MOLECULAR AND CELLULAR BIOLOGY, Aug. 1996, p. 4215–4221 Vol. 16, No. 8
0270-7306/96/$04.00+0

SIN3-Dependent Transcriptional Repression by Interaction with the Mad1 DNA-Binding Protein

MARGARET M. KASTEN,[1] DONALD E. AYER,[1,2] AND DAVID J. STILLMAN[1]*

Division of Molecular Biology and Genetics, Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah Health Sciences Center, Salt Lake City, Utah 84132,[1] and Division of Basic Sciences, Fred Hutchinson Cancer Research Center, Seattle, Washington 98104[2]

Received 20 March 1996/Returned for modification 30 April 1996/Accepted 21 May 1996

The *SIN3* gene in *Saccharomyces cerevisiae* encodes a negative regulator of transcription of a large number of genes. Mouse homologs of *SIN3* have been identified through screens for proteins interacting with the mammalian Mad1 protein, a transcriptional repressor. We find that yeast Sin3 (ySin3) interacts with Mad1 and that, as for mouse Sin3, the N terminus of Mad1 interacts with the PAH2 domain of ySin3. Although Mad1 (a basic helix-loop-helix leucine zipper [bHLH-Zip] protein) forms a heterodimer with the Max bHLH-Zip protein, LexA-Mad1 and VP16-Max do not activate transcription of a reporter gene in a two-hybrid assay. This failure in activation is due to direct repression by ySin3, as LexA-Mad1 and VP16-Max are able to activate the two-hybrid reporter in a *sin3* mutant. This inhibition of activation by LexA-Mad1 and VP16-Max requires the PAH2 domain of ySin3 and the N-terminal interaction region of Mad1. These data demonstrate that ySin3 functions as a transcriptional repressor by being brought to promoters by interacting with proteins bound to DNA.

The *Saccharomyces cerevisiae SIN3* gene was first identified as a negative regulator of the yeast *HO* gene, which encodes a site-specific endonuclease that initiates mating-type switching (30, 37). Subsequently, *SIN3* has been found to negatively regulate a wide variety of yeast genes involved in many different cell functions. These genes include *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 (8, 17, 38, 42, 49). Although it is clear that *SIN3* is involved in transcriptional regulation, the yeast Sin3 (ySin3) protein itself does not directly bind to DNA (46). It has been suggested that ySin3 may interact with sequence-specific DNA-binding proteins in order to function as a transcriptional repressor of specific promoters (47), but these proteins have not yet been identified.

Mouse genes encoding proteins homologous to ySin3 have been identified recently. The mouse Sin3 (mSin3) homologs were identified as interacting with either the mouse Mad1 or the mouse Mxi protein (6, 33). (Recently Mad3 and Mad4 were identified [19], and the original Mad was renamed Mad1.) Mad1 and Mxi are related basic helix-loop-helix leucine zipper (bHLH-Zip) proteins which form heterodimers with the Max bHLH-Zip protein (4, 50). It has been shown that the Mad1-Max heterodimer binds to DNA, that it functions as a transcriptional repressor (4), and that Mad1, Max, and mSin3 form a ternary complex (6). Studies with mutant Mad1 proteins suggest that the ability to interact with mSin3 is required for repression by the Mad1-Max DNA-binding heterodimer (6).

Max interacts with a number of proteins. In addition to interacting with Mad1 and Mxi, Max is an obligate dimerization partner for the Myc proto-oncogene family of bHLH-Zip proteins (1, 28). Myc-Max heterodimers recognize the same E-box sequence (CACGTG) as do the Mad1-Max dimers, in a sequence-specific manner (4, 7). However, while Mad1-Max heterodimers repress transcription (4), the Myc-Max heterocomplex activates transcription of a minimal promoter bearing the CACGTG binding site (2, 26). It has been proposed that switching from Myc-Max to Mad1-Max complexes may result in the downregulation of the Myc target genes and lead from cell proliferation to differentiation (3, 4). Mad1 also appears to play a role in differentiation beyond antagonizing Myc, since Myc and Mad1 have distinct spatial localizations in the intestinal epithelium and in developing neural tubes (12, 19). Because mSin3 mediates Mad1-induced repression of reporter constructs in mammalian cells, it seems likely that the mSin3 proteins would play an important role in driving differentiation. It is likely that mSin3 plays a broader role, beyond that in proliferating cells, as it is also expressed in differentiated cells.

The yeast *SIN3* gene encodes a 175-kDa protein that contains four paired amphipathic helix (PAH) motifs (44). Each PAH motif consists of two amphipathic helices separated by 20 amino acids, with the hydrophobic amino acids being highly conserved in each amphipathic helix. Similar motifs have been described for the HLH and tetratrico pptide repeat (TPR) proteins (22, 27), and it is believed that these regions mediate protein-protein interactions. The two mSin3 genes encode somewhat smaller proteins, at 120 or 143 kDa, which show approximately 35% amino acid identity to ySin3 (6). The level of conservation is highest in the PAH domains. Interestingly, it is the PAH2 region of mSin3 that shows the highest level of conservation with ySin3 and it is PAH2 of mSin3 that interacts with Mad1. The PAH motif is similar to an HLH domain, suggesting that mSin3 would interact with Mad1 via its HLH. However, it is the amino-terminal region of Mad1, distinct from the DNA-binding bHLH region, that interacts with mSin3 (6). This N-terminal region contains a predicted amphipathic alpha helix, and a proline substitution mutation that disrupts the alpha helix blocks interaction with mSin3.

In this report, we demonstrate by using the yeast two-hybrid

* Corresponding author. Mailing address: Division of Molecular Biology and Genetics, Department of Oncological Sciences, Huntsman Cancer Institute, University of Utah Health Sciences Center, Salt Lake City, UT 84132. Phone: (801) 581-5429. Fax: (801) 581-3607. Electronic mail address: stillman@bioscience.utah.edu.

