggest that *STB1*, STB4 and *STB5* may encode transcriptional activators or proteins involved in transcription.

A role for *STB1* and *STB5* in transcription activation is also supported by experiments with LexA fusions. LexA-Stb1p and LexA-Stb5p fusions function as transcriptional activators of promoters containing a LexA binding site. LexA-Stb4p does not activate transcription, but immunoblot analysis suggests that this fusion protein is not stably expressed in yeast.

The *STB* genes

STB1, *STB2*, and *STB3* were cloned and their DNA sequences were determined. The sequences of *STB4* and *STB5* were obtained from the yeast genome sequencing project. The genes were physically mapped onto the yeast genome by Southern blot hybridization to yeast chromosome blots and prime clone blots (Riles et al. 1993). Table 2 lists the chromosome, ORF designation, prime clone number and accession number for each of the *STB* genes.

STB4 and *STB5* are predicted to encode proteins bearing a C6 zinc cluster DNA-binding motif (Marmorstein et al. 1992). Neither protein is predicted to have the dimerization motif present in some C6 zinc cluster proteins (Marmorstein et al. 1992; Marmorstein and Harrison 1994). In addition to the C6 zinc cluster motifs, Stb4p and Stb5p are both asparagine rich.

We identified a yeast gene with strong similarity to *STB2* on chromosome XI. This open reading frame, YKL072, was identified in the course of the yeast genome sequencing project and we have designated this gene *STB6*. Stb2p and Stb6p share 46% identity and 66% similarity at the amino acid level, and this homology extends across the entire peptide sequence. It should be noted that we have not tested whether Stb6p interacts with Sin3p.

STB1 and *STB3* have no significant homology or motifs to known proteins as determined by database searches. Both Stb1p and Stb3p, like Stb4p and Stb5p, are asparagine rich, while Stb3p is also rich in serine and glutamine.

STB gene disruptions

Strains bearing disruptions of any one of the six *STB* genes are viable, as are *stb2 stb6*, and *stb4 stb5* double mutants. We also determined that a *stb4 stb5 ume6* triple mutant is viable, as the *UME6* gene, which also encodes a C6 zinc cluster protein, has been implicated in regulation of some *SIN3*-dependent promoters (Bowdish and Mitchell 1993; Hudak et al. 1994; Strich et al. 1994; Kadosh and Struhl 1997). While none of the genes is essential, *stb5* mutants are slow growing. All *stb* mutants are able to grow on various carbon sources, although the growth defect of a *stb5* mutant is accentuated on several carbon sources other than glucose. An *stb5* mutant is very slow growing on medium in which galactose, sucrose, or glycerol/ethanol is used as the sole carbon source. A *stb4* mutant grows normally on alternative carbon sources, and the growth defect of a *stb4 stb5* double mutant is no worse than that of a *stb5* single mutant.

STB1 contributes to transcriptional activation of *HO*::*lacZ*

The *STB* genes were analyzed with respect to a possible role in transcriptional regulationof *HO*. A *sin3* mutation overcomes the requirement for the *SWI5* activator for *HO* expression, as *HO*::*lacZ* is not expressed in a *swi5* mutant, but *HO*::*lacZ* expression is observed in a *swi5 sin3* double mutant. Strains bearing mutations in each of the six *STB* genes were examined for an increase in *HO*::*lacZ* transcription in a *swi5* background, similar to the effect of a *sin3* mutation. Likewise, *stb2 stb6* double mutants and *stb4*, *stb5*, and *ume6* double and triple mutants were examined. None of the *stb* single, double or triple mutants examined showed an increase in the *HO* reporter activity in a *swi5* background (data not shown).

