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The *Saccharomyces cerevisiae SIN3* Gene, a Negative Regulator of *HO*, Contains Four Paired Amphipathic Helix Motifs

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The *SIN3* gene (also known as *SDI1*) is a negative regulator of the yeast *HO* gene. Mutations in *SIN3* suppress the requirement for the *SWI5* activator for expression of the yeast *HO* gene and change the normal asymmetric pattern of *HO* expression in mother and daughter cells. Furthermore, the in vitro DNA-binding activity of several DNA-binding proteins is reduced in extracts prepared from *sin3* mutants. We have cloned the *SIN3* gene and determined that a haploid strain with a *SIN3* gene disruption is viable. We determined the sequence of the *SIN3* gene, which is predicted to encode a 175-kDa polypeptide with four paired amphipathic helix motifs. These motifs have been identified in the *myc* family of helix-loop-helix DNA-binding proteins and in the TPR family of regulatory proteins. The *SIN3* transcript was mapped, and it was determined that the *SIN3* transcript was absent in stationary-phase cells. Immunofluorescence microscopy with anti-SIN3 antibody demonstrated that SIN3 protein was present in nuclei. A comparison of restriction map and sequence data revealed that *SIN3* is the same as regulatory genes *UME4* and *RPD1*.

The yeast *HO* gene has a unique pattern of differential expression. It is expressed in one of the two cells resulting from mitotic division, the mother cell, but it is not expressed in daughter cells (24). *HO* encodes an endonuclease which initiates mating-type interconversion, and the transcriptional regulation of *HO* is sufficient to explain the observed asymmetry in mating-type switching (15, 45). Genetic analysis has identified six genes, *SWI1* to *6*, required for the transcriptional activation of *HO* (4, 42); among these genes, only *SWI5* has been shown to play a role in the mother-daughter asymmetry of *HO* expression (25, 26). The *SWI5* gene encodes a DNA-binding protein that binds *in vitro* to a site in the *HO* promoter (44).

Mutations in the *SIN3* gene (also known as *SDI1*) were isolated as suppressors that allow the expression of *HO* in the absence of the *SWI5* gene product (27, 43). The *sin3* mutation causes a significant change in the pattern of *HO* regulation, with *HO* expression now observed in daughter cells. *SIN3* is required to repress *HO* expression in daughter cells and can therefore be described as a negative transcriptional regulator. In addition, a DNA-binding protein that recognizes a site in the *HO* promoter in the vicinity of the *SWI5* binding site is missing in extracts prepared from *sin3* mutants. It is believed that this *SIN3*-dependent DNA-binding protein acts as a repressor. Finally, we have identified another *SIN3*-dependent DNA-binding protein, REB1, which binds to many yeast promoters (52).

In this report, we characterize the *SIN3* gene in molecular detail. We have determined the sequence of the *SIN3* gene, determined the phenotype of null mutations, and used antisera to determine the intracellular location of the SIN3 protein.

MATERIALS AND METHODS

Cloning of *SIN3*. *Saccharomyces cerevisiae* strain S596-3D (*HO:lacZ swi5Δ::LEU2 sin3-1 ura3-52 MAT***a**) was transformed by the method of Ito et al. (14) with eight pools of a YCp50-based genomic library (kindly supplied by M. Rose). Because the *sin3-1* mutation suppresses the requirement of the *HO:lacZ* promoter for *SWI5*, the parent strain will have high levels of β-galactosidase activity relative to the activity of a similar strain bearing a functional copy of *SIN3*. A total of 10^4 Ura^+ transformants were selected on SD-uracil plates and screened for β-galactosidase production on SD-uracil plates containing 50 mM KPi (pH 7.0) and 600 μg of 5-bromo-4-chloro-3-indolyl-β-D-galactoside (X-Gal) per ml. White colonies were selected and counterscreened for **a**-factor production by a halo assay (10). Plasmid DNA was prepared from transformants that could produce **a**-factor, and the transformants were tested for the ability to complement *sin3-1* when reintroduced into strain S596-3D. One transformant, A4.16, contained a plasmid, pA4.16, which conferred *SWI5* dependence on the *HO:lacZ* promoter when introduced into S596-3D.

γδ insertional mutagenesis. Insertions of the transposon γδ into pA4.16 were obtained by using a method adapted from Guyer (11). Bacterial strain E8037 (F′ *lac* Str^s Amp^s) was transformed with plasmid pA4.16, thus conferring ampicillin resistance. This strain was grown at 37°C to an optical density at 600 nm of 0.7, as was the recipient strain MH6 (F^- *strA* Amp^s). The two strains were mixed in a 1:1 ratio and shaken gently at 37°C for approximately 2 h in a 25-ml tube. Cells were then plated at various dilutions onto LB plates containing ampicillin (100 μg/ml) and streptomycin (250 μg/ml). Colonies resistant to both drugs represent transfers of pA4.16, which encodes ampicillin resistance, to the Str^r MH6 recipient. Such transfer can occur only by γδ-mediated formation of a cointegrate between pA4.16 and the F′ plasmid; the cointegrate spontaneously resolves after transfer, leaving a copy of the γδ transposon in pA4.16. Transconjugants were obtained at frequencies of 10^{-4} to 10^{-5} per donor cell. Plasmid DNA was then prepared from several of these transconjugants, and the positions of the transposed γδ element were mapped by restriction digestion. Insertional derivatives were then introduced into strain

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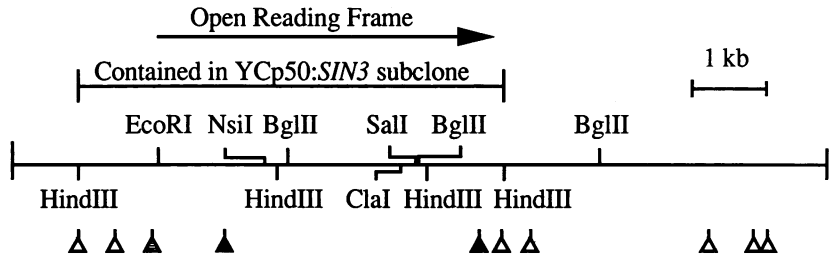


FIG. 1. Restriction map of *SIN3* and γδ insertions. Locations of γδ insertions into the cloned *SIN3* gene are indicated by triangles. These insertional derivatives were introduced into strain S596-3D (*HO:lacZ swi5Δ::LEU2 sin3-1 ura3-52*), and β-galactosidase levels were measured to determine SIN3 activity. Open triangles indicate *SIN3*$^+$ insertions; filled triangles indicate *sin3* insertions. The insertion represented by the shaded triangle had only partial SIN3 function and may be due to insertion within the *SIN3* promoter. The open reading frame derived from the DNA sequence and the region contained in the YCp50:*SIN3* subclone are indicated.