TABLE 1. Plasmids used in this study

Plasmid	Description	Type	Marker	Source or reference
M1153	LexA-ySin3	YEp	*HIS3*	47
M1776	LexA-ySin3(PAH2)	YEp	*HIS3*	This study
M2582	LexA-Mad1	YEp	*TRP1*	6
M2644	LexA-Mad1:Pro	YEp	*TRP1*	6
M2985	LexA-Max	YEp	*TRP1*	This study
M2807	VP16-Mad1	YEp	*LEU2*	This study
M2974	VP16-Max	YEp	*LEU2*	6
M2631	GAD-ySin3(PAH2)	YEp	*LEU2*	This study
M2608	GAD-Mad1	YEp	*LEU2*	This study
pRS425	Prey control plasmid	YEp	*LEU2*	35
M3016	GST-Mad1	YCp	*URA3*	This study
M3017	GST-Mad1:Pro	YCp	*URA3*	This study
pRD56	GST vector	YCp	*URA3*	31
pRS313	Control plasmid	YCp	*HIS3*	35
M1635	Wild-type ySin3	YCp	*HIS3*	47
M1636	ySin3ΔPAH1	YCp	*HIS3*	47
M1637	ySin3ΔPAH2	YCp	*HIS3*	47
M1638	ySin3ΔPAH3	YCp	*HIS3*	47
M1639	ySin3ΔPAH4	YCp	*HIS3*	47

assay that the ySin3 protein can interact with the mammalian Mad1 protein. We also show that the interaction between ySin3 and Mad1 can result in transcriptional repression. These experiments provide direct evidence that ySin3 can repress transcription through interactions with DNA-binding proteins.

MATERIALS AND METHODS

Strains, media, and culture conditions. Four yeast strains were used in this work. DY150 (*ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-1*) has been described previously (47). DY984 (*sin3*::*ADE2*) is isogenic to DY150 and has been described previously (47). Strain DY150 was transformed with plasmid pSH18-34Δspe that had been cleaved with *Stu*I, resulting in strain DY1641 which has a *lexA-lacZ* reporter integrated at the *URA3* locus. Plasmid pSH18-34Δspe contains the *lacZ* gene under control of the *CYC1* promoter containing eight LexA operators and was provided by R. Brent. Strain DY2516 (*sin3*::*ADE2 URA3*::8 *lexA-lacZ*) was constructed by transforming DY1641 with the *sin3* disruption plasmid M1142. The gene disruption was confirmed by Southern blot analysis. Strains were transformed as described elsewhere (21), except that 50 μg of herring sperm DNA was used as the carrier nucleic acid instead of yeast tRNA. Strains were grown on selective complete media (34) containing 2% glucose, except when galactose was used as indicated, and supplemented with adenine, uracil, and amino acids as appropriate but lacking the essential components to select for plasmids. A *sin3* mutation results in a lag period much longer than that for wild-type strains (18), and care was taken that log-phase cultures were used for transformations or β-galactosidase determinations. Protein extracts were prepared for quantitative measurement of β-galactosidase levels in triplicate as described previously (9).

Plasmids. 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 upon request. Plasmids pSH2-1 (obtained from R. Brent) (15) and pBTM116 (obtained from S. Fields) were used to construct the LexA bait plasmids. Plasmids pVP16 (obtained from A. Vojtek) (43) and pGAD424 (obtained from S. Fields) were used to create the VP16 and *GAL4* activation domain fusion plasmids, respectively. Plasmid pRD56 (obtained from R. Deshaies) (31) was used to construct the glutathione *S*-transferase (GST) fusion plasmids.

GST copurifications. Strain DY150 containing the indicated plasmids was used for GST copurifications. Cells were grown in 100 ml of synthetic complete medium lacking uracil and tryptophan (to select for plasmids) with 2% galactose as a carbon source. The preparation of protein extracts and the GST copurification were performed as described elsewhere (20), with the following modifications. Four milligrams of total yeast protein was chromatographed on a 1.5-ml glutathione-agarose column. Samples of the total unfractionated yeast protein loaded on the column, the final-wash fraction, and the eluted fraction were electrophoresed on sodium dodecyl sulfate (SDS)-polyacrylamide gels and electroblotted. The Western blots (immunoblots) were incubated with anti-LexA serum (obtained from E. Golemis) and visualized with the ECL (Amersham) enhanced chemiluminescence kit.

RESULTS

ySin3 and Mad1 interact through PAH2 of ySin3 and the N terminus of Mad1. The mouse homolog of y*SIN3* was identified by using a two-hybrid screen to identify proteins which interact with Mad1 (6). The interaction occurs through the PAH2 region of mSin3 and the N terminus of Mad1. Since the PAH2 regions of mSin3 and ySin3 are highly conserved, we decided to investigate the interaction of ySin3 with Mad1 using a yeast two-hybrid system (11, 14). This system could prove to be a useful model for a complex transcription system such as that used in mammalian cells, and it may also provide insight into the mechanisms by which ySin3 can function as a transcriptional repressor in *S. cerevisiae*. To measure two-hybrid interactions, we used a strain with an integrated *lacZ* reporter under the control of a promoter containing a LexA binding site (*lexA-lacZ*); thus, specific interaction results in the production of β-galactosidase.

The two-hybrid data in Table 2 demonstrate that Mad1 and ySin3 interact in vivo. Although expression of a LexA-ySin3 fusion protein does not activate transcription of the reporter, coexpression of a VP16-Mad1 fusion (the VP16 activation domain fused to Mad1) stimulates transcription. Expression of VP16-Mad1 in the absence of LexA-ySin3 does not activate transcription (data not shown). The PAH2 region of ySin3 is sufficient for this interaction, since the LexA-ySin3(PAH2) bait construct containing only the PAH2 region of ySin3 is able to interact with the VP16-Mad1 fusion and activate the *lacZ* reporter over 3,000-fold. The two-hybrid activation still occurs when the positions of ySin3(PAH2) and Mad1 are switched as fusions to DNA-binding and activation domains. The interaction requires the amino terminus of Mad1, as the two-hybrid activation is abolished by proline mutations in Mad1 (Mad1:Pro) which disrupt the predicted alpha-helical region in the amino terminus of Mad1. This Mad1:Pro mutant contains two proline substitutions (leucine at position 12 changed to proline [L12P] and alanine at position 16 changed to proline [A16P]) in the N-terminal alpha-helical region essential for Mad1's interaction with mSin3 (6).