However, an *stb1* mutation is associated with a decrease in *HO*::*lacZ* expression (Table 3). The effect of

Table 2 The *STB* genes

Gene	Chrom.	Prime Clone Numbers	Accession Number	ORF Name	Size[a]	Sequence features
STB1	XIV	6742, 5413	U33439	YNL309w	459	No homology
STB2	XIII	3574, 9796	U33438	YMR053c	850	Homologous to *STB6*
STB3	IV	5879, 5219	U33440	YDR169c	513	No homology
STB4	XIII	4178, 3524	Z49211	YMR019w	949	C6 zinc cluster DNA-binding
STB5	VIII	9986	U00027	YHR178w	743	C6 zinc cluster DNA-binding
STB6	XI	n.d.	X75780	YKL072w	766	Homologous to *STB2*

[a] Size is given as the number of amino acid residues encoded by the ORF
n.d. = not determined

Table 3 Effect of a *stb1* mutation on expression of an integrated *HO*::*lacZ* reporter in wild-type, *swi5*, *sin3*, and *swi5 sin3* mutants

Genotype	*HO*::*lacZ* activity[a]	Activity (% of wild type)[b]	Activity (% of *STB1*)[c]
Wild type	330 ± 20	100%	100%
stb1	250 ± 5	76%	76%
swi5	5 ± 1	2%	100%
swi5 stb1	4 ± 1	1%	80%
swi5 sin3	70 ± 2	21%	100%
swi5 sin3 stb1	24 ± 2	7%	34%
sin3	1100 ± 15	333%	100%
sin3 stb1	770 ± 20	223%	70%

[a] β-Galactosidase levels were determined for three independent spores for each genotype, and expressed as units ± standard error. These isogenic strains have an integrated *HO*::*lacZ* reporter
[b] β-Galactosidase levels are normalized to wild type (*SWI5 SIN3 STB1*)
[c] β-Galactosidase levels for each *stb1* mutant are normalized to the corresponding *STB1* strain

stb1 can be seen in a number of specific genotypes, but perhaps most strikingly in the *swi5 sin3* double mutants. The *swi5 sin3 stb1* strains have 7% of wild type *HO*::*lacZ* activity as compared to 21% of wild type activity in the *swi5 sin3* double mutant. In this situation there is a three-fold reduction in *HO*::*lacZ* activity due to the *stb1* mutation. This effect is specific for the *HO* promoter, as expression from the *CYC1* promoter is unaffected by a *stb1* mutation (data not shown). These results suggest a model for how Sin3p and Stb1p may be interacting and affecting the *HO* promoter. According to this model, one role of Sin3p as a negative regulator is to inactivate or sequester the Stb1p activator from the *HO* promoter. This model is based on the observation that Stb1p can be considered an activator of *HO* in a *swi5 sin3* background, but a *stb1* mutation has little effect on *HO*::*lacZ* in a *SWI5 SIN3* strain. Sin3p sequesters Stb1p from the *HO* promoter in a *swi5* background, thereby preventing Stb1p from activating *HO*. A *swi5 sin3* double mutant does not have the Sin3p protein to sequester the Stb1p activator, and so the double mutant expresses *HO*::*lacZ* partly as a result of activation by Stb1p. Stb1p is not the only means by which *HO*::*lacZ* is activated in a *swi5 sin3* strain, however, as there is still weak activation of the *HO* promoter in a *swi5 sin3 stb1* triple mutant (reduced three-fold compared to the *swi5 sin3* mutant). Northern analysis of the effect of *stb1* mutations on the endogenous *HO* gene support the idea that *STB1* is a weak activator of *HO* transcription which is inhibited by *SIN3* (data not shown). The model does not require Stb1p to bind to the *HO* promoter, nor interact with DNA directly, to function as an activator of *HO*.

Additional support for the notion that *STB1* is an activator of *HO* comes from overexpression studies. In one set of experiments the *STB1* gene on a multicopy YEp plasmid causes a modest increase in *HO*::*lacZ* expression, and this increase is seen in *SWI5 SIN3*, *swi5 SIN3*, and *swi5 sin3* strains (Table 4). In a second set of experiments, a LexA-Stb1p fusion protein is overproduced from the strong *ADH1* promoter. As there are no known LexA binding sites in the *HO* promoter, the LexA region of the protein is not expected to contribute to changes in *HO* expression; the vector expressing only LexA is used as a control. High-level expression of LexA-Stb1p from the *ADH1* promoter causes a significant increase in *HO*::*lacZ* expression, particularly in the *swi5 SIN3* strain, where there is a 17-fold increase over the vector control. This effect is specific for the *HO* promoter, as expression from the *CYC1* promoter is unaffected by *STB1* overexpression (data not shown). These results indicate that *STB1* can augment activation of the *HO* promoter when expressed from a strong promoter, and further support a role for *STB1* in *HO* regulation.