S596-3D (*HO:lacZ swi5Δ::LEU2 sin3-1 ura3-52*) and assayed for the ability to complement *sin3-1* by measuring β-galactosidase activity.

Subcloning of *SIN3*. The 5.8-kb region determined to contain *SIN3* was subcloned into the Bluescript SK(+) vector in three stages. In the first stage, a central 3.6-kb *Eco*RI-*Sal*I fragment of pA4.16 was ligated to the Bluescript vector at the *Eco*RI and *Sal*I sites to create plasmid pIC24, which contains *Bam*HI and *Xho*I sites on the left and right, respectively, in the polylinker. The 1.1-kb *Hin*dIII-*Eco*RI fragment, which is at the left end of the final YCp50 subclone (Fig. 1), was subcloned into pUC18, and the *Hin*dIII site was converted to a *Bam*HI site with a linker. This 1.1-kb *Bam*HI-*Eco*RI fragment was then cloned into *Bam*HI- and *Eco*RI-cleaved pIC24, generating pIC27. In a similar fashion, an *Xho*I site was created adjacent to the *Hin*dIII site at the right end of the final YCp50 subclone, generating the subclone pIC26. The 1.2-kb *Sal*I-*Xho*I insert from pIC26 was then inserted between the *Sal*I and *Xho*I sites of pIC27 to yield pIC28, with the proper orientation of the fragment verified with internal restriction sites. pIC28 contains the *SIN3* gene on a 5.8-kb fragment with *Bam*HI and *Xho*I ends.

To confirm that *SIN3* was entirely contained within the pIC28 insert, the 5.8-kb *Bam*HI-*Sal*I insert was cloned into YCp50 cleaved with *Bam*HI and *Sal*I, to create YCp50:*SIN3*. YCp50:*SIN3* was then introduced into strain S596-3D (*HO:lacZ swi5Δ::LEU2 sin3-1 ura3-52*) and shown to complement *sin3-1*, as measured by β-galactosidase activity.

Other plasmid constructions. YIp*SIN3*L was constructed by cleaving pA4.16 with *Sal*I and *Cla*I, purifying the 5.6-kb fragment, and ligating the fragment into *Sal*I-*Cla*I-digested YIp5. The 5.6-kb fragment extends from the *Sal*I site in YCp50 to the *Cla*I site in the *SIN3* gene. This construction was inserted at the *SIN3* locus, creating a *SIN3*$^+$ gene linked to *URA3*. The *SIN3::LEU2* disruption was made by replacing an 2.0-kb internal *Nsi*I-*Sal*I fragment of *SIN3* with a 3.4-kb *Pst*I-*Xho*I fragment of *LEU2* from plasmid YEp13. The chromosomal copy of *SIN3* was replaced by this disrupted version by the one-step gene replacement method (35). Plasmid YCp50:pGAL-*SIN3* was constructed by first creating a *Bam*HI site just upstream of the initiator ATG codon and then inserting *SIN3* as a 4.8-kb *Bam*HI-*Xho*I fragment into pBM272 (16) cleaved with *Bam*HI and *Sal*I.

Sequencing of *SIN3*. The 5.8-kb insert of pIC28 was cloned into the SK(−) version of the Bluescript vector, generating pIC29. Sets of overlapping, unidirectional deletions were made from pIC28 and pIC29 by using the exonuclease III-S1 nuclease method of Henikoff (12). The sequence was determined by the dideoxy-chain termination method, using Sequenase (U.S. Biochemical Corp.) from double-stranded templates.

Primer extension assays. The experiment to map the 5′ end of the *SIN3* message used a complementary oligonucleotide (5′-GCGGTCAATAGCGTAGCCTGTTGTGGAGGG-3′) labeled with [γ-^{32}P]ATP and polynucleotide kinase (18). This labeled oligonucleotide was hybridized to 40 μg of RNA from strain DY150 (*ho SWI5*$^+$ *SIN3*$^+$) and treated with reverse transcriptase as described previously (20). RNA was prepared as described previously (24). The labeled oligonucleotide was also used as a primer in a DNA sequencing reaction using Sequenase (U.S. Biochemical Corp.) and a pIC28 double-stranded template, but without any labeled deoxynucleoside triphosphates. The primer extension and DNA sequencing reactions were run in adjacent lanes on a 4% polyacrylamide–8 M urea sequencing gel.

The experiment measuring *SIN3* mRNA levels in stationary phase and during the cell cycle used RNA prepared from strain DY150 synchronized by inoculating stationary-phase-arrested cells into fresh medium as described previously (24). RNA (10 μg) was hybridized to each of three oligonucleotides, *SIN3*, *HO* (5′-GGGATCTAACCTACCAGGTT CACC-3′), and *HIS3* (5′-ATCGAGTGCTCTATCGCTAG GGGACCACCC-3′), treated with reverse transcriptase, phenol extracted, ethanol precipitated, and electrophoresed on a 6% polyacrylamide–8 M urea gel.