Endogenous ySin3 inhibits activation by Mad1-Max in a two-hybrid assay. It was demonstrated that Mad1 and Max can form heterodimers (4). Thus, it is surprising that the two-hybrid screen conducted with LexA-Mad1 did not identify Max as a partner (6). In fact, mSin3 was the only protein identified interacting with LexA-Mad1 in the two-hybrid screen. Because Mad1 can interact with ySin3, a transcriptional repressor, we hypothesized that endogenous ySin3 may interact with LexA-

TABLE 2. Interaction between Mad1 and ySin3 through PAH2 of ySin3 and the amino terminus of Mad1[a]

Bait	Prey	Mean LacZ activity (U) ± SD	Fold activation[b]
LexA-ySin3	Vector	9 ± 1	1
	VP16-Mad1	7,300 ± 2,500	810
LexA-ySin3(PAH2)	Vector	3 ± 3	1
	VP16-Mad1	11,000 ± 6,300	3,600
LexA-Mad1	Vector	35 ± 15	1
	GAD-ySin3(PAH2)	7,700 ± 1,700	220
LexA-Mad1:Pro	Vector	47 ± 5	1
	GAD-ySin3(PAH2)	30 ± 67	0.6

[a] The wild-type strain DY1641, with an integrated *lexA-lacZ* reporter, was transformed with the indicated plasmids, and three independent transformants were assayed for β-galactosidase activity.

[b] The fold activation was determined by normalizing to the level for the prey vector control for each bait construct.

TABLE 3. Inhibition by endogenous ySin3 of activation in a Mad1-Max two-hybrid assay[a]

Bait	Prey	Wild type		*sin3* mutant	
		Mean LacZ activity (U ± SD)	Fold activation[b]	Mean LacZ activity (U ± SD)	Fold activation[b]
LexA-Mad1	Vector	2.6 ± 1.7	1	4.2 ± 1.4	1
	VP16-Max	4.2 ± 0.7	1.6	130 ± 10	31
LexA-Mad1:Pro	Vector	10 ± 1.8	1	5.8 ± 0.7	1
	VP16-Max	1,300 ± 270	130	91 ± 7.4	16
LexA-Max	Vector	17 ± 3.7	1	9.2 ± 1.2	1
	GAD-Mad1	15 ± 6.3	0.9	340 ± 8.3	37

[a] The wild-type strain DY1641 and the *sin3* strain DY2516, each containing an integrated *lexA-lacZ* reporter, were transformed with the indicated plasmids, and three individual transformants were assayed for β-galactosidase activity. Plasmid LexA-Mad1:Pro is a LexA fusion protein with Mad1 containing two proline mutations in a predicted N-terminal alpha helix. VP16-Max has Max fused to the VP16 activation domain. In GAD-Mad1, Mad1 is fused to the *GAL4* activation domain.

[b] The fold activation was determined by normalizing to the level for the prey vector control.

Mad1 and inhibit activation by an interacting fusion protein containing Max fused to the VP16 activation domain.

We assayed for transcription activation by LexA-Mad1 and VP16-Max in wild-type and *sin3* strains that contained an integrated *lexA-lacZ* reporter (Table 3). We found that LexA-Mad1 and VP16-Max are not able to activate transcription in the wild-type strain (Table 3). When interaction is assayed in a *sin3* strain, however, LexA-Mad1 and VP16-Max stimulate transcription 30-fold, suggesting that endogenous ySin3 is interfering with activation in the wild-type strain. If endogenous ySin3 represses transcription of the LexA-Mad1 and VP16-Max complex by directly contacting Mad1, we would predict that disrupting the Mad1-ySin3 interaction would restore transcriptional activation of the *lexA-lacZ* reporter in a wild-type strain. As anticipated, a LexA-Mad1:Pro construct demonstrates activation with VP16-Max in wild-type *S. cerevisiae*. This provides further support for the model that ySin3 is repressing transcription of the reporter and is doing so through the N-terminal alpha helix of Mad1. Unexpectedly, activation by LexA-Mad1:Pro and VP16-Max is reduced in a *sin3* mutant, in comparison with activation in the wild type. This effect of a *sin3* mutation on activation by VP16 is discussed below.

In further support of this model, we find that by switching the DNA-binding and activation domains on the hybrid proteins, fusing Max to LexA and Mad1 to the activation domain, we are also able to activate transcription in a *sin3* mutant-dependent manner: activation occurs in a *sin3* strain but not in a wild-type strain (Table 3).

An alternative explanation for the lack of activation by Mad1 and Max in a two-hybrid assay is that ySin3 prevents the physical interaction between Mad1 and Max, disallowing formation of heterodimers. Although it is clear that Max, Mad1, and mSin3 can form a ternary complex in vitro (6), we wished to demonstrate that Max and Mad1 can interact when ySin3 is present in yeast cells.

To address this question, we used a copurification assay. Yeast plasmids which direct the expression of fusion proteins containing GST fused to either Mad1 or Mad1:Pro were constructed. Expression of the GST-Mad1 and GST-Mad1:Pro fusion constructs is under the control of the galactose-inducible *GAL1* promoter. Wild-type *S. cerevisiae* coexpressing one of the GST fusions and LexA-Max (driven by the constitutive *ADH1* promoter) was grown in selective medium with galactose as the carbon source, and protein extracts were prepared. The GST-Mad1 protein was purified by glutathione chromatography, and immunoblots with these fractions were probed with antibody to LexA to determine whether LexA-Max associated with the GST-Mad1 protein (Fig. 1). The results demonstrate that Max interacts with GST-Mad1:Pro and with GST-Mad1, but not with the GST-only control. Thus, Mad1 and Max physically interact in *S. cerevisiae* independently of the presence of ySin3. Therefore, the lack of activation by LexA-Mad1 and VP16-Max in the wild-type yeast strain cannot be explained by a simple lack of interaction and strongly indicates that ySin3 is interacting with the DNA-bound Mad1-Max complex to repress transcription.

A *sin3* mutation relieves the toxicity of LexA-Mad1. Colony size on plates is a sensitive indicator of growth rate and can be used to show that ectopic expression of foreign proteins can inhibit cell growth. Cells expressing a LexA-Mad1 fusion protein form small colonies, indicating that LexA-Mad1 is toxic. However, cells expressing the LexA-Mad1:Pro fusion protein grow at a normal rate, suggesting that ySin3 interaction contributes to the LexA-Mad1 toxicity. To test this hypothesis, we transformed plasmids expressing LexA-Mad1 and LexA-Mad1:Pro into a *sin3* mutant and determined growth rates on plates (Fig. 2). Although LexA-Mad1 is toxic in wild-type cells, it is not toxic in *sin3* mutants; equivalent growth rates were seen for *SIN3* and *sin3* strains carrying the LexA-Mad1:Pro plasmid. These results strengthen the hypothesis that Mad1 interacts with ySin3.

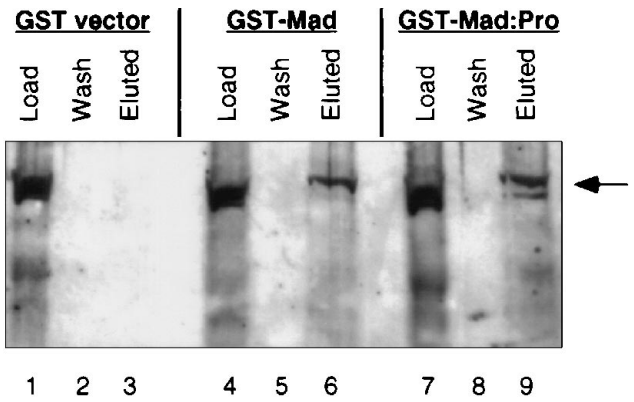

FIG. 1. Mad1 and Max physically interact in a *SIN3*$^+$ strain. Strain DY150 (wild type) containing a LexA-Max plasmid and a second plasmid as indicated (the GST vector, the GST-Mad1 plasmid, or the GST-Mad1:Pro plasmid) was grown in synthetic complete medium (lacking uracil and tryptophan) with 2% galactose as a carbon source. The galactose induces expression of the GST fusion proteins. Protein extracts were prepared and fractionated by glutathione-agarose chromatography. A 50-μg amount of unfractionated total yeast protein (Load) and 70-μl samples of the last-wash (Wash) and eluted (Eluted) fractions were separated on an SDS gel and analyzed by immunoblotting with anti-LexA antibody. The arrow indicates the LexA-Max protein.

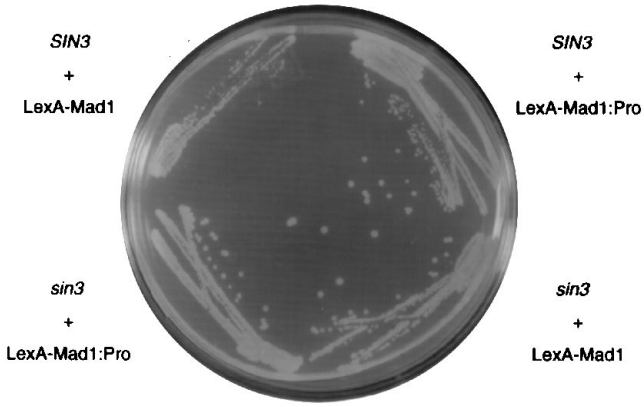

FIG. 2. The toxicity of LexA-Mad is relieved by a *sin3* mutation. Strains DY150 (*SIN3*) and DY984 (*sin3*) were transformed with either LexA-Mad or LexA-Mad:Pro mutant plasmids, with selection for tryptophan prototrophy. Transformed strains were grown on selective medium lacking tryptophan for 3 days at 30°C.

Inhibition of VP16-Max by ySin3 requires PAH2. We used *sin3* mutants (47) that had various PAH domains deleted to determine which region(s) of ySin3 is required for repression of VP16-Max activation. Plasmids containing these ySin3 PAH deletions were transformed into a *sin3* mutant strain with the integrated *lexA-lacZ* reporter and the LexA-Mad1 and VP16-Max plasmids. Transformants were grown under selection conditions to maintain the plasmids, and β-galactosidase levels were measured to determine the ability of these ySin3 PAH deletions to repress VP16-Max activation. Deletion of PAH1, PAH3, or PAH4 has no effect on the ability of ySin3 to inhibit activation by VP16-Max. Deletion of the PAH2 region, however, abolishes ySin3 repression (Table 4). The situation is actually more complex (see below), as LexA-Mad1 and VP16-Max is a more potent activator in the ySin3ΔPAH2 strain than in the *sin3* strain. Nonetheless, this experiment demonstrates that the PAH2 region of ySin3 is required to inhibit the transcriptional activation by LexA-Mad1 and VP16-Max.

Reduced activation by VP16-Max in a *sin3* mutant. This sixfold increase in activation by LexA-Mad1 and VP16-Max in the ySin3ΔPAH2 strain (Table 4) suggests that ySin3 affects two-hybrid activation by VP16-Max and LexA-Mad1 in two distinct ways. First, ySin3 interacts with Mad1 via PAH2 and inhibits the VP16 activation domain. Second, the increase in activation in the ySin3ΔPAH2 strain compared with the level in the strain with the *sin3* null mutation suggests that either ySin3 is required for full VP16 activation or that some other factor affects activation by LexA-Mad1 and VP16-Max, and that the activity of this factor is altered in a *sin3* mutant. According to this scenario, either ySin3 represses a second protein that represses VP16-Max or ySin3 activates an activator.