Table 4 Overexpression of *STB1* increases *HO*::*lacZ* expression

Plasmid construct	*HO*::*lacZ* activity					
	SWI5 SIN3		*swi5 SIN3*		*swi5 sin3*	
	lacZ activity	Activation level	lacZ activity	fold Activation level	lacZ activity	fold activation
YEp vector	260	1×	33	1×	310	1×
YEp-*STB1*	340	1.3×	83	2.5×	700	2.2×
Yep-p*ADH1*:LexA vector	350	1×	53	1×	330	1×
Yep-p*ADH1*:LexA-*STB1*	880	2.5×	920	17×	2400	7.2×

[a] The YEp vector is pRS425, the YEp-*STB1* plasmid is M2517, the YEp-p*ADH1*:LexA vector is pSH2-1, and the YEp-p*ADH1*:LexA-*STB1* plasmid is M2708. Yeast strains DY131 (*SWI5 SIN3*), DY409 (*swi5 SIN3*), and DY1025 (*swi5 sin3*) were transformed with plasmids and three independent transformants were assayed for β-galactosidase activity. Standard errors were < 20%

Effect of *stb* mutations on other *SIN3*-regulated promoters

SIN3 also negatively regulates the genes *IME2*, *INO1*, and *SPO13*, as these genes are derepressed in a *sin3* mutant. To determine the effect of *stb* mutations on repression of these *SIN3*-dependent genes, plasmids with *IME2*::*lacZ*, *INO1*::*lacZ*, or *SPO13*::*lacZ* reporters were transformed into isogenic *STB* and *stb* strains and promoter activity determined by blue/white assays in the presence of X-Gal. For the *INO1*::*lacZ* reporter, the growth medium was supplemented with 1 mM choline chloride and 75 mM inositol to repress the *INO1* promoter.

Mutations in *STB1*, *STB2*, and *STB3* show no effect on any of the three reporters. A *stb5* disruption has a minor effect on *IME2*::*lacZ*; the reporter is activated approximately 5 fold (data not shown). However, a *sin3* mutation has a much greater effect, a 1000-fold derepression of *IME2*::*lacZ* expression. Blue/white filter assays with X-Gal showed that *stb4* and *stb6* mutations caused derepression of the *IME2*::*lacZ* and *INO1*::*lacZ* reporters.

Stb1p and Stb2p are present in the Sin3p protein complex

A co-purification assay was used to demonstrate that Sin3p physically interacts with Stb1p and Stb2p. In these experiments we used a plasmid that expresses Sin3p fused to glutathione-S-transferase (GST). The high affinity of GST for glutathione allows one to purify the GST-fusion protein, along with any associated proteins, by chromatography on glutathione-agarose. We have previously shown that Sin3p is present in a large, multiprotein complex (Kasten et al. 1997). The GST-Sin3p complex can be purified in two steps: after size fractionation on a Sephacryl S-400 column, proteins eluting in the 2–3 MDa size range, including GST-Sin3p, can be isolated by chromatography on a glutathione-agarose resin.

In the first experiment, protein extracts prepared from a yeast strain expressing both GST-Sin3p and LexA-Stb1p, were chromatographed on the Sephacryl S-400 column and the fractions containing GST-Sin3 (corresponding to approximately 2–3 MDa) were applied to the glutathione-agarose resin. Lacking an antibody to Stb1p, we used antisera to LexA to identify the LexA-Stb1p fusion protein. The load, the final wash, and the eluate fractions from the glutathione affinity chromatography were analyzed on immunoblots probed with antibodies recognizing either GST (for Sin3p) or LexA (for LexA-Stb1p). Both the GST-Sin3p and LexA-Stb1p proteins are present in the eluate from the glutathione column, demonstrating that Stb1p associates with Sin3p (Fig. 3A, lanes 1–3).

