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In vitro regulation of a *SIN3*-dependent DNA-binding activity by stimulatory and inhibitory factors

(transcriptional regulation/protein–protein interactions/formamide/paired amphipathic helices)

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ABSTRACT **The yeast *SIN3* gene (also known as *SDI1*) is a known negative regulator of the yeast *HO* gene. A DNA-binding activity, called SDP1, which binds to the *HO* promoter, is absent in extracts prepared from *sin3* mutants and has been proposed to function as a repressor. We show that *SIN3* does not encode SDP1 and that SDP1 DNA-binding activity is modulated *in vitro* by two factors, an inhibitory factor, I-SDP1, and a stimulatory factor, S-SDP1. I-SDP1 acts as an *in vitro* inhibitor of the SDP1 DNA-binding activity. Restoration of the DNA-binding activity is achieved by inclusion of a stimulatory factor, S-SDP1, which copurifies with the SIN3 protein. SDP1 DNA-binding activity was restored by treating a protein fraction containing SDP1 and I-SDP1 with the dissociating agent formamide.**

SIN3 (also known as *SDI1*) was first identified as a suppressor mutation that permits *HO* expression in a *swi5* mutant strain (1, 2). The yeast *HO* gene encodes an endonuclease that initiates mating-type interconversion (3, 4). *HO* is expressed in mother cells but not in daughters (5), leading to an asymmetry observed in mating-type switching: only mothers can switch (6). *SWI5* encodes a transcriptional activator that recognizes a site in the *HO* promoter and plays a major role in determining the mother/daughter asymmetry of *HO* expression (7, 8). In a *swi5* mutant strain, *HO* expression is reduced at least 20-fold. A mutation in the *SIN3* gene overcomes the requirement for *SWI5*, and *HO* expression in a *swi5 sin3* mutant is restored to 30% of the wild-type level. Two additional phenotypes of the *sin3* mutation are significant. First, the *sin3* mutation alters the mother/daughter regulation of *HO* expression. *HO* is expressed in daughters, and daughters can switch mating types. Second, a DNA-binding protein is missing in extracts produced from *sin3* mutants (1). This protein recognizes a site in the *HO* promoter adjacent to the SWI5-binding site.

We believe that this *SIN3*-dependent DNA-binding protein, which we call SDP1, functions as a repressor in daughter cells. It is known that SWI5 is a transcriptional activator (7). The fact that the *sin3* mutation overcomes the requirement for the SWI5 activator implies that *SIN3* negatively regulates *HO*. A simple model proposes that, in the absence of a repressor, the activator is no longer needed. We believe that SDP1, which binds to *HO* close to the SWI5-binding site, is that repressor. SDP1 is absent in *sin3* mutants, and *HO* is therefore expressed even in the absence of SWI5. It is believed that SWI5 is absent in daughters and that this determines the differential expression of *HO* in mothers and daughters (8, 9). The *sin3* mutation permits *HO* expression in daughter cells, then, because the absence of the repressor eliminates the requirement for the SWI5 activator.

It has been demonstrated recently that *SIN3* transcriptionally regulates other yeast genes in addition to *HO*. A comparison of restriction maps and sequence data has revealed that *SIN3* is the same gene as *UME4* and *RPD1* (ref. 10; M. Vidal, R. Strich, R. E. Esposito, and R. F. Gaber, personal communication). Strich *et al.* (11) identified *UME4* as a negative regulator of *SPO13*, and Vidal *et al.* (12) identified *RPD1* as a negative regulator of *TRK2*. Recent work indicates that *SIN3* (*SDI1* = *UME4* = *RPD1*) may be involved in the regulation of a host of yeast genes (M. Vidal, R. Strich, R. E. Esposito, and R. F. Gaber, personal communication).

METHODS

Bandshift Assays. Labeled double-stranded DNA probes were prepared from complementary oligonucleotides containing the SDP1 binding site (TCGACTGCCGGTGCCTGCGATGAGATACG and TCGACGTATCTCATCGCAGGCACCGGCAG) synthesized on an Applied Biosystems synthesizer. The oligonucleotides were labeled and used in gel-retardation assays as described (13).