TABLE 4. Requirement for PAH2 for repression of VP16-Max by Sin3p[a]

ySin3 construct	Activation by LexA-Mad1 and VP16-Max	
	Mean LacZ activity (U) ± SD	% of control LacZ activity
Vector (*sin3*$^-$)	150 ± 40	100
ySin3	10 ± 4	7
ySin3ΔPAH1	7 ± 3	5
ySin3ΔPAH2	940 ± 450	630
ySin3ΔPAH3	10 ± 1	7
ySin3ΔPAH4	11 ± 2	7

[a] The various *SIN3* deletion constructs on YCp plasmids were transformed into strain DY2516 (*sin3*$^-$ *lexA-lacZ*) with plasmids for LexA-Mad1 and VP16-Max. β-Galactosidase activity was determined as a measure of promoter activity from three independent transformants.

We favor a model where ySin3 represses a repressor, for several reasons. First, ySin3 can function as a repressor, as LexA-ySin3 represses transcription from promoters with a LexA binding site (47). Second, although Vidal et al. (42) showed that expression of certain yeast genes decreases in *sin3* mutants and suggested that ySin3 functions as an activator, we believe this effect on activation is indirect. *STE6* expression is reduced in a *sin3* mutant, and we demonstrated that this is due to decreased activity of Mcm1 and Ste12, transcriptional activators of *STE6* (45). These results are consistent with the hypothesis that ySin3 represses a protein that inhibits transcriptional activators. Phosphorylation of transcription factors in *S. cerevisiae* is associated with increased activity as a transcriptional activator (29, 36). We have suggested that ySin3 may repress expression of a protein phosphatase, as phosphorylation of the Ste12 transcription factor is altered in a *sin3* mutant (45).

Our model is that ySin3 represses a second protein, possibly a protein phosphatase, that represses VP16-Max. In a *sin3* mutant, the level or activity of this putative repressor increases and VP16 is a less potent activator. Thus, two factors contribute to the increased activation by VP16-Max and LexA-Mad1 in the ySin3ΔPAH2 strain. The ySin3ΔPAH2 deletion protein has lost the ability to directly interact with Mad1 and thus cannot repress LexA-Mad1 and VP16-Max. However, ySin3ΔPAH2 retains the ability to negatively regulate this putative second repressor.

The data on two-hybrid activation by LexA-Mad1:Pro and VP16-Max support the idea that there is an additional repressor inhibiting activation by VP16-Max. The LexA-Mad1:Pro fusion protein contains amino acid substitutions that block the interaction of Mad1 with Sin3. Thus, we predicted that ySin3 would not inhibit activation by LexA-Mad1:Pro and VP16-Max in a wild-type strain, and this prediction was borne out (Table 3). As the LexA-Mad1:Pro bait no longer interacts with ySin3, we had expected equal levels of activation in wild-type and *sin3* strains. However, activation by LexA-Mad1:Pro and VP16-Max shows an eightfold decrease when assayed in a *sin3* strain (Table 3, compare wild-type and *sin3* strains). This result is consistent with the proposal that there is an additional repressor negatively regulated by *SIN3* which interferes with activation by the VP16-Max construct. This effect is not specific to ySin3-interacting proteins, as a *sin3* mutation reduces transcriptional activation by other two-hybrid activation partners (23).

What regions of ySin3 are required to repress this putative repressor? To address this question, a two-hybrid experiment was performed with LexA-Mad1:Pro and VP16-Max in a *sin3* mutant strain. This strain was transformed with plasmids, either the vector, wild-type ySin3, or one of the ySin3 PAH deletion constructs. LexA-Mad1:Pro does not interact with ySin3, and two-hybrid activation by LexA-Mad1:Pro and VP16-Max is not inhibited by ySin3. The two-hybrid activation data (Table 5) show that the *SIN3*$^+$ strain has an activation level 18-fold higher than does the *sin3* strain. High-level activation is also seen with the ΔPAH1, ΔPAH2, and ΔPAH4 deletion constructs, suggesting that these regions are not required for repression of the repressor. The ΔPAH2 result is important, as PAH2 is the region that interacts with native Mad1. In contrast, the ΔPAH3 deletion mutant displays two-

TABLE 5. Effect of a *sin3* mutation on activation by VP16[a]

ySin3 construct	Activation by LexA-Mad1:Pro and VP16-Max	
	Mean LacZ activity (U) ± SD	Fold activation
Vector (*sin3*$^-$)	110 ± 6	1
ySin3	2,000 ± 790	18
ySin3ΔPAH1	2,300 ± 200	21
ySin3ΔPAH2	1,200 ± 270	11
ySin3ΔPAH3	210 ± 30	2
ySin3ΔPAH4	2,100 ± 560	19

[a] The various *SIN3* deletion constructs on YCp plasmids were transformed into strain DY2516 (*sin3*$^-$ *lexA-lacZ*) with plasmids for LexA-Mad1:Pro and VP16-Max. β-Galactosidase activity was determined as a measure of promoter activity from three independent transformants.

hybrid activation by LexA-Mad1:Pro and VP16-Max at a level similar to that seen with the *sin3* null mutant. The PAH3 region of ySin3 has been previously shown to play an important role in transcriptional repression of *SIN3*-dependent genes in *S. cerevisiae* (47).

Deletions of PAH2 and of PAH3 of ySin3 have very different effects on two-hybrid activation involving LexA-Mad1 or LexA-Mad1:Pro. *ySIN3* represses activation by LexA-Mad1 and VP16-Max, but deletion of PAH2 eliminates this repression (Table 4). In contrast, deletion of PAH3 leads to decreased two-hybrid activation by LexA-Mad1:Pro and VP16-Max, where the Mad1:Pro mutation eliminates ySin3 interaction. We suggested that this decrease in VP16 activation is an indirect effect, proposing that ySin3 represses a repressor. What is the effect of deleting both PAH2 and PAH3 regions of ySin3? The ySin3ΔPAH2ΔPAH3 mutant causes two-hybrid activation by LexA-Mad1:Pro and VP16-Max to resemble that seen to occur in the *sin3* null mutant (data not shown), as deletion of PAH3 should derepress the repressor of VP16 activation. Two-hybrid activation by LexA-Mad1 and VP16-Max in the presence of the ySin3ΔPAH2ΔPAH3 mutant also resembles *sin3* activation (data not shown). In this mutant, deletion of PAH2 eliminates Mad1 binding and transcriptional repression of the reporter but deletion of PAH3 allows expression of the putative repressor of VP16. Thus, the two-hybrid activation resembles the level of activation for the *sin3* null mutant and not the higher levels seen with ySin3ΔPAH2.