To demonstrate the specificity of this interaction, extracts prepared from several control strains were analyzed. Examination of an extract prepared from a strain expressing GST-Sin3p and LexA (instead of LexA-Stb1p) shows that the LexA DNA-binding domain does not interact with GST-Sin3p (Kasten et al. 1997). An extract prepared from a strain expressing GST (instead of GST-Sin3p) and LexA-Stb1p was used to demonstrate that LexA-Stb1p does not interact with the GST domain. After gel filtration chromatography, two protein pools from this size-fractionated GST/LexA-Stb1p extract, one at 2 MDa (the size of the GST-Sin3p complex) and one at 100 kDa (the native size of GST), were then fractionated by glutathione affinity chromatography. The GST protein is clearly present in the glutathione column eluate loaded with the 100 kDa pool (Fig. 3A, lane 5). Importantly, LexA-Stb1p did not associate with GST (Fig. 3A, lanes 4–5). We conclude that the interaction between Sin3p and Stb1p is specific.

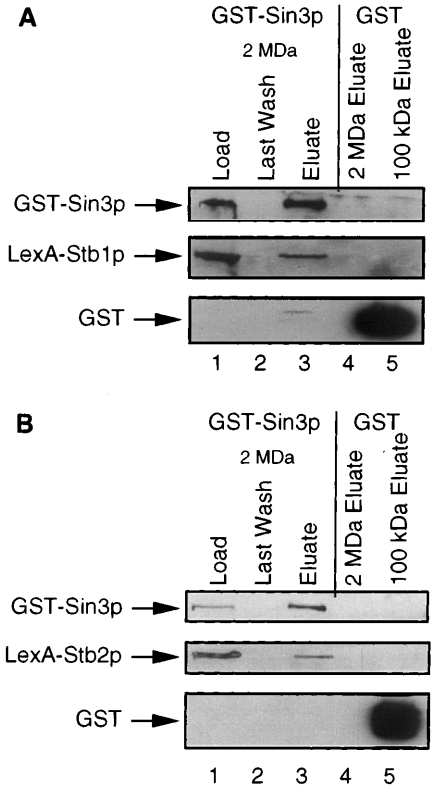


Fig. 3A, B Sin3p is physically associated with Stb1p and Stb2p. Extracts prepared from strain DY984 (*sin3*) containing two expression plasmids were first fractionated by size and then subjected to glutathione affinity chromatography. For each panel extracts were analyzed from yeast expressing either GST-Sin3p or GST only. For the GST-Sin3p experiments, proteins in the 2-MDa size range (containing GST-Sin3p) were chromatographed on the glutathione resin, and samples from the glutathione Load, Wash, and Eluate fractions were analyzed on immunoblots with antisera recognizing either GST or LexA. For the experiments with GST only, proteins in the 2-MDa (where GST-Sin3p elutes) and the 100-kDa (where GST elutes) size range were chromatographed on glutathione and the eluate fractions analyzed. **A** Yeast strains expressing LexA-Stb1p, together with either GST-Sin3p or GST. **B** Yeast strains expressed LexA-Stb2p, along with either GST-Sin3p or GST

A similar experiment was conducted with extracts prepared from a yeast strain expressing both GST-Sin3p and LexA-Stb2p (Fig 3B). The experiments show that the LexA-Stb2p fusion protein associates with GST-Sin3p. This experiment could not be conducted with Stb3p, Stb4p, or Stb5p, as the LexA-Stb3p, LexA-Stb4p and LexA-Stb5p plasmids did not produce stable fusion proteins detectable by immunoblot analysis.

Discussion

The yeast Sin3p protein functions as a transcriptional repressor. We have conducted a two-hybrid screen using a portion of Sin3p as bait, with the expectation that we could identify distinct types of proteins. First, we expected to identify DNA-binding proteins, as Sin3p does not bind directly to DNA, but is targeted to promoters via interactions with DNA-binding proteins. Two genes identified in this screen, *STB4* and *STB5*, encode C6 zinc cluster proteins, and these are likely to be DNA-binding proteins. We also expected to recover a second class of proteins, those that are complexed with Sin3p, or that act downstream, effecting transcriptional repression. *STB1*, *STB2*, and *STB3* fit into this second class. We have shown previously that Sin3p is part of a large multiprotein complex, and in this report we show that Stb1p and Stb2p are present in this complex, which is required for transcriptional repression.

The fact that a LexA-Sin3p fusion protein blocks expression of promoters that carry a LexA binding site (Wang and Stillman 1993) could create problems in a two-hybrid screen, as the repression activity in Sin3p could interfere with the transcriptional activation by any potential two-hybrid partner. We therefore tested a LexA-Sin3-Gal4-AD triple fusion, consisting of the LexA DNA-binding domain fused to Sin3p fused to the Gal4p activation domain, and the fact that this did not activate suggested that repression by Sin3p was dominant over the Gal4p activation domain. We then tested smaller regions of Sin3p, and found that a triple fusion with the PAH2 region of Sin3p was competent for activation. The properties of the triple fusion with PAH2 suggested that the PAH2 region of Sin3p may interact with *HO* DNA-binding proteins, and we decided to conduct the two-hybrid screen using the PAH2 region of Sin3p as bait.

As just described, one of the reasons for using only part of Sin3p as bait in a two-hybrid screen was the fact that the LexA-Sin3-Gal4-AD triple fusion was unable to activate a two-hybrid reporter. At a later time, however, immunoblot experiments were performed which showed that the LexA-Sin3-Gal4-AD triple fusion is unstable in cells (data not shown), suggesting a trivial cause for the failure of this triple fusion to activate. We therefore tested the various *STB* clones with a LexA-Sin3p bait that contained full-length Sin3p. None of these *STB* clones produced positive two-hybrid interactions with LexA-Sin3(full length), despite the fact that they are positive when tested with the LexA-Sin3(PAH2) bait (data not shown). One possible explanation is that while the Stb proteins are able to interact with an isolated region of Sin3p, they may not be able to associate with full-length Sin3p. However, we have clearly demonstrated that Stb1p and Stb2p remain physically associated with full-length Sin3p during gel filtration and affinity chromatography. Taken together, these results suggest that the repression properties of Sin3p are dominant over the activation function of the Gal4p activation domain.

We also observed that a *sin3* mutation results in reduced transcriptional activation by several pairs of two-hybrid partners (Fig 2). How is this possible, if Sin3p functions as a transcriptional repressor? We suggest that Sin3p represses the expression of a transcriptional inhibitor, and thus reduces transcriptional activation. For the purpose of this discussion let us suggest that this transcriptional inhibitor is a protein phosphatase. Thus, in a *sin3* mutant there is increased expression of a protein phosphatase which dephosphorylates activation domains and thus decreases their activity. A *sin3* mutation causes decreased expression of the *STE6* gene, and this is probably an indirect effect (Vidal et al. 1991; Wang et al. 1994). A *sin3* mutation results in decreased activity of Mcm1p and Ste12p, the transcriptional activators of *STE6*. Phosphorylation of the Ste12p transcription factor is altered in a *sin3* mutant, and phosphorylation of transcription factors in yeast correlates with increased activity as a transcriptional activator (Wang et al. 1994). We suggest that Sin3p represses expression of a second protein, possibly a protein phosphatase, and that in a *sin3* mutant the level or activity of this putative repressor increases, and activation by GAD and VP16 is less effective.

The two-hybrid screen identified five genes, *STB1*–*STB5*, encoding proteins that interact with Sin3p (Table 2). The yeast genome contains a gene that is similar to *STB2*, and we have named this paralogous gene *STB6*. Gene disruption experiments allow us to conclude that none of these genes is essential, and all of the double, triple and quadruple mutant strains constructed were viable. *STB4* and *STB5* are homologous to C6 zinc cluster proteins, and thus they are likely DNA-binding proteins (see below). For *STB1*, *STB2*, *STB3*, and *STB6*, computer searches did not reveal any homologies or protein motifs that might provide information as to function.

Several lines of evidence suggest that Stb1p, Stb4p, and Stb5p may be transcriptional activators. In the clones identified from the two-hybrid library as interacting with LexA-Sin3(PAH2), these open reading frames were not fused in frame to the Gal4p activation domain. We describe these genes as GAD independent, because when they are overexpressed from a simple multicopy yeast plasmid they can, with LexA-Sin3-(PAH2), activate a promoter containing a LexA binding site. This result suggests that these proteins contain their own activation domains. GAD-independent activators

have been identified by other investigators in two-hybrid screens (Treich et al. 1995; Lesage et al. 1996; Pagé et al. 1996; Wotton et al. 1996). LexA-Stb1p and LexA-Stb5p fusion proteins activate transcription of promoters containing LexA binding sites, supporting the idea that these proteins contain activation domains. Finally, Stb1p contributes to the transcriptional activation of *HO*, as *HO*::*lacZ* expression is reduced in an *stb1* mutant (Table 3) and overexpression of Stb1p increases *HO*::*lacZ* expression (Table 4).

The Stb4p and Stb5p proteins contain a C6 zinc cluster DNA-binding motif. All C6 zinc cluster proteins bind to CGG sequences. Interestingly, the URS1 sequence implicated in some *SIN3*-dependent repression contains CGG sequences (Strich et al. 1994; Vidal et al. 1995; Kadosh and Struhl 1997). Some C6 zinc cluster proteins, such as Gal4p and Ppr1p, bind DNA as a homodimer, via a coiled-coil dimerization domain downstream of the zinc cluster domain (Marmorstein et al. 1992; Marmorstein and Harrison 1994; Liang et al. 1996). Other C6 zinc cluster family members, such as ArgR2p and Mal63p, do not have these coiled-coil regions, and it is not clear what mechanism these proteins use for binding to DNA. It has been suggested that these proteins may bind DNA as monomers or heterodimers (Reece and Ptashne 1993). Stb4p and Stb5p belong to this latter group of C6 zinc cluster proteins. Apart from the C6 zinc cluster motifs, Stb4p and Stb5p have no apparent similarities to each other or to other known proteins, except that both proteins are asparagine rich.

It is quite possible that genetic redundancy could explain the failure to uncover a *sin3*-like phenotype in *stb4* and *stb5* mutants. Based on this idea of redundancy, we examined whether combining a *ume6* mutation with *stb4* and *stb5* would affect *HO* regulation. The *UME6* gene has been identified in many of the same genetic screens as *SIN3*, and *UME6*, like *SIN3*, is a negative regulator of *IME2*, *INO1*, and *SPO13* (Bowdish and Mitchell 1993; Hudak et al. 1994; Strich et al. 1994). However, *UME6* is not a negative regulator of *HO* gene expression (Stillman et al. 1994). All of the genes regulated by *UME6* contain the GC-rich URS1 element in their promoters, as does *HO*. Although these observations suggest that *STB4* and *STB5* might well show genetic redundancy with *UME6*, a *stb4 stb5 ume6* triple mutant did not affect *HO* regulation. A search of the YPD database (Garrels 1995) shows that the yeast genome contains 52 genes encoding C6 zinc cluster proteins, and thus there are other gene products that could contribute to *SIN3*-dependent repression in place of Stb4p and Stb5p.

Sin3p, which is part of a large multiprotein complex, appears to play a critical role in transcriptional repression. Sin3p can recognize sequence-specific DNA-binding proteins, and thus it can recruit other proteins to specific promoters. One of the proteins present in the yeast Sin3p complex is Rpd3p (Kadosh and Struhl 1997; Kasten et al. 1997), and the recent work demonstrating that Rpd3p is a histone deacetylase (Rundlett et al. 1996; Taunton et al. 1996) suggests that repression of transcription requires modification of histones. Stb1p and Stb2p are present in the Sin3p complex, and further work is needed to determine their role in transcriptional repression.

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