Mol Gen Genet (1994) 245:724–733

ORIGINAL PAPER

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The yeast *SIN3* gene product negatively regulates the activity of the human progesterone receptor and positively regulates the activities of GAL4 and the HAP1 activator

Received: 5 April 1994 / Accepted: 7 June 1994

Abstract The activation of gene transcription in eukaryotic organisms is regulated by sequence-specific DNA-binding proteins as well as by non-DNA-binding proteins. In this report we describe the modulatory functions of a non-DNA-binding protein, SIN3 (also known as SDI1, UME4, RPD1, and GAM2) on the transactivation properties of the human progesterone receptor (hPR), GAL4, and the HAP1 activator in yeast. Our data suggest that SIN3 is a dual function protein. It negatively regulates the transcriptional activities of hPR-A and hPR-B by affecting the N-terminal activation domain (AF1). SIN3 positively regulates the transcriptional activities of GAL4 and the HAP1 activator. However, it has no effect on the transcriptional activities of the human glucocorticoid receptor (hGR) and GCN4. The SIN3 protein contains four copies of a paired amphipathic helix (PAH) motif. Deletion analysis of the *SIN3* PAH motifs shows that the PAH3 motif is essential for *SIN3*-mediated regulation of hPR, GAL4, and the HAP1 activator. In constrast, the PAH1, PAH2, and PAH4 motifs are not required for *SIN3*-mediated regulation of these activators. Additionally, we examined the mechanism(s) by which the SIN3 protein modulate the activities of various activators. We are unable to demonstrate the direct interaction of SIN3 protein with these activators using the yeast two-hybrid system or co-immunoprecipitation. These data suggest that SIN3 regulates the transactivation functions of hPR, GAL4, and the HAP1 activator by an indirect mechanism.

Communicated by D. Y. Thomas

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Key words SIN3 protein · Human progesterone receptor · GAL4 · HAP1 · Gene regulation Transcription

Introduction

The rate and pattern of gene transcription are regulated in eukaryotic organisms by various mechanisms, which include alterations in the chromatin structure and/or the specific interplay of regulatory proteins (Gross and Garrard 1987; Ptashne 1986, 1988; Roth et al. 1990; Straka and Horz 1991). These regulatory proteins form a multiprotein complex with the components of the RNA polymerase II transcriptional machinery in the promoter regions of target genes (Lewin 1990; Lillie and Green 1989; Ptashne 1988; Ptashne and Gann 1990; Pugh and Tjian 1991). Both sequence-specific DNA-binding proteins and non-DNA-binding proteins may function as regulatory proteins. The DNA-binding proteins interact with *cis*-acting DNA promoter elements and modulate the transcription of target genes. However, the non-DNA-binding proteins exert their effects on transactivation via protein-protein interactions (Berger et al. 1992; Pugh and Tjian 1991; Vidal et al. 1991; Weinzierl et al. 1993). The non-DNA-binding proteins that play roles in gene transcription and its regulation have been identified by both biochemical and genetic methods. Genetic methods in yeast have led to the identification of the *SIN3* gene. This gene codes for a non-DNA-binding regulatory protein that negatively regulates the yeast *HO* gene (Nasmyth et al. 1987; Sternberg et al. 1987). *HO encodes* a site-specific endonuclease necessary for cell type switching (Kostriken et al. 1983). Six genes (*SWI1–SWI6*) required for the transcription of *HO*, and five genes (*SIN1–SIN5*) which code for negative regulators of this gene have been identified (Herskowitz et al. 1992; Nasmyth 1993). The *SIN3* gene was identified as a suppressor mutation because, after inactivation of this gene, the requirement for the *SWI5*

gene product for *HO* transcription is relieved (Nasmyth 1993; Sternberg et al. 1987).