DISCUSSION

We have used a two-hybrid assay to demonstrate that the mammalian Mad1 protein interacts with the yeast Sin3 protein. This interaction occurs through PAH2 of ySin3 and the amino-terminal alpha helix of Mad1, the same regions previously identified as required for interaction between mSin3 and Mad1 (6). These data suggest that the structure of PAH2 has been conserved between the ySin3 and mSin3 proteins. mSin3 is unable to complement a yeast *sin3* defect in repressing transcription of three different promoters regulated by *SIN3* (23); thus, it was not certain that Mad1 and ySin3 would physically interact. This suggests that other regions of mSin3 are unable to interact with the yeast transcriptional apparatus.

Our data indicating an interaction between Mad1 and ySin3 extend this interaction to endogenous ySin3. We have shown that endogenous ySin3 inhibits activation of the *lexA-lacZ* reporter by LexA-Mad1 and VP16-Max in a two-hybrid assay. LexA-Mad1 and VP16-Max physically interact in *SIN3*$^+$ *S. cerevisiae*, yet they do not activate transcription; thus, the interference with activation cannot be explained by a loss of Mad1-Max interaction caused by ySin3. Additionally, LexA-Mad1 expression is somewhat toxic in *S. cerevisiae*, but this toxicity can be alleviated either by mutating residues in Mad1 that interact with Sin3 or by a genomic *sin3* mutation.

Repression by endogenous mSin3 is dominant over the VP16 activation domain fused to the Gal4 DNA-binding domain (5), and we show here that this is also true for ySin3. It is not clear what precise conditions are necessary for ySin3's repression to be dominant over the activation domain; however, our data indicate that inhibition by endogenous ySin3 in a two-hybrid system is not restricted solely to the Mad1-Max heterodimer. A two-hybrid screen conducted by using LexA-Mad1:Pro as bait identified positive clones which do not show transactivation with wild-type LexA-Mad1 (data not shown), suggesting that ySin3 may be inhibiting transactivation. We expect that similar results would be obtained if the library screens were conducted with wild-type LexA-Mad1 in a *sin3* strain.

We favor the model that endogenous ySin3 inhibits activation by LexA-Mad1 and VP16-Max heterodimers by directly contacting this DNA-bound complex. Although Mad1 is not a yeast protein, these experiments demonstrate that ySin3 at endogenous levels can interact with DNA-binding proteins to repress transcription. In this particular case, the protein-protein interaction and repression require the PAH2 domain of ySin3. Our data provide the first direct evidence that ySin3 can repress transcription by interacting with DNA-bound proteins, although they do not eliminate the possibility that ySin3 affects transcription through additional mechanisms.

ySin3 has four PAH motifs, and we have suggested that each of these putative protein-protein interaction domains may interact with distinct partners (47). ySin3 derivatives that have deletions of one or more PAH domains have been expressed in *S. cerevisiae*, with the result that deletion of PAH3 has the strongest effect on transcription of *SIN3*-dependent genes (47). LexA-ySin3 represses transcription from heterologous promoters containing a LexA binding site, and deletion of PAH3 from LexA-ySin3 eliminates this repression (47). However, the mutant ySin3 derivative lacking PAH3 was still able to block activation by LexA-Mad1 and VP16-Max (Table 4). These differences in requirements for PAH3 suggest that ySin3 is working somewhat differently in repressing transcription from native yeast promoters relative to repression of LexA-Mad1 and VP16-Max. The PAH2 region of ySin3 interacts with the mammalian Mad1 protein, but what is the role of the PAH2 region in transcriptional regulation in *S. cerevisiae*? Deletion of PAH2 has only a modest effect on repression of *SIN3*-dependent genes in vivo (45). We have identified five proteins that interact with the PAH2 region of ySin3 (23), and experiments to determine the role of these proteins are in progress. On the basis of the interaction between ySin3 and Mad1, we suggest that PAH2 is probably important for targeting ySin3 to specific promoters, but these promoters have not yet been identified.

A similar mechanism of repression has been established for the yeast Ssn6 (same as Cyc8) and Tup1 proteins. Genetic evidence indicates that like ySin3, Ssn6 and Tup1 function as transcriptional repressors, and LexA fusions with ySin3, Ssn6, or Tup1 repress transcription of promoters containing LexA binding sites (24, 40, 47). Ssn6 and Tup1 form a large complex (48), and like ySin3, this complex does not bind to DNA directly. It is proposed that this complex is recruited to promoters through sequence-specific DNA-binding proteins, such as Mig1 at glucose-repressible genes, Matα2 and Mcm1 at Matα2-repressible genes, and Rox1 at oxygen-repressible genes (24, 25, 41). It has recently been demonstrated that Mig1

represses transcription in an *SSN6*- and *TUP1*-dependent manner (39), further supporting the model in which Mig1 recruits Ssn6-Tup1 to glucose-repressible promoters.

The conservation of protein sequence between ySin3 and mSin3 suggests that the mechanisms of transcriptional repression are conserved between yeast and mammalian cells. This idea is supported by our observation that ySin3 can block transcription in *S. cerevisiae* via interaction with the mammalian Mad1 protein. Metazoan systems provide other examples of transcriptional corepressor proteins, like ySin3, that do not bind directly to DNA. The mammalian SMRT and N-CoR proteins are corepressors that inhibit transcriptional activation by nuclear hormone receptors (10, 16). Groucho is a *Drosophila melanogaster* protein that does not bind DNA but interacts with bHLH DNA-binding proteins to repress transcription (13, 32). We expect that additional instances of transcriptional repression requiring factors that do not bind to DNA but interact with DNA-binding proteins will be found, and we hope that continued study of ySin3 will help us understand the mechanisms of this type of transcriptional regulation.