Yeast Protein Extracts and Fractionation. Yeast extracts were prepared from strain DY699 (*MATa*, *leu2*, *trp1Δ63*, *ura3-52*, *pep4-3*, *prb1-1133*, *prc1-403*) and dialyzed into AN100 buffer [10% (vol/vol) glycerol/20 mM Tris·HCl, pH 8.0/0.5 mM EDTA/0.5 mM dithiothreitol/100 mM NaCl] as described (13). All chromatography steps were carried out at 4°C on an FPLC apparatus (Pharmacia). One hundred fifty milligrams of protein was loaded onto a 50-ml heparin-Sepharose (BRL) column equilibrated in AN100 buffer, and proteins were eluted from the column with a linear NaCl gradient (0.1–1.0 M). Fractions were collected, dialyzed into AN100 buffer, and then assayed for SDP1 DNA-binding activity with a bandshift assay and for SIN3 by immunoblotting. Fractions containing SDP1 activity, which was eluted from the column at about 0.3 M NaCl, were pooled and chromatographed on a 20-ml native salmon sperm DNA-cellulose column. Protein was eluted from the column with a linear NaCl gradient, and fractions containing SDP1 DNA-binding activity (eluting at about 0.15 M NaCl) were pooled and dialyzed (fraction iii). The DNA-cellulose column flowthrough was loaded on a 1-ml FPLC Mono S column, which was eluted with a linear NaCl gradient. The flowthrough fraction contained an inhibitory factor, I-SDP1 (fraction iv), while a stimulatory activity, S-SDP1 (fraction v), is a pool of fractions eluting from the column at about 0.45 M NaCl. It should be noted that different protein preparations were used in different figures, and since the specific activities are not the same, quantitative comparisons cannot be made between experiments.

Formamide Treatment. Four microliters of the DNA-cellulose fraction containing SDP1 activity was mixed with 1

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Abbreviations: PAH, paired amphipathic helices; HLH, helix–loop–helix; TPR, tetratricopeptide.

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μl of deionized formamide and incubated for 15 min on ice. This was then added to a reaction mixture containing labeled DNA and poly(dI-dC)·poly(dI-dC), which was incubated and electrophoresed as described (13).

Immunoblot Analysis. SIN3 protein was detected on blots with an affinity-purified rabbit polyclonal antibody to a TrpE–SIN3 fusion protein as described (13).

RESULTS

***SIN3* Does Not Encode the *SIN3*-Dependent DNA-Binding Protein SDP1.** We previously have identified a protein that binds to the *HO* promoter and demonstrated that this protein, SDP1, is absent in extracts prepared from *sin3* mutants (1). We have now fractionated yeast proteins on a heparin-Sepharose column and assayed for SDP1 DNA-binding activity with a bandshift assay. Immunoblot analysis with anti-SIN3 polyclonal antibody revealed that the heparin-Sepharose column fractions with SDP1 DNA-binding activity also contain SIN3 protein (data not shown). The SDP1 and SIN3 peaks are not coincident: there are fractions containing SIN3 protein without detectable SDP1 activity. The fractions with the SDP1 DNA-binding activity were pooled and run on a DNA-cellulose column. As shown in Fig. 1, the SDP1 activity was retained on the column (fraction iii). The SIN3 protein, however, flowed through the DNA-cellulose column. Therefore SDP1 is not encoded by *SIN3*.

Two additional activities that influence the SDP1 DNA-binding activity were discovered (see below). The DNA-cellulose column flowthrough was fractionated on a Mono S column. Fraction v, eluted from Mono S, contains a stimulatory activity, called S-SDP1. Fraction iv, the Mono S column flowthrough, contains an inhibitory activity, I-SDP1. Fractions iii, iv, and v all derive from the same heparin-Sepharose column pool.

A Stimulator of the SDP1 DNA-Binding Activity. The DNA-cellulose flowthrough, which contains SIN3, was chromatographed on a Mono S column. The immunoblot shows that SIN3 was retained on the column and that SIN3 was present in fractions 12–16, with maximal antigen in fraction 14 (Fig. 2*A*). The Mono S fractions containing SIN3 can stimulate the SDP1 DNA-binding activity. When a small quantity of fraction iii from the DNA-cellulose column, containing SDP1, was used in a bandshift assay, weak DNA-binding activity was seen (Fig. 2*B*, lane 1). However, when Mono S fractions were mixed with SDP1, a marked stimulation of SDP1 activity was seen (Fig. 2*B*, lanes 6–9). These fractions do not contain a DNA-binding activity in the bandshift assay when assayed without fraction iii (Fig. 2*B*, lanes 2–5). The stimulatory activity is present in fractions 12–16 (fraction v), with a peak in fraction 14. This corresponds closely to the fractions that contain SIN3, as determined immunologically, and leads to the suggestion that *SIN3* either encodes S-SDP1 or interacts with it.

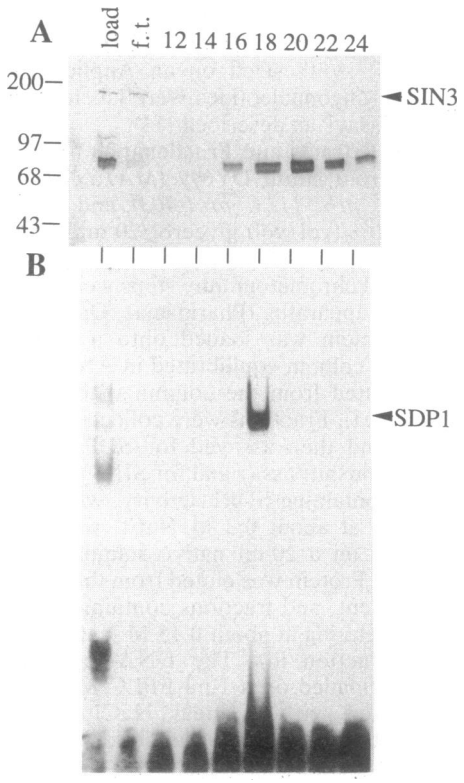

FIG. 1. DNA-cellulose column chromatography. (*A*) Immunoblot. DNA-cellulose column fractions were probed with anti-SIN3 antibody. Numbers indicate column fractions. Protein markers (in kDa) are indicated. The arrow indicates the position of the 175-kDa SIN3 protein. The ≈80-kDa cross-reactive protein species is not a breakdown product of SIN3 since it is present in extracts prepared from strains bearing a *SIN3* gene disruption. f.t., Flowthrough. (*B*) Bandshift assay. DNA-cellulose column fractions were assayed for SDP1 DNA-binding activity with a bandshift assay. Numbers indicate column fractions. The arrow indicates position of the SDP1 protein–DNA complex.

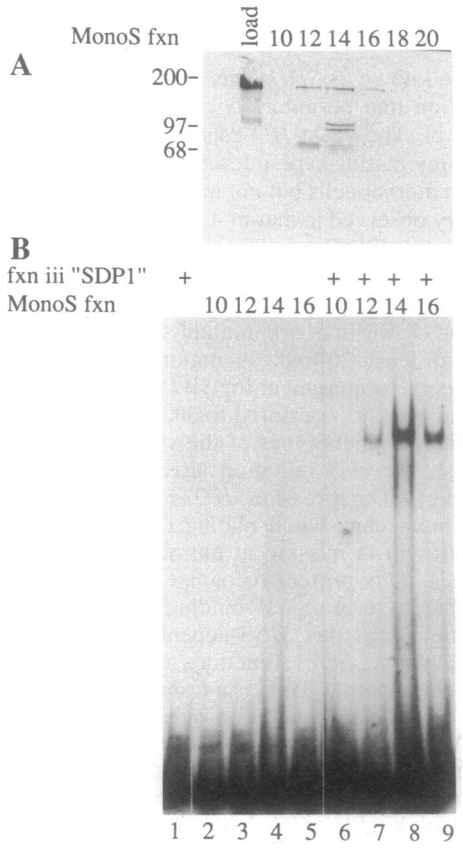

FIG. 2. Identification of stimulatory activity in Mono S column fractions. (*A*) Immunoblot of Mono S column fractions with anti-SIN3 antibody. The ≈80-kDa cross-reactive protein species seen in fractions 12 and 14 is not a breakdown product of SIN3 since it is present in extracts prepared from strains bearing a *SIN3* gene disruption. The ≈100-kDa species in fractions 14 and 16 may be a SIN3 breakdown product. Molecular markers (in kDa) are indicated. (*B*) Bandshift assay. Lane 1, SDP1 DNA-binding activity (fraction iii from DNA-cellulose column); lanes 2–5, Mono S column fractions 10, 12, 14, and 16; lanes 6–9, SDP1 DNA-binding activity (fraction iii) and Mono S column fractions 10, 12, 14, and 16. fxn, Fraction.

Fraction iii, containing SDP1, was incubated with increasing amounts of the S-SDP1 stimulatory activity from the Mono S column (Fig. 3, lanes 1–5). The SDP1 DNA-binding activity was stimulated in a dose-dependent fashion. Again, no DNA-binding activity was seen with only S-SDP1 (Fig. 3, lane 6).

An Inhibitor of the SDP1 DNA-Binding Activity. An inhibitor of SDP1 DNA-binding activity, called I-SDP1, was identified in the flowthrough of the Mono S column (fraction iv). When fraction iv was incubated with fractions iii and v (SDP1 and S-SDP1) and run on a bandshift gel, the DNA-binding activity was lost (Fig. 3, lanes 8–10). Addition of the I-SDP1 fraction to the SDP1/S-SDP1 mixture eliminated the DNA-binding signal in a dose-dependent fashion. No bandshift with the mobility of SDP1 is seen after incubation of only I-SDP1 with the labeled DNA probe (Fig. 3, lanes 11 and 12), although a rapidly migrating DNA–protein complex can be seen.

To determine if I-SDP1 inhibits yeast DNA-binding activities besides SDP1, radiolabeled DNA probes containing binding sites for the following proteins were prepared: RAP-1 (also known as GRFI and TUF) (14–16), ABF-1 (15–17), and MCM1 (also known as PRTF and GRM) (18–20). When I-SDP1 was added to binding reactions containing any one of these DNA-binding proteins and the appropriate DNA probe, inhibition of the DNA-binding activities was not observed (data not shown). Thus, I-SDP1 does not prevent the formation of protein–DNA complexes nonspecifically.

Stimulation of SDP1 Activity by Formamide. Baeuerle and Baltimore (21, 22) identified an inhibitor of a mammalian DNA-binding protein, NF-κB. The inhibitor, called IκB, associates with NF-κB and prevents NF-κB from binding to DNA. They demonstrated that protein-dissociating agents such as formamide and sodium deoxycholate promote the separation of IκB from NF-κB and permit NF-κB to bind to DNA. We asked whether protein-dissociating agents would relieve SDP1 inhibition by I-SDP1. Fraction iii gives a weak DNA-binding signal when incubated with probe (Fig. 4, lane 1). Incubation of fraction iii with formamide significantly stimulated the DNA-binding activity (Fig. 4, lane 3). The level of stimulation of SDP1 binding activity by formamide treatment did not reach that achieved by addition of the S-SDP1 stimulatory activity (Fig. 4, lane 2). SDP1 activity

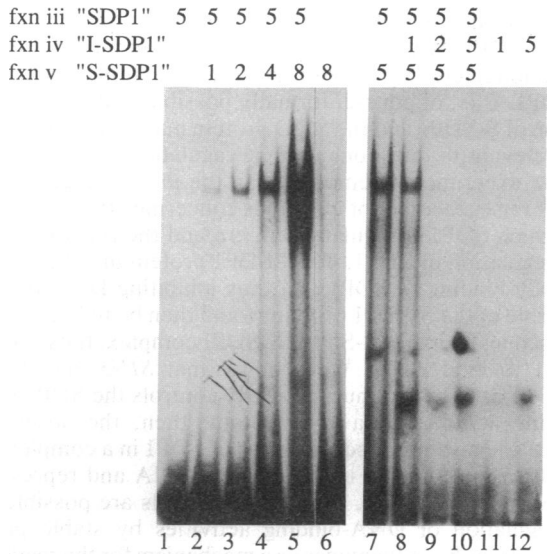

FIG. 3. Dose-dependent stimulation and inhibition of SDP1 DNA-binding activity. The volumes (in μl) of fractions (fxn) iii, iv, and v shown in the figure were incubated with the labeled DNA probe and analyzed by electrophoresis with a gel-retardation assay.

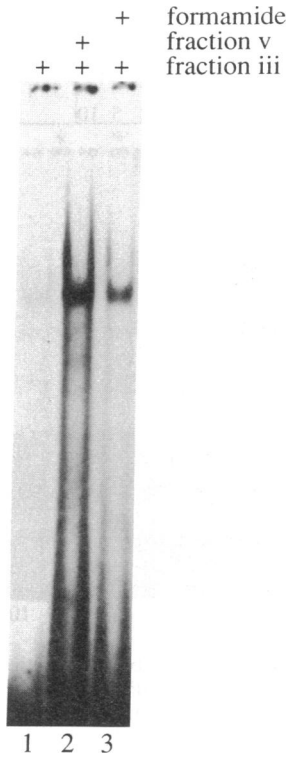

FIG. 4. Formamide treatment leads to an increase in the SDP1 DNA-binding activity. Lanes: 1, fraction iii only; 2, fractions iii and iv; 3, fraction iii only, but treated with formamide.

was not stimulated by the addition of sodium deoxycholate and it was not stimulated by treatment with urea or guanidine hydrochloride (data not shown). This experiment indicates that in addition to SDP1 fraction iii contains a disassociable inhibitor, possibly I-SDP1.

The Stimulatory and Inhibitory Activities Are Not Catalytic. The experiment in Fig. 5 demonstrates that the stimulatory and inhibitory activities act in a reversible manner. For the sample in Fig. 5, lane 3, SDP1 was incubated with S-SDP1 and I-SDP1 and then loaded onto a bandshift gel. For other samples (Fig. 5, lanes 7 and 9, indicated with an asterisk), SDP1 was incubated with only S-SDP1 or only I-SDP1 for 10 min; then the other component was added, and the incubation was continued for an additional 10 min before electrophoresis. These experiments demonstrate that the effects of either S-SDP1 or I-SDP1 on SDP1 are reversible. Thus, for instance, I-SDP1 is not simply a protease.

The stimulator and inhibitor might be enzymes that modify SDP1 in opposing ways. For example, S-SDP1 could be a kinase and I-SDP1 could be a phosphatase. Kinetic experiments were performed to determine if S-SDP1 and I-SDP1 function catalytically. If S-SDP1 were an enzyme, we would expect that the yield of SDP1 activity would increase with time of incubation with S-SDP1. However, prolonged incubation of SDP1 with a subsaturating amount of S-SDP1 does not lead to an increase in SDP1 activity (data not shown). Similarly, incubation of SDP1 and S-SDP1 with a subsaturating quantity of I-SDP1 for various periods of time does not lead to a progressive change in the amount of the SDP1 DNA-binding activity recovered in a bandshift experiment. We conclude that S-SDP1 and I-SDP1 do not act catalytically.

DISCUSSION

A DNA-binding activity, SDP1, which binds to the *HO* promoter, was not detected in extracts prepared from *sin3*

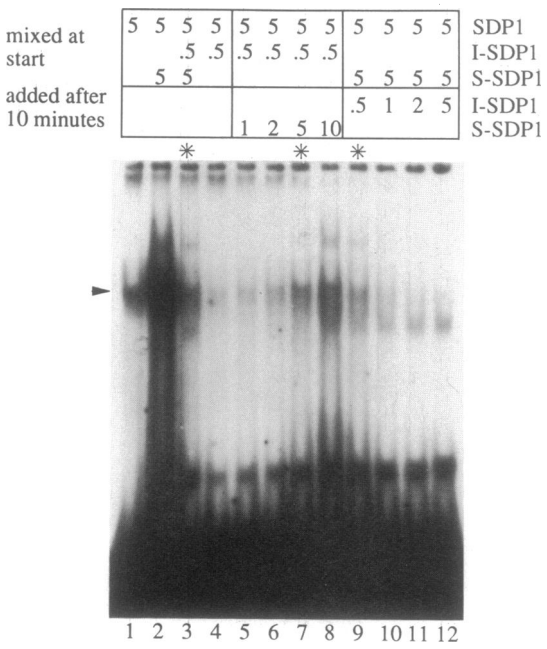

FIG. 5. The actions of the inhibitory and stimulatory components act in a reversible manner. SDP1 (fraction iii) was added to all reaction mixtures. I-SDP1 (fraction iv) and S-SDP1 (fraction v) were added as indicated. Lanes 1–4, all components were added and mixed at the start of the incubation. Lanes 5–8, DNA, SDP1, and I-SDP1 were incubated for 10 min, then S-SDP1 was added and incubated for an additional 10 min before loading on the gel. Lanes 9–12, DNA, SDP1, and S-SDP1 were incubated for 10 min, then I-SDP1 was added and incubated for an additional 10 min before loading on the gel. The same amounts of I-SDP1, SDP1, and S-SDP1 were added to the reactions in lanes 3, 7, and 9 (indicated by asterisks), although the order in which the components were added was different.

mutant strains (1). We have postulated that SDP1 acts as a transcriptional repressor, since the pattern of *HO* regulation is altered in a *sin3* mutant. In a *sin3* mutant strain, *HO* is inappropriately expressed in daughters, and the SWI5 transcriptional activator is no longer required for *HO* expression. Here we have demonstrated that the *SIN3*-dependent DNA-binding activity SDP1 is not encoded by *SIN3*. SDP1 activity can be inhibited by I-SDP1 and stimulated by S-SDP1.

A number of observations have led us to suggest that stable protein interactions regulate SDP1 DNA-binding activity *in vitro*. In titration experiments, a direct correspondence was seen between the amount of I-SDP1 added and the extent of inhibition and also between the amount of S-SDP1 added and the extent of stimulation. The effects of I-SDP1 and S-SDP1 on the SDP1 DNA-binding activity are saturable and reversible. Varying the time of incubation had no effect on either the stimulatory or the inhibitory reaction, suggesting that I-SDP1 and S-SDP1 do not act catalytically.

Recently there have been several reports of regulation of DNA-binding activity by protein–protein interactions. Baeuerle and Baltimore (21, 22) identified an inhibitor, IκB, which associates with NF-κB and prevents NF-κB from binding to DNA. The protein-dissociating agent formamide promoted the separation of IκB from NF-κB and permitted NF-κB to bind to DNA. We have found that formamide treatment of a fraction containing SDP1 and I-SDP1 also leads to an increase in DNA-binding activity. Picard *et al.* (23) recently demonstrated that the steroid binding domain of the glucocorticoid receptor regulates nuclear localization and DNA-binding activity of the receptor. They suggest that the DNA-binding activity of the receptor is inhibited by the binding of the heat shock protein hsp90 and that glucocorticoid hormone promotes DNA-binding through the release of the hsp90 protein. Both IκB and hsp90 prevent DNA binding by sequestering proteins in the cytoplasm. In contrast, SIN3 is present in the nucleus (10).

Benezra *et al.* (24) recently have identified a protein, called Id, which inhibits the DNA-binding activity of MyoD and the enhancer binding proteins E12 and E47. These DNA-binding proteins contain a conserved cluster of basic residues adjacent to the helix–loop–helix (HLH) region, and they normally bind DNA as homodimeric or heterodimeric complexes. It is believed that the HLH region is involved in protein–protein interactions (25, 26). Id, which contains an HLH region but lacks a basic region, forms heterodimers with these DNA-binding proteins. However, these heterodimers are unable to bind DNA.

The predicted amino acid sequence of the SIN3 protein indicates that it is very large (175 kDa) and contains four copies of a repeated motif (10). This motif consists of paired amphipathic helices (PAH) separated by a 20-amino-acid "loop" segment, with conservation of the hydrophobic residues in the amphipathic helices. The structure of these repeats is like that of the tetratricopeptide (TPR) (27, 28) and HLH motifs (25). The TPR genes (*Saccharomyces cerevisiae CDC16*, *CDC23*, *SKI3*, and *SSN6* as well as *Schizosaccharomyces pombe nuc1*) each contain multiple PAH motifs and are involved in cell cycle control or transcriptional regulation. The HLH proteins (such as MyoD and myc), in contrast, contain a single PAH motif, preceded by a conserved basic region that is involved in DNA binding (29, 30). The HLH proteins bind to DNA as dimers, and the PAH region is involved in dimer formation (26). Within each of these three families of PAH repeats (SIN3, TPR, and HLH), the hydrophobic residues in the amphipathic helices are strongly conserved. These hydrophobic residues are not conserved between families, however. It has been suggested that the paired amphipathic helixes are involved in specific protein–protein interactions (25, 30). The presence of PAH motifs in SIN3 is consistent with our model of *SIN3* by means of protein–protein interactions.

SIN3 and the SDP1 DNA-binding activity are linked by two observations: the SDP1 activity is missing in extracts prepared from *sin3* mutant strains, and the SIN3 protein copurifies with S-SDP1. The copurification of the stimulatory factor S-SDP1 with the SIN3 protein prompts us to suggest two possibilities, which at present we cannot distinguish: (*i*) *SIN3* encodes S-SDP1, or (*ii*) the SIN3 protein complexes with S-SDP1. We have been unsuccessful in our attempts to immunodeplete SIN3 protein from fractions containing S-SDP1. It is, of course, formally possible that the copurification of S-SDP1 and the SIN3 protein may be fortuitous and not relevant to questions of gene regulation.

The experiments demonstrating the *in vitro* regulation of SDP1 raise a number of questions concerning the mechanism by which SDP1 is regulated *in vitro* and the role of *SIN3* in the regulation of SDP1. The I-SDP1 protein may function *in vivo* by binding to SDP1 and thus inhibiting DNA binding. The role of the S-SDP1 protein would then be to bind I-SDP1 and sequester it in an I-SDP1/S-SDP1 complex, thus allowing SDP1 to bind DNA. We propose that *SIN3* encodes the S-SDP1 protein and thus indirectly controls the SDP1 DNA-binding activity. In a *sin3* mutant, then, the absence of S-SDP1 leads to the sequestration of SDP1 in a complex with I-SDP1, and SDP1 is unable to bind DNA and repress *HO* transcription. Clearly, many other models are possible.

Regulation of DNA-binding activities by stable protein interactions has advantages as a mechanism for the regulation of gene expression. It permits the cell to make rapid adjustments in the level of active transcriptional regulators without synthesizing new proteins or degrading existing proteins and to integrate multiple regulatory signals in making these ad-

justments. SIN3 may determine the activity of SDP1 (and other proteins) by differentially binding or releasing I-SDP1 (and other factors?) in response to regulatory signals. There may be other analogous inhibitors and stimulators that modulate specific DNA-binding proteins, with each of these DNA-binding proteins being regulated by several regulatory proteins. If each inhibitor and stimulator interacts with a distinct collection of DNA-binding proteins in the cell, a complex regulatory network can be built that regulates gene expression by means of stable protein interactions. If we suppose that each of these regulatory proteins (i.e., SIN3) can sense a particular aspect of cellular metabolism, such as cell cycle position or the level of a nutrient or metabolite, then the level of a specific DNA-binding protein could be fine tuned by integrating information from a number of these sensory/stimulatory proteins.

We have suggested that *SIN3* regulates the activity of DNA-binding proteins in addition to SDP1. We have identified another DNA-binding protein, REB1, which is *SIN3* dependent (13). We believe that one function of SIN3 is to sense information on intracellular sugar metabolism, since REB1 levels in a *sin3* mutant are drastically affected by a change in the carbon source (13). The specific mechanism by which REB1 is made to depend on *SIN3* is not yet understood, although the fact that the REB1 protein contains a single PAH motif is intriguing (ref. 31; D.J.S., unpublished observations). *SIN3* transcriptionally regulates a number of yeast genes including *HO*, *SPO11*, *SPO13*, and *TRK2* (1, 2, 11, 12). Although further work is required to determine by what mechanism *SIN3* regulates these various genes, it is possible that expression of these genes is regulated by a *SIN3*-dependent DNA-binding protein (SDP1, REB1, or another, as yet unidentified, protein) and that the decreased level of specific DNA-binding proteins in a *sin3* mutant leads to altered regulation of multiple genes. There may be other proteins like SIN3, which effect gene regulation by controlling the level of active DNA-binding proteins. In this regard it is noteworthy that the TPR family of genes with multiple PAH motifs (*Saccharomyces cerevisiae CDC16*, *CDC23*, *SKI3*, and *SSN6* as well as *Schizosaccharomyces pombe nuc1*) are involved in either cell cycle control or transcriptional regulation (27, 28).

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