Antibody to SIN3. A TrpE-SIN3 fusion protein was produced in *Escherichia coli* from a plasmid containing a 1.8-kb internal *Bgl*II fragment from *SIN3* cloned into the pATH2 vector (8). The fusion protein was purified from sodium dodecyl sulfate-gels and used to immunize rabbits. The antiserum to SIN3 was affinity purified on a β-galactosidase–SIN3 affinity column and stored in 10 mM Tris–0.2 M glycine (pH 7.0) containing 1 mg of bovine serum albumin per ml. The β-galactosidase–SIN3 fusion protein was produced in *E. coli* from a plasmid containing a 1.8-kb internal *Bgl*II fragment from *SIN3* cloned into the pUR278 vector (37). The β-galactosidase–SIN3 fusion protein was purified on a *p*-aminobenzyl 1-thio-β-D-galactopyranoside–agarose column (Sigma) and then coupled to Affigel-10 (Bio-Rad). The affinity-purified antiserum is specific for SIN3 on Western immunoblots.

Immunoblot analysis. Protein (150 μg) from strains DY150 (*ho SWI5*$^+$ *SIN3*$^+$) and DY773 (*ho SWI5*$^+$ *sin3::LEU2*) were separated by electrophoresis in a 7.5% polyacrylamide gel and transferred to nitrocellulose filters as described previously (48). Prestained molecular weight standards (Bethesda Research Laboratories, Inc.) were electrophoresed in adjacent lanes. The filters were blocked for 30 min with Tris-

buffered saline containing 3% nonfat dry milk and incubated overnight at 4°C with 1/5,000-diluted affinity purified rabbit polyclonal antibody to TrpE-SIN3 fusion protein. The filter was washed three times in Tris-buffered saline, and the immunoreactive bands were visualized with an alkaline phosphatase-conjugated secondary antibody, nitro blue tetrazolium chloride, and 5-bromo-4-chloro-3-indolylphosphate D-toluidine salt (Bethesda Research Laboratories).

Immunofluorescence. Immunofluorescent staining of yeast cells was performed as described previously (1), with modifications. Cells (strain DY150 with plasmid YCp50:pGAL-*SIN3*) were grown to mid-log phase under selection to maintain the plasmid, treated with formaldehyde for 1.5 h, and then harvested by centrifugation. The cell walls were digested with β-glucuronidase (Sigma) for 2 h at 37°C, washed three times by centrifugation with solution C (35 mM potassium phosphate [pH 6.8], 0.5 mM $MgCl_2$, 1.2 M sorbitol), and suspended in phosphate-buffered saline (PBS). Cell suspensions (30 μl) were placed on polylysine (Sigma)-coated microscope slides and incubated for 15 min at room temperature. The cells affixed to the slides were then fixed first in cold methanol (−20°C) for 7 min and then in cold acetone (−20°C) for 1 min, and finally were allowed to air dry. The slides were blocked with 3% nonfat dry milk in PBS for 30 min at room temperature. A 30- to 50-μl sample of affinity-purified anti-SIN3 antibody (50 μg/ml) was applied, and the sample was incubated overnight in a moist chamber at 4°C. The slides were washed three times in PBS for 10 min to remove unbound primary antibody, and the excess liquid was removed by blotting the edge of the slide with a Kimwipe. A 30- to 50-μl sample of secondary antibody (rhodamine-conjugated goat anti-rabbit immunoglobulin G; Calbiochem) was applied, and the samples were incubated for 4 h at 4°C in the dark. The slides were then washed twice in PBS, stained with 4,6-diamidino-2-phenylindole (DAPI) (0.5 μg/ml; Sigma) in PBS for 10 min, and then washed again in PBS. The excess liquid was removed, and 10 μl of 50% glycerol containing a 1-mg/ml concentration of *p*-phenylenediamine (Sigma) was placed on top of the samples. After being covered and sealed with cover slips, the samples were examined with a fluorescent microscope. Photographs were taken with Kodak Tri-X Pan film, using appropriate filters for rhodamine antibody staining and for DAPI staining.

Computer analysis. Primary sequence data were assembled by using the Intelligenetics GEL program from the BIONET computer resource. The CHARGPRO, PESTFIND, and PROSITE programs from the PC/GENE package (Intelligenetics) were used to determine the isoelectric point, to search for PEST sequences, and to search for specific protein signatures (glycosylation sites, kinase domains, etc.), respectively. The DNA Strider program (19) was used to determine the codon adaptation index. GenBank release 64.0, EMBL release 23.0, SWISS-PROT release 14.0, and GenPept release 63.0 were searched with the FASTA program (30). The paired amphipathic helix (PAH) motifs were identified by visual inspection.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the EMBL, GenBank, and DDBJ nucleotide sequence data bases under accession number M36822.

RESULTS

Cloning of the *SIN3* gene. We cloned the *SIN3* gene from a genomic library in the centromeric shuttle vector YCp50. A *MAT***a** HO:*lacZ swi5*Δ::*LEU2 sin3-1 ura3-52* strain (which forms blue colonies on X-Gal) was transformed with the library, and approximately 10^4 transformants were screened for white colonies by replica plating onto medium containing X-Gal. We reasoned that white colonies would result from plasmids containing the *SIN3* gene or the *MAT*α locus. Presence of the *SIN3* gene would render the *HO* promoter dependent on *SWI5*, which is defective in the recipient strain. A plasmid containing the *MAT*α locus would lead to production of the **a**1-α2 repressor, and *HO* expression would be blocked. We eliminated white colonies that contained the *MAT*α locus because they do not produce **a**-factor. Plasmid DNA was prepared from the remaining white transformants and reintroduced into the parent strain. One transformant, A4.16, contained a plasmid, pA4.16, that yielded white transformants when reintroduced into the parent strain (Table 1).

TABLE 1. Complementation of a *sin3* mutation by plasmid-borne *SIN3*[a]

Genotype	β-Galactosidase activity[b]	
	Control plasmid	pA4.16 (*SIN3*)
HO:lacZ SWI5 SIN3	100	114
HO:lacZ swi5 SIN3	<2	ND
HO:lacZ swi5 sin3-2	63	6.5

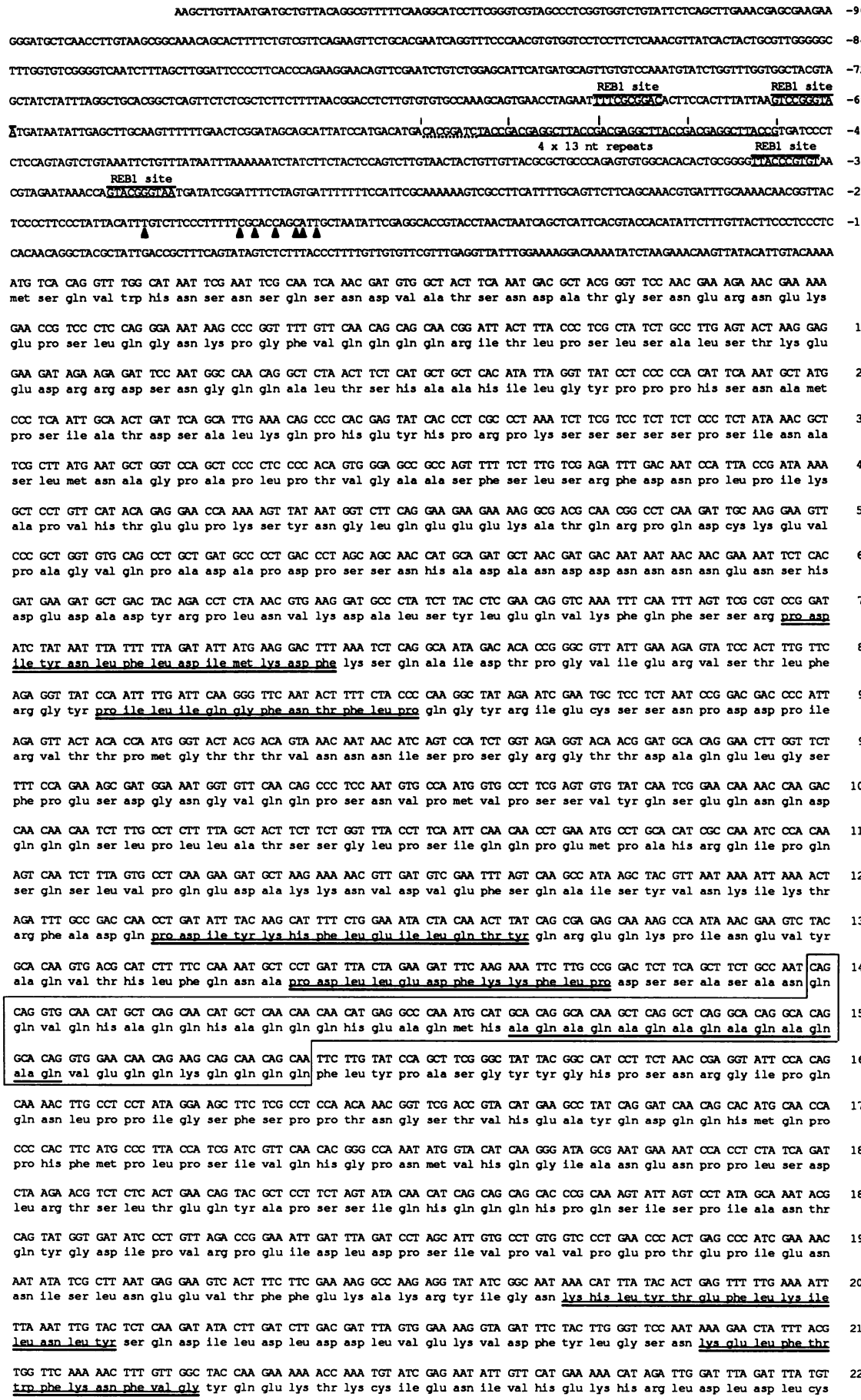
[a] Strains of indicated genotype containing either the control plasmid (p*ADE3*) or the plasmid carrying *SIN3* (pA4.16) were assayed for β-galactosidase as described in Materials and Methods. The *swi5* mutations were *swi5*::*LEU2*.

[b] Normalized to the value for the wild-type strain with a control plasmid. ND, Not done.

To prove that we had indeed cloned the *SIN3* gene, we targeted integration of a YIp5 subclone of pA4.16 to the homologous region of the chromosome. Specifically, YIp*SIN3*L was linearized with *Bgl*II and introduced into strain D246-2B (*HO:lacZ swi5*Δ::*LEU2 SIN3*$^+$). Integration of this plasmid into the chromosome does not inactivate *SIN3*. This URA$^+$ white transformant was then crossed to a *sin3* strain, S488-10D (*HO:lacZ swi5*Δ::*LEU2 sin3-1 ura3-52*), and progeny were scored for *URA3* and β-galactosidase activity (as a marker for *SIN3*). All of the 15 tetrads were parental ditypes, and no recombinants were observed among 64 total segregants. Thus, pA4.16 contains the *SIN3* gene.

Localization of the *SIN3* gene on pA4.16 was accomplished by constructing a number of deletion derivatives, as well as insertional derivatives with the γδ transposon (see Materials and Methods). These derivatives were then introduced into an *HO:lacZ swi5*Δ::*LEU2 sin3-1* strain (S596-3D) and assayed for β-galactosidase activity to assess complementation of *sin3-1*. This analysis showed that the *SIN3* gene resided within a 5.8-kb region of pA4.16 (Fig. 1). This 5.8-kb region was subsequently subcloned into YCp50 and shown to complement *sin3-1*.

Disruption of the *SIN3* gene. To determine the phenotype of a null mutation at the *SIN3* locus, we disrupted the chromosomal copy of *SIN3*. A *sin3*::*LEU2* disruption was created by removing a 1.8-kb internal fragment of *SIN3* and replacing it with the yeast *LEU2* gene. The wild-type chromosomal *SIN3* gene was replaced with this construction by integrative transformation, and the structure of the gene replacement was confirmed by Southern blot analysis. A haploid yeast bearing the *sin3*::*LEU2* disruption was viable and showed the same quantitative level of *HO* expression in a *swi5* mutant as did the *sin3* missense alleles (data not shown).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4 x 13 nt repeats
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TTG GAC AGC TCT ACT GCA AGT GTA CTG CCT GCA TCC ATT ACT ACT GTA CCA CAA GAT GAT AAT ATA GAA ACG ACT GGG AAT ACT GAA TCT  4590
leu asp ser ser thr ala ser val leu pro ala ser ile thr thr val pro gln asp asp asn ile glu thr thr gly asn thr glu ser

TCA GAC AAG GGG GCT AAG ATT CAA TAA GTAGTTTTAACTAGATTTAGAACAAAAATCTTTAGTACGACAGGGTCTTTCTTCTGATGTGTACCTTTAATTAATGTCAACGAT  4680
ser asp lys gly ala lys ile gln ***

ATAACATTGTAATTTTAAAATTCTAAGTGTTTTTGAAGCTT                                                                              4721
```

FIG. 2. Sequence of the *SIN3* gene. The DNA sequence of the 5.8-kb region containing *SIN3* and flanking regions is shown, with +1 at the initiator ATG codon. In the promoter region, the four REB1-binding sites are indicated, as are the 13-nt repeats. The 5′ ends of mRNA transcripts determined by primer extension are shown by arrowheads. In the coding region, the glutamine-rich region is boxed, and the alanine-glutamine repeats are underlined. The amphipathic helices of the PAH repeats are indicated by double underline. Potential splicing signals are indicated with a dotted underline. The consensus transcription termination signal is underlined.

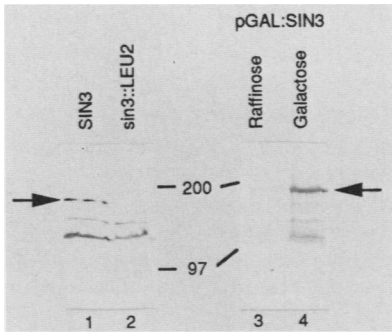


FIG. 3. Immunoblot with anti-SIN3 antibody. Lanes: 1, protein from strain DY150 (*ho SIN3 ura3-52*); 2, protein from the isogenic strain DY773 (*ho sin3::LEU2 ura3-52*); 3, protein from strain DY150 with the plasmid YCp50:pGAL-*SIN3* grown on raffinose; 4, protein from strain DY150 with the plasmid YCp50:pGAL-*SIN3* grown on galactose. The two blots were developed for different periods of time.

Sequence of the *SIN3* gene. Figure 2 shows the sequence of the 5.8-kb fragment that contains the *SIN3* gene. One large open reading frame of 4,614 nucleotides (nt) is seen, which could encode a protein of 1,538 amino acids. Another open reading frame starts at nt 300, extending in the opposite direction and continues beyond the sequenced region. Two observations indicate that the 4,614-nt open reading frame encodes *SIN3*. First, γδ disruptions within this open reading frame led to a *sin3* phenotype. In addition, we constructed a plasmid, YCp50:pGAL-*SIN3*, which directs the synthesis of the 175-kDa open reading frame under the control of the inducible *GAL1* promoter. This plasmid was transformed into strain DY768 (*HO:lacZ swi5Δ::hisG sin3Δ::LEU2*), and *HO* promoter activity was determined by measuring β-galactosidase levels. This strain was phenotypically *SIN3*$^+$ on galactose medium and *sin3* on glucose medium.

The SIN3 protein was identified on immunoblots with antibody to SIN3 (Fig. 3). A TrpE-SIN3 fusion protein was produced in *E. coli* with a pATH vector and used to immunize rabbits, and the antibody was affinity purified. The antibody recognizes several proteins on immunoblots, but only a protein of approximately 170 kDa is absent in an isogenic strain bearing the *SIN3::LEU2* gene disruption. An increase in the quantity of the 170-kDa band was seen when SIN3 was overproduced from the *GAL1* promoter (Fig. 3).

The predicted amino acid sequence of the SIN3 protein indicates that it is a large polypeptide, 174,930 Da. The protein is acidic, with a predicted isoelectric point of 5.23. The amino acid sequence NX(S/T), which is a potential site for glycosylation (17), appears eight times in SIN3. PEST regions, rich in P, E, S, and T residues, are proposed to signal proteins for rapid degradation (34). SIN3 contains two regions with significant PEST scores, amino acids 1089 to 1113 and 1495 to 1532, which have PEST scores of 16.7 and 11.3, respectively. Yeast genes that are highly expressed usually have a biased codon usage (3). The calculated codon adaptation index (40) of the *SIN3* open reading frame is 0.150, indicative of a protein that is not highly expressed in the cell. No obvious DNA-binding motifs (helix-turn-helix, homeo domain, zinc finger, or leucine zipper), kinase domains, membrane-spanning regions, or ATP-binding sites were identified in the SIN3 sequence.

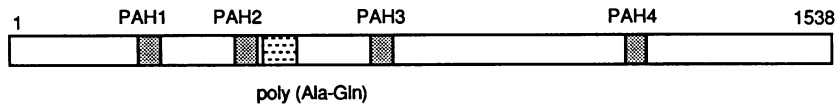
The predicted amino acid sequence of SIN3 contains four copies of an internal amino acid repeat (Fig. 4). The repeat consists of two amphipathic helices separated by a spacer of about 20 amino acids. Several recent reports have described similar motifs consisting of two amphipathic helices separated by a spacer or loop, and it has been suggested that this paired amphipathic helix (PAH) motif is involved in protein-protein interactions. The TPR repeat, which has two amphipathic helices separated by six amino acids, has been found in the *S. cerevisiae CDC16*, *CDC23*, *SKI3*, and *SSN6* genes and the *Schizosaccharomyces pombe nuc2* gene (13, 41). Members of the helix-loop-helix (HLH) (*myc*) family of proteins contain two linked amphipathic helices separated by 10 to 24 amino acids, and this region of the protein is crucial for dimerization (7, 22). The repeat structure observed in SIN3 has the same overall structure (two putative amphipathic alpha-helical regions separated by a spacer) but does not share amino acid similarity with the TPR or HLH (*myc*) repeats.

A search was made for possible splicing signals in the *SIN3* gene. The highly conserved sequence TACTAACA is present at the branch site in yeast intervening sequences, with the underlined adenosine forming the branch point (53). Upstream 200 to 400 nt from the TACTAACA branch point is the 5′ splice site, with the conserved sequence GTAPyGT, and the 3′ splice site PyAG usually is 20 to 40 nt downstream. The sequence TACTAACA can be found in the *SIN3* gene at nt 4248 to 4255, and 5′ (GCATGT; nt 3936 to 3941) and 3′ (CAG; nt 4303 to 4305) splice sites are also present (Fig. 2). Although the 5′ splice site (GCATGT) deviates by 1 nt from the consensus GTAPyGT, it has been demonstrated that the yeast *COX5B* gene, which contains the same atypical splice site, is spliced in vivo. We do not expect this potential intron to be removed from *SIN3* for two reasons, although we have not established this fact directly. First, intron removal would lead to a change in reading frame, and a stop codon occurs three codons after the 3′ splice site. Translation of a spliced message would produce a truncated protein of 1,316 amino acids (150 kDa). Second, although the anti-SIN3 antibody detects several species on Western blots, only the 170 kDa species is absent in the *SIN3* disruption strain. Yeast intervening sequences are located very close to the 5′ end of the gene, usually before codon 20 (53), whereas this potential *SIN3* intervening sequence is some 4,000 nt from the ATG codon. It is tempting to speculate that these splicing signals might operate efficiently if present at the 5′ end of a transcription unit.

Genes similar to *SIN3*. Computer searches of protein and nucleic acid data bases found significant matches to only one region of *SIN3*, the glutamine-rich segment indicated in Fig. 2. Polyglutamine runs have been identified in a number of genes encoding mammalian or *Drosophila* transcription factors (see reference 21 for a review). Yeast regulatory genes with polyglutamine runs include *PHO2* (39), *HAP1* (31), *HAP2* (32), *ADR6* (29), *SSN6* (38, 49), and *GAL11* (47).

SIN3 also contains a run of alternating alanine and glutamine residues. Three genes were identified with poly(Ala-Gln) runs: the *Drosophila* zeste gene (33) and the yeast genes *SSN6* (38, 49) and *GAL11* (47). In addition to containing the poly(Ala-Glu) runs, each of these genes contains polyglutamine runs. The zeste gene is involved in the regulation of the white gene, can be an activator or a repressor, and is involved in the phenomenon of interchromosomal complementation known as transvection (54). *SSN6* and *GAL11* mutants are pleiotropic. *SSN6* (also known as *CYC8*) mutations were identified as mutations that overcame the glucose repression of iso-2-cytochrome *c* (36) and of invertase (5). *GAL11* (also known as *SPT13*) is involved in regulating the galactose-metabolizing enzymes (28, 47).

A.



B.

```
                HELIX A                              HELIX B
             12345678901234                       123456789012
HLH1 FSSRPDIYNLFLDIMKDFKSQAIDTPGVIE--RVSTLFRGYPILIQGFNTFLPQGYR
HLH2 FADQPDIYKHFLEILQTYQREQKPINEVYA--QVTHLFQNAPDLLEDFKKFLPDSSA
HLH3 YIGNKHLYTEFLKILNLYSQDILDLDDLVE--KVDFYLGSNKELFTWFKNFVGYQEK
HLH4 FRHWTTIYERLLEIKQMNERVTKEINTRSTVTFAKDLDLLSSQLSEMGLDFVGEDAY
        IY  FL I*  *                         L*  F  F*
```

C.

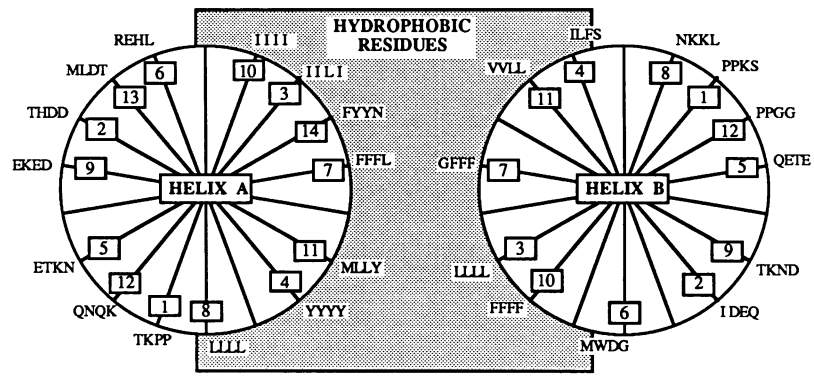


FIG. 4. PAH motifs in the *SIN3* gene. (A) Map of the *SIN3* gene showing positions of the PAH motifs and the poly(Ala-Gln) region. (B) Amino acids, in one-letter code, present in each of the PAH motifs. The numbers correspond to the positions in panel C. Conserved amino acids are shown below the numbers, with * indicating nonidentical hydrophobic residues. (C) Amino acid sequences of helix A and helix B displayed on Edmundson helical wheels. The sequence from PAH1 is closest to the wheel. The sequences for PAH2, PAH3, and PAH4 are in subsequent positions. Note the strong conservation of hydrophobic residues, which are on one side of each helix.

It is intriguing to compare the phenotypes of *SIN3* mutants with those of *SSN6* and *GAL11* mutants. First, mutations in each of these genes permit inappropriate expression of certain promoters. *SIN3* was identified as a bypass mutant, suppressing the *SWI5* requirement for *HO* activation. *SSN6/CYC8* also acts as a bypass mutant, suppressing the *SNF1* requirement for *SUC2* activation (5). Insertion of a Ty element in the promoter of the *HIS4* or *LYS2* genes prevents expression of these genes, but a mutation in *GAL11/SPT13* permits expression of these promoters (9). In addition, *SIN3*, *SSN6*, and *GAL11* mutants are all sporulation defective when homozygous diploid.

A comparison of unpublished restriction map and sequence data has revealed that *SIN3* is the same gene as *UME4* and *RPD1* (M. Goebl, personal communication; R. Strich and R. E. Esposito, personal communication; M. Vidal and R. Gaber, personal communication). The *UME4* gene was identified by a mutation that permitted inappropriate expression of the *SPO13* gene (46). *RPD1* was identified as a mutation that increased expression of the *TRK2* gene (50). Vidal et al. (50) have mapped *RPD1* to 0.2 centimorgans from the *PHO80* gene on chromosome XV, very close to the centromere. We have observed that sporulation of a *SIN3/sin3 TRP1/trp1* diploid yielded 10 parental ditype: 9 nonparental ditype: 0 tetratype asci, also indicating that *SIN3* is centromere linked.

Transcriptional mapping. The 5′ end of the *SIN3* gene was mapped by primer extension (Fig. 5) and RNase protection experiments (data not shown). The primer extension experiments demonstrate the existence of multiple 5′ ends for the *SIN3* transcript, with starts at −221 to −197 from the initiator ATG codon (Fig. 2). This is a relatively long 5′ untranslated region for a yeast gene (6). The consensus element TAG. . .TAGT. . .TTT has been proposed as a key element in transcriptional termination (55). This sequence element is present 3′ to the *SIN3* termination codon (Fig. 2).

There are several noteworthy features in the DNA sequence 5′ to the coding region. A 13-nt sequence is tandemly repeated four times from nt −540 to −488. Whereas the first repeat is inexact, the others contain the identical sequence ACGAGGCTTACCG. The promoter also contains one weak and three strong binding sites for the REB1 DNA-binding protein (52). The binding sites are in pairs, and the relative positions and orientations of the sites are conserved. The binding sites in each pair are in opposite orientations, with exactly the same spacing between the binding sites for each pair.

***SIN3* is not expressed in stationary-phase cells.** An experi-

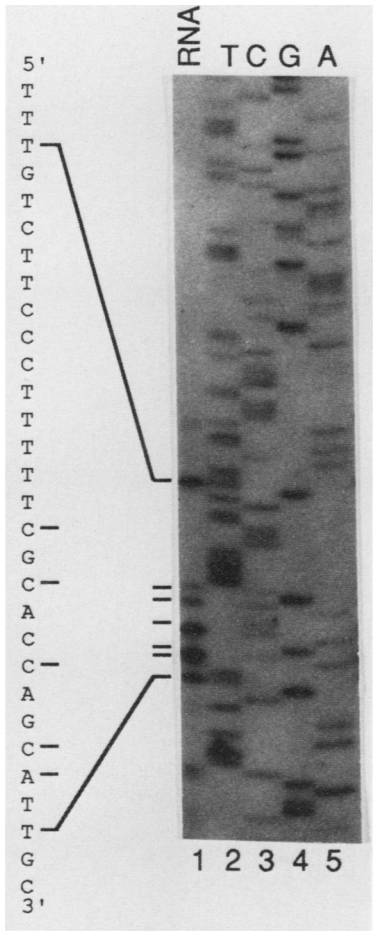


FIG. 5. Primer extension mapping of *SIN3* mRNA. Lanes: 1, primer extension products; 2 to 5, dideoxy sequencing reactions using the labeled primer and dATP, dGTP, dCTP, and dTTP, respectively. The sequencing lanes are labeled with the complementary nucleotides so that the sequence corresponds to the strand shown in Fig. 2.

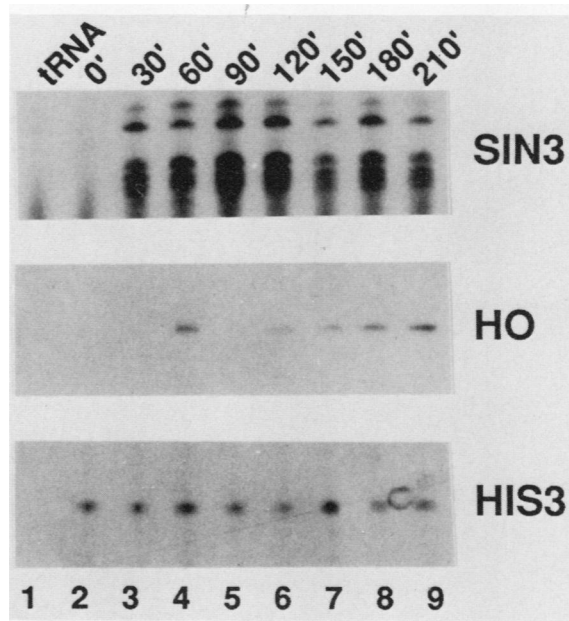


FIG. 6. Absence of *SIN3* mRNA in stationary-phase cells. Primer extension reactions were performed with RNA as follows: lane 1, tRNA only; lane 2, RNA from stationary-phase cells; and lanes 3 to 9, RNA from stationary-phase cells grown for the indicated times (in minutes) in fresh medium. Primers were used that are complementary to *SIN3*, *HO*, and *HIS3* mRNAs.

ment was performed to determine whether *SIN3* expression is cell cycle regulated. Cells were synchronized by inoculating stationary-phase-arrested cells into fresh medium (24). At various times, cells were harvested and RNA was prepared for primer extension analysis with probes for *SIN3*, *HO*, and *HIS3* (Fig. 6). Although *HO* transcript levels changed during the cell cycle, we found no evidence that *SIN3* was differentially expressed during the cell cycle. We did note that the *SIN3* transcript was absent in stationary-phase cells, whereas the *HIS3* control transcript was still present. This observation is of interest since *SIN3* (=*UME4*) is a negative regulator of *SPO13*, a sporulation-specific gene (46).

***SIN3* is present in the nucleus.** Immunofluorescence was used to determine the subcellular localization of SIN3, using antibody prepared against SIN3. Yeast cells were grown to mid-log phase, fixed with formaldehyde, and prepared for immunofluorescence with affinity-purified antibody to SIN3. Faint nuclear staining was seen in wild-type cells, and no staining was seen in strains bearing a *SIN3* chromosomal disruption (data not shown). When SIN3 was overproduced from the *GAL1* promoter, bright immunofluorescent staining was seen (Fig. 7). Comparison with the DAPI staining pattern indicates that SIN3 is present in the nucleus. We noted that not all cells were stained with antibody. We believe that this failure to stain is attributable to the fact some cells were not permeabilized, since a similar fraction of cells was stained with an anti-β-galactosidase antibody. Importantly for the asymmetric expression of *HO*, immunofluorescent staining of SIN3 protein was approximately equal in mother and daughter cells, whether overproduced from the *GAL1* promoter or produced in low quantity from its own promoter.

DISCUSSION

The yeast *SIN3* gene was identified as a negative regulator of the *HO* gene. *HO* is normally expressed only in mother cells, and the *SWI5* activator is required for expression. In a *sin3* mutant, however, *HO* is expressed in mother and daughter cells, and expression occurs in the absence of the *SWI5* gene product. We have cloned and sequenced the *SIN3* gene and determined that the 175-kDa *SIN3* gene product is present in the nucleus.

The SIN3 protein contains four repeats of a motif whose structure is like that of the helix-loop-helix (HLH) and TPR motifs. These repeats consist of paired amphipathic helix (PAH) motifs separated by a 10- to 30-amino-acid segment that is thought to form a loop. The HLH proteins are a set of DNA-binding proteins related to *myc* (22), which bind to DNA as either hetero- or homodimers and contain a conserved basic region N terminal to the PAH motif. It is believed that the basic region is involved in DNA binding and that the PAH domain is involved in dimerization, analogous to leucine zipper proteins (7, 22, 23, 51). The TPR repeat (approximately 34 residues in length) was recently identified in several *S. cerevisiae* and *Schizosaccharomyces pombe* genes, and it has been proposed that this motif mediates protein-protein interactions (13, 41). The TPR

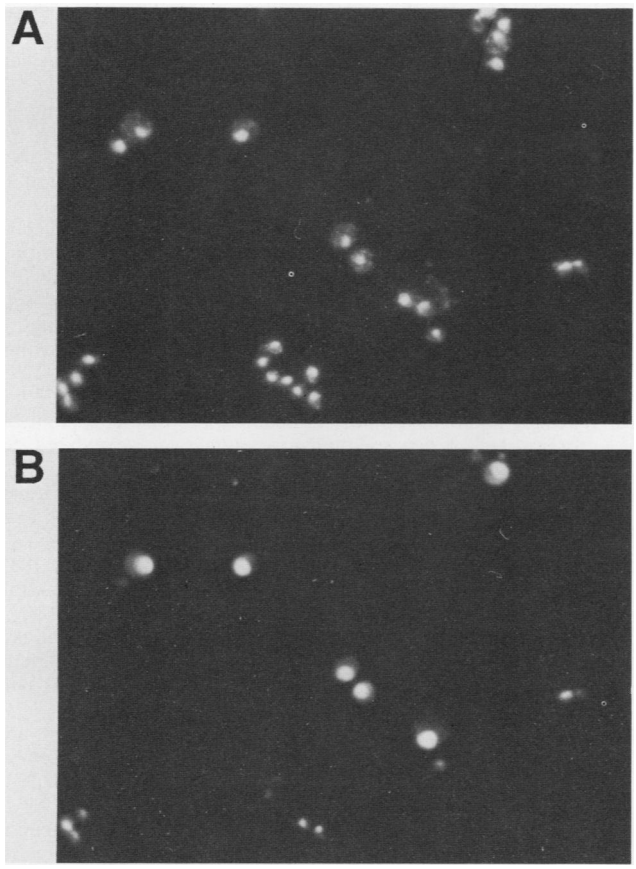


FIG. 7. Immunolocalization of SIN3 protein. (A) DAPI staining of nuclei; (B) fluorescence staining with antibody to SIN3.

proteins are like SIN3 in that they contain multiple PAH domains and lack a linked basic region, whereas the *myc* family proteins contain a single PAH. There is strong conservation of the hydrophobic residues in the amphipathic helices within each of the three PAH families, the *SIN3*, TPR, and HLH (*myc*) families. Although these three families all contain similar amphipathic helix motifs, the hydrophobic residues are not conserved among them. It is tempting to speculate that these hydrophobic residues provide the specificity in protein-protein interactions.

Some recent biochemical observations on a *SIN3*-dependent DNA-binding activity could be nicely explained by our observations on *SIN3*. A protein, called SDP1, that binds to the *HO* promoter is absent in extracts prepared from *SIN3* mutants (27). (We postulate that SDP1 functions as a repressor.) *SIN3* does not encode SDP1 but rather regulates the in vitro DNA-binding activity of SDP1 by protein-protein interactions involving an additional protein called I-SDP1 (H. Wang and D. J. Stillman, submitted for publication). In vitro experiments indicate that the I-SDP1 protein inhibits the DNA-binding activity of SDP1 and that SIN3 overcomes this inhibitory effect. If these interactions occur in vivo, then the levels of free SDP1 would be reduced in a *sin3* mutant, and repression would be decreased. Perhaps the PAH repeats observed in SIN3 mediate interaction with I-SDP1 and thus ultimately govern the activity of SDP1. The gene coding for I-SDP1 has not been identified, but it would be striking if it contains a PAH repeat. The function of SIN3 could thus be analogous in some respects to that of the Id protein (2), which contains a PAH of the HLH (*myc*) family but lacks a basic region. Id can form heterodimers with HLH proteins, but these heterodimers do not bind to DNA.

Finally, it recently has been discovered that *SIN3* affects transcription of several other genes. *SIN3* is the same gene as *UME4* and *RPD1*, which were identified as genes regulating transcription of *SPO13* and *TRK2*, respectively (46, 50). Interestingly, *SIN3* functions at each of these genes as a negative regulator. *SIN3* has also been shown to affect the transcriptional level of a number of other yeast genes, including *RME1*, *PHO5*, and *BAR1* (Strich and Esposito, personal communication; Vidal and Gaber, personal communication). It remains to be seen whether *SIN3*-dependent regulation of DNA-binding proteins mediates the transcriptional regulation of these various genes.

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