ACKNOWLEDGMENTS

We gratefully acknowledge Kim Hudak, John Lopes, and Susan Henry for communicating unpublished results, Yi Wei Jiang for advice on GST coprecipitation, and Erica Golemis for the gift of LexA antibody. We also thank Roger Brent, Ray Deshaies, Stan Fields, Carol Laherty, and Anne Vojtek for generously providing plasmids.

This work was supported by National Institutes of Health grants CA57138 to Robert N. Eisenman and GM39067 to D.J.S.

REFERENCES

1. **Amati, B., M. W. Brooks, N. Levy, T. D. Littlewood, G. I. Evans, and H. Land.** 1993. Oncogenic activity of the c-Myc protein requires dimerization with Max. Cell **72:**233–245.
2. **Amati, B., S. Dalton, M. W. Brooks, T. D. Littlewood, G. I. Evan, and H. Land.** 1992. Transcriptional activation by the human c-Myc oncoprotein in yeast requires interaction with Max. Nature (London) **359:**423–426.
3. **Ayer, D. E., and R. N. Eisenman.** 1993. A switch from Myc:Max to Mad:Max heterocomplexes accompanies monocyte/macrophage differentiation. Genes Dev. **7:**2110–2119.
4. **Ayer, D. E., L. Kretzner, and R. N. Eisenman.** 1993. Mad: a heterodimeric partner for Max that antagonizes Myc transcriptional activity. Cell **72:**211–222.
5. **Ayer, D. E., C. D. Laherty, Q. A. Lawrence, A. Armstrong, and R. N. Eisenman.** Mad proteins contain a dominant transcription repression domain. Submitted for publication.
6. **Ayer, D. E., Q. A. Lawrence, and R. N. Eisenman.** 1995. Mad-Max transcriptional repression is mediated by ternary complex formation with mammalian homologs of yeast repressor Sin3. Cell **80:**767–776.
7. **Blackwood, E. M., B. Lüscher, and R. N. Eisenman.** 1992. Myc and Max associate in vivo. Genes Dev. **6:**71–80.
8. **Bowdish, K. S., and A. P. Mitchell.** 1993. Bipartite structure of an early meiotic upstream activation sequence from *Saccharomyces cerevisiae*. Mol. Cell. Biol. **13:**2172–2181.
9. **Breeden, L., and K. Nasmyth.** 1987. Cell cycle control of the yeast *HO* gene: cis- and trans-acting regulators. Cell **48:**389–397.
10. **Chen, J. D., and R. M. Evans.** 1995. A transcriptional co-repressor that interacts with nuclear hormone receptors. Nature (London) **377:**454–457.
11. **Chien, C., P. L. Bartel, R. Sternglanz, and S. Fields.** 1991. The two-hybrid system: a method to identify and clone genes for proteins that interact with a protein of interest. Proc. Natl. Acad. Sci. USA **88:**9578–9582.
12. **Chin, L., N. Schreiber-Agus, I. Pellicer, K. Chen, H.-W. Lee, M. Dudast, C. Cordon-Cardo, and R. A. DePinho.** 1995. Contrasting roles for Myc and Mad proteins in cellular growth and differentiation. Proc. Natl. Acad. Sci. USA **92:**8488–8492.
13. **Dawson, S. R., D. L. Turner, H. Weintraub, and S. M. Parkhurst.** 1995. Specificity for the hairy/enhancer of split basic helix-loop-helix (bHLH) proteins maps outside the bHLH domain and suggests two separable modes of transcriptional repression. Mol. Cell. Biol. **15:**6923–6931.
14. **Fields, S., and O. Song.** 1989. A novel genetic system to detect protein-protein interactions. Nature (London) **340:**245–246.
15. **Hanes, S. D., and R. Brent.** 1989. DNA specificity of the bicoid activator protein is determined by homeodomain recognition helix residue 9. Cell **57:**1275–1283.
16. **Hörlein, A. J., A. M. Näär, T. Heinzel, J. Torchia, B. Gloss, R. Kurokawa, A. Ryan, Y. Kamei, M. Söderström, C. K. Glass, and M. G. Rosenfeld.** 1995. Ligand-independent repression by the thyroid hormone receptor mediated by a nuclear receptor co-repressor. Nature (London) **377:**397–404.
17. **Hudak, K., J. Lopes, and S. Henry.** 1994. A pleiotropic phospholipid biosynthetic regulatory mutation in *Saccharomyces cerevisiae* is allelic to *sin3* (*sdi1*, *ume4*, *rpd1*). Genetics **136:**475–483.
18. **Hudak, K., J. Lopes, and S. Henry.** Personal communication.
19. **Hurlin, P. J., C. Queva, P. J. Koskinen, E. Steingrimsson, D. E. Ayer, N. G. Copeland, N. A. Jenkins, and R. N. Eisenman.** 1995. Mad3 and Mad4: novel Max-interacting transcriptional repressors that suppress c-myc dependent transformation and are expressed during neural and epidermal differentiation. EMBO J. **14:**5646–5659.
20. **Jiang, Y. W., P. R. Dohrmann, and D. J. Stillman.** 1995. Genetic and physical interactions between yeast *RGR1* and *SIN4* in chromatin organization and transcriptional regulation. Genetics **140:**47–54.
21. **Jiang, Y. W., and D. J. Stillman.** 1995. Regulation of *HIS4* expression by the *Saccharomyces cerevisiae SIN4* transcriptional regulator. Genetics **140:**103–114.
22. **Jones, N.** 1990. Transcriptional regulation by dimerization: two sides to an incestuous relationship. Cell **61:**9–11.
23. **Kasten, M. M., and D. J. Stillman.** Unpublished data.
24. **Keleher, C. A., M. J. Redd, J. Schultz, M. Carlson, and A. D. Johnson.** 1992. Ssn6/Tup1 is a general repressor of transcription in yeast. Cell **68:**709–719.
25. **Komachi, K., M. J. Redd, and A. D. Johnson.** 1994. The WD repeats of Tup1 interact with the homeo domain protein alpha 2. Genes Dev. **8:**2857–2867.
26. **Kretzner, L., E. M. Blackwood, and R. N. Eisenman.** 1992. Myc and Max proteins possess distinct transcriptional activities. Nature (London) **359:**426–429.
27. **Lamb, J. R., S. Tugendreich, and P. Hieter.** 1995. Tetratrico peptide repeat interactions: to TPR or not to TPR? Trends Biochem. Sci. **20:**257–259.
28. **Mukherjee, B., S. D. Morgenbesser, and R. A. DePinho.** 1992. Myc family oncoproteins function through a common pathway to transform normal cells in culture: cross-interference by Max and trans-acting dominant mutants. Genes Dev. **6:**1480–1492.
29. **Mylin, L. M., J. P. Bhat, and J. E. Hopper.** 1989. Regulated phosphorylation and dephosphorylation of GAL4, a transcriptional activator. Genes Dev. **3:**1157–1165.
30. **Nasmyth, K., D. Stillman, and D. Kipling.** 1987. Both positive and negative regulators of *HO* transcription are required for mother-cell-specific mating-type switching in yeast. Cell **48:**579–587.
31. **Park, H. O., J. Chant, and I. Herskowitz.** 1993. BUD2 encodes a GTPase-activating protein for Bud1/Rsr1 necessary for proper bud-site selection in yeast. Nature (London) **365:**269–274.
32. **Paroush, Z., R. L. Finley, Jr., T. Kidd, S. M. Wainwright, P. W. Ingham, R. Brent, and D. Ish-Horowicz.** 1994. Groucho is required for Drosophila neurogenesis, segmentation, and sex determination and interacts directly with hairy-related bHLH proteins. Cell **79:**805–815.
33. **Schreiber-Agus, N., L. Chin, K. Chen, R. Torres, G. Rao, P. Guida, A. I. Skoultchi, and R. A. DePinho.** 1995. An amino-terminal domain of Mxi1 mediates anti-Myc oncogenic activity and interacts with a homolog of the yeast transcriptional repressor SIN3. Cell **80:**777–786.
34. **Sherman, F.** 1991. Getting started with yeast. Methods Enzymol. **194:**1–21.
35. **Sikorski, R. S., and P. Hieter.** 1989. A system of shuttle vectors and yeast host strains designed for efficient manipulation of DNA in *Saccharomyces cerevisiae*. Genetics **122:**19–27.
36. **Song, D., J. W. Dolan, Y. L. Yuan, and S. Fields.** 1991. Pheromone-dependent phosphorylation of the yeast STE12 protein correlates with transcriptional activation. Genes Dev. **5:**741–750.
37. **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.
38. **Strich, R., M. R. Slater, and R. E. Esposito.** 1989. Identification of negative regulatory genes that govern the expression of early meiotic genes in yeast. Proc. Natl. Acad. Sci. USA **86:**10018–10022.
39. **Treitel, M. A., and M. Carlson.** 1995. Repression by SSN6-TUP1 is directed by MIG1, a repressor/activator protein. Proc. Natl. Acad. Sci. USA **92:**3132–3136.
40. **Tzamarias, D., and K. Struhl.** 1994. Functional dissection of the yeast Cyc8-Tup1 transcriptional co-repressor complex. Nature (London) **369:**758–761.
41. **Tzamarias, D., and K. Struhl.** 1995. Distinct TPR motifs of Cyc8 are involved in recruiting the Cyc8-Tup1 corepressor complex to differentially regulated promoters. Genes Dev. **9:**821–831.
42. **Vidal, M., R. Strich, R. E. Esposito, and R. F. Gaber.** 1991. *RPD1* (*SIN3*/*UME4*) is required for maximal activation and repression of diverse yeast genes. Mol. Cell. Biol. **11:**6306–6316.
43. **Vojtek, A. B., S. M. Hollenberg, and J. A. Cooper.** 1993. Mammalian Ras interacts directly with the serine/threonine kinase Raf. Cell **74:**205–214.
44. **Wang, H., I. Clark, P. R. Nicholson, I. Herskowitz, and D. J. Stillman.** 1990. The *Saccharomyces cerevisiae SIN3* gene, a negative regulator of *HO*, contains four paired amphipathic helix motifs. Mol. Cell. Biol. **10:**5927–5936.
45. **Wang, H., L. Reynolds-Hager, and D. J. Stillman.** 1994. Genetic interactions between *SIN3* and the *Saccharomyces cerevisiae MCM1*, *STE12*, and *SWI1* transcriptional activators. Mol. Gen. Genet. **245:**675–685.

46. **Wang, H., and D. J. Stillman.** 1990. *In vitro* regulation of a *SIN3*-dependent DNA-binding activity by stimulatory and inhibitory factors. Proc. Natl. Acad. Sci. USA **87:**9761–9765.
47. **Wang, H., and D. J. Stillman.** 1993. Transcriptional repression in *Saccharomyces cerevisiae* by a SIN3-LexA fusion protein. Mol. Cell. Biol. **13:**1805–1814.
48. **Williams, F. E., U. Varanasi, and R. T. Trumbly.** 1991. The *CYC8* and *TUP1* proteins involved in glucose repression in *Saccharomyces cerevisiae* are associated in a protein complex. Mol. Cell. Biol. **11:**3307–3316.
49. **Yoshimoto, H., M. Ohmae, and I. Yamashita.** 1992. The *Saccharomyces cerevisiae GAM2*/*SIN3* protein plays a role in both activation and repression of transcription. Mol. Gen. Genet. **233:**327–330.
50. **Zervos, A. S., J. Gyuris, and R. Brent.** 1993. Mxi1, a protein that specifically interacts with Max to bind Myc-Max recognition sites. Cell **72:**223–232.