SIN3 is a 175 kDa non-DNA-binding nuclear protein which contains four repeats of a paired amphipathic helix (PAH) motif. Similar motifs have also been identified in the *myc* family of helix-loop-helix (HLH) DNA-binding proteins and in the tetratricopeptide repeat (TPR) family of regulatory proteins. It has been suggested that the amphipathic helix motifs of the SIN3 protein are involved in protein-protein interactions (Wang et al. 1990) that regulate gene transcription via stable protein-protein interactions (Wang and Stillman 1993).

Despite the complexity of gene transcription and its regulation, these processes are conserved among different eukaryotic species. We and others have previously shown that steroid hormone receptors and other mammalian transcription factors can function in yeast (Giguere et al. 1986; Mak et al. 1989; Metzger et al. 1988; Nawaz et al. 1992; Ptashne 1988; Vegeto et al. 1992; Yoshinaga et al. 1992). The ability of the mammalian transcription factors to function in yeast suggests that the yeast-based system can be utilized to identify the gene products essential for the function of steroid hormone receptors and other transcriptional activators.

The yeast SWI proteins positively regulate the transcriptional activities of the steroid hormone receptors in yeast (Yoshinaga et al. 1992; Z.N., unpublished observations). Since the SIN proteins antagonize the effects of SWI proteins, in the present study we focused on the biochemical mechanisms by which the yeast SIN3 protein modulates the transactivation functions of steroid hormone receptors. For this purpose, we chose the human progesterone receptor (hPR) and the human glucocorticoid receptor (hGR) as our models. hPR and hGR are members of the steroid hormone receptor superfamily of transcriptional activators whose functions are regulated by their cognate ligands (for reviews see Beato 1989; Carson-Jurica et al. 1987, 1990; Greene and Chambon 1986; O'Malley 1990). Structurally these molecules are similar, consisting of a ligand-binding domain located in the C-terminus, a well-defined DNA-binding domain located centrally, and the N-terminal domain which is required for maximal transactivation (Conneely et al. 1986; O'Malley 1990). The mechanisms of activation of these transactivators are related. Ligand binding converts these receptors from an inactive to an active form (Carson-Jurica et al. 1990; O'Malley 1990) and activated receptors then bind to specific DNA-response elements and regulate transcription (Payvar et al. 1983; Scheidereit et al. 1989).

The transactivation functions of hPR and hGR appear to be mediated by negatively charged amino acids, this feature is also characteristic of the activation domains of the yeast GAL4, HAP1, and GCN4 transcriptional factors (Hollenberg and Evans 1988; Hope et al. 1988; Ptashne 1988; Sigler 1988). In the present study, we have also focused on the mechanisms by which the SIN3 protein modulates the transcriptional activities of these yeast activators. The GAL4, GCN4, and HAP1 proteins activate transcription in yeast by binding to specific sequences located in the promoter regions of their target genes. GAL4 and GCN4 activate the transcription of sets of genes involved in galactose catabolism and amino acid biosynthesis, respectively (Carey et al. 1989; Oliphant et al. 1989). HAP1 is also a yeast transcriptional activator that binds to the *CYC1* upstream activation site (UAS1). *CYC1 enco*des the major isolog of cytochrome c in yeast, and its transcription is induced by heme (Peter et al. 1987).

We show in this study that the SIN3 protein is a dual function protein. It negatively regulates the transcriptional activity of hPR-A and hPR-B and positively regulates the transcriptional activities of GAL4 and the HAP1 activator. However, it has no effect on the transcriptional activities of hGR and GCN4. We also show that the same region of the SIN3 protein (the PAH3 motif) is required for both positive and negative regulation.

Materials and methods

Materials

Enzymes for DNA manipulation and modification were purchased from Boehringer Mannheim (Indianapolis, Ind.), Promega Biotec (Madison, Wis.) or New England Biolabs (Bethesda, Md.). The Immobilon-P (PVDF) transfer membranes (IPVM 30uRo) were obtained from Millipore (Bradford, Mass.). Rabbit anti-rat and rabbit anti-mouse antibodies (IgG) were purchased from Zymed (San Francisco, Calif.). The ECL western blotting detection kit, the anti-rabbit and anti-mouse antibodies labeled with horseradish peroxidase (HRP) as well as the Rainbow protein molecular weight markers were acquired from Amersham (Arlington Heights, Ill.). The Bio-Rad protein assay system and high-pressure liquid chromatography-grade ammonium persulfate were obtained from Bio-Rad (Richmond, Calif.), while dithiothreitol was purchased from Boehringer Mannheim (Indianapolis, Ind.). Progesterone, triamcinolone acetonide, adenine sulfate, O-nitrophenyl β-D-galactopyranoside, mercaptoethanol, protease inhibitors, uracil and general reagents were purchased from Sigma (St. Louis, Mo.). Casamino acids, yeast nitrogen base without amino acids, dextrose, and other medium components were obtained from Difco-BRL (Bethesda, Md.). Glass beads (0.45–0.50 μm) were obtained from Thomas Scientific (Swedeshore, N.J.).

Buffers

The transcriptional buffer for β-galactosidase assays contained 0.12 M Na_2HPO_4, 0.04 M NaH_2PO_4, 10 mM KCl, 1 mM $MgSO_4$, and 2.7% 2-mercaptoethanol, pH 7.0. For analysis of receptors, the cells were homogenized in transcription buffer with 0.3 M NaCl. Protein extraction buffer for analysis of GAL4 and the SIN3 contained 0.2 M TRIS-HCl pH 8.0, 10 mM $MgCl_2$, 1 mM DTT, 10% glycerol, 0.5 M NaCl, 1.74 mg phenylmethylsulfonyl fluoride, 0.013 mg leupeptin, and 0.003 mg pepstatin.

Yeast strains

The *Saccharomyces cerevisiae* strains DY150 (*MAT***a**, *ura* 3-1, *leu* 2-3, 112 *trp* 1-1, *his* 3-11, 15 *ade* 2-1, *can* 1-100) and DY984 (*MAT*

a, *ura* 3-1, *leu* 2-3, 112 *trp* 1-1, *his* 3-11, 15 *ade*2-1, *can* 1-100, *sin3* Δ:: *ADE*2) were used throughout this study. All transformations into these strains were performed by following the lithium acetate-polyethylene glycol transformation protocol (Itao et al.1983).

DNA constructions

The expression vector YEPhGR was constructed in the following manner. The human glucocorticoid receptor plasmid pRShGR (Giguere et al. 1986) was digested with *Kpn*I and *Eco*RI and ligated to a linker which contained an internal *Nco*I site. The sequence of the linker was as follows: 5′-GGTACCGGATCCATGGACTCCAAAGAATCATTAACTCCTGG-3′.

The *Nco*I-*Xho*I (latter site filled-in with Klenow enzyme) fragment of this vector was purified and cloned into the *Nco*I-*Asp*718 (latter site repaired with Klenow enzyme) sites of the yeast expression vector YEPE2 (McDonnell et al. 1991), yielding the plasmid YEPhGR. YEPhGR is a high-copy-number vector that uses the copper-responsive yeast metallothionein (CUP1) promoter to drive synthesis of hGR mRNA.

The progesterone-responsive/glucocorticoid-responsive reporter plasmid was constructed as follows. Plasmid YRpPC3GS$^+$ (Mak et al. 1989) was digested with *Tth*III1, the ends were repaired by Klenow enzyme, and then this plasmid was redigested with *Xho*I. The *Tth*III1 (end filled)-*Xho*I fragment of plasmid YRpPC3GS$^+$ was purified. Plasmid YEPE2 (McDonnell et al. 1991) was digested with *Mlu*I, and the ends were repaired using the Klenow enzyme. Then, plasmid YEPE2 was redigested with *Xho*I and the vector fragment was ligated with the gel-purified fragment (*Tth*III1-*Xho*I) of plasmid YRpPC3GS$^+$, yielding plasmid YEPEG2LEU2. Plasmid YEPEG2LEU2 contains two copies of progesterone-responsive/glucocorticoid-responsive elements upstream of the yeast iso-1-cytochrome *c* minimal promoter fused to the *Escherichia coli lacZ* gene.

To construct a high-copy-number GAL4 expression plasmid, the *Bam*HI fragment of plasmid PCL2 (gift from Jutta Koenigsfeld, Giessen, Germany), containing the alcohol dehydrogenase (ADH) promoter and terminator sequences fused to GAL4 coding sequences, was cloned into the corresponding sites of the yeast expression vector PCB-UB stop. Plasmid PCB-UB stop was generated by the insertion of a linker, containing an internal *Bam*HI site, into the *Nco*I-*Asp*718 sites of plasmid YEPE2 (McDonnell et al. 1991). The sequence of the linker used was as follows: 5′ CATGGCAGCTGGATCCTAAGTAGCTGAAGCTTGGTACC 3′.

To construct the expression vector YEPEN-hPR, the *Nco*I-*Dra*I fragment of plasmid YEPhPR-B (Vegeto et al. 1992), containing amino acids 1–638 of hPR-B, was cloned into the *Nco*I-*Pvu*II sites of the yeast expression vector PCB-UB stop.

The expression vector YEPEhPR-C was constructed as follows. The DNA sequences of amino acids 537–934 of hPR were amplified by the polymerase chain reaction (PCR). The PCR fragment containing amino acids 537–934 of hPR was digested with *Acc*I and *Sal*I and then this fragment was cloned into the *Sma*I-*Pvu*II sites of the vector PCB-UB stop, yielding plasmid YEPEh-PR-C. The expression plasmids for progesterone receptors are also under the control of the CUP1 promoter.

The GAL4 reporter plasmid pLGSD5LEU2 and the HAP1 reporter plasmid pLGΔAlu*Xho*LEU2 are similar to the plasmids pLGSD5 (Guarente et al. 1982), and pLGΔAlu*Xho* (Guarente et al. 1984), respectively. The only difference is that pLGSD5LEU2 and pLGΔAlu*Xho*LEU2 contain *LEU2* as a selection marker instead of *URA3*. The GCN4 reporter plasmid 14 × 2 has been described previously (Hinnebusch et al. 1985). In all reporter plasmids, UASs are fused to the *CYC1-lacZ* reporter gene.

Transcriptional assays

Yeast cells transformed with the appropriate expression and reporter plasmids were grown overnight at 30°C in selective medium. These cultures were used to inoculate fresh medium at a density of 10^4 cells/ml. When the cells reached an optical density of 1.0 at 600 nm, they were harvested, lysed by glass-bead homogenization in the transcription buffer, and the level of β-galactosidase produced was measured as described previously (Miller 1972; Nawaz et al. 1992).

Preparation of yeast extracts

Yeast cells transformed with the appropriate expression plasmids were grown overnight at 30°C in selective medium. These cultures were used to inoculate fresh medium (10 ml) at a density of 10^4 cells/ml. When the cells reached an optical density of 1-3 at 600 nm, they were harvested, and lysed by glass-bead homogenization in ice-cold protein extraction buffer.

Immunodot and western blot analysis

The same cytosol extracts used for β-galactosidase assays were dotted directly onto nylon membranes. For western blot analysis, proteins from yeast were resolved in a 7.5% SDS-polyacrylamide gel and were transferred to Immobilon membranes as described previously (Carson-Jurica et al. 1987). The membranes were then incubated with appropriate antibodies and immunodetection was carried out using the ECL detection method (Amersham).

Results

Effect of the *sin3* mutation on the transcriptional activity of the human progesterone receptor

Evidence suggests that the transcriptional activation mediated by steroid hormone receptors and other transcriptional activators involves additional factors which modulate their activities. Based on this evidence we decided to address the question of what role the SIN3 protein plays in the signal transduction pathways of various activators. In order to study the role of the SIN3 protein on the transcriptional activity of hPR, the yeast expression plasmid carrying the human progesterone receptor B cDNA (YEPhPR-B) (Vegeto et al. 1992), together with the progesterone-responsive reporter plasmid (YEPEG2LEU2) were transformed into the yeast DY150 (wild type) and DY984 (a *sin3* mutant strain) strains by standard techniques (Ito et al. 1983). The transformants were selected by tryptophan and leucine prototrophy.

The transcriptional activity of hPR-B in these transformants was determined by measuring the β-galactosidase activities of the transformants grown overnight in the presence of progesterone. As shown in Fig. 1A, hPR-B activity in the *sin3* mutant strain was three- to four fold higher than in the wild-type strain, and this activity was strictly hormone dependent. This effect of the *sin3* mutation on the transcriptional activity of hPR is maintained even at lower hormone levels (data not shown). To confirm that the higher transcriptional activity of hPR in the *sin3* mutant strain was not due to higher expression levels of hPR in the *sin3* mutant strain, the extracts used for transcriptional assays were also analyzed by western immunoblot analysis (data not

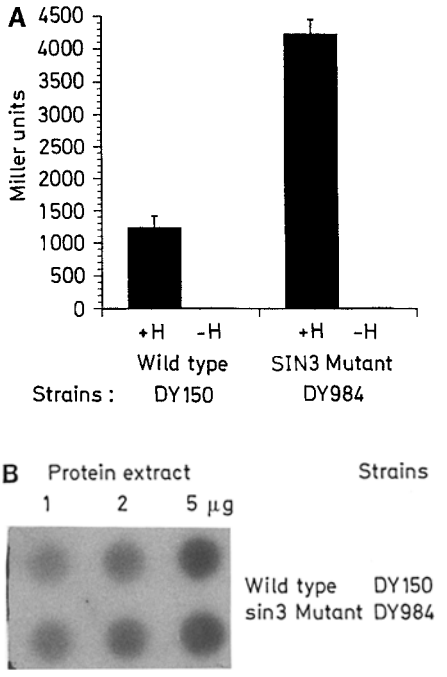

Fig. 1A, B Transcriptional activity of the human progesterone receptor (hPR) in the wild-type and in the *sin3* mutant yeast strains. **A** The yeast expression plasmid carrying the human progesterone receptor B cDNA (YEPhPR-B) and a progesterone-responsive *CYC1-lacZ* fusion reporter plasmid (YEPEG2LEU2) were transformed into yeast DY150 (wild type) and DY984 (*sin3* mutant) strains by following the lithium acetate-polyethylene glycol transformation protocol. The transformants were then grown overnight in minimal medium containing 2% glucose in the presence of 10^{-6} M progesterone. The β-galactosidase activities were measured (in Miller units). **B** Dot blot analysis showing hPR expression levels in DY150 and DY984. The extracts used for β-galactosidase assays in **1A** were immuno-assayed for hPR levels in wild-type and *sin3* mutant strains. The strains and proteins are indicated

shown) and by quantitative immuno-dot blot analysis (Fig. 1B). This analysis shows that hPR-B expression levels in the wild-type strain and in the *sin3* mutant strain are similar.

hPR is present in target tissues as two distinct subtypes, PR-B and PR-A. The PR-B isoform contains a N-terminal fragment of 164 amino acids which is absent in PR-A. It is likely that these two forms arise as a result of alternative initiation of translation (Carson-Jurica et al. 1987; Christensen et al. 1991). Like hPR-B, the transcriptional activity of the A form of PR was also negatively regulated by the SIN3 protein (data not shown). These results suggest that the SIN3 protein acts as a negative regulator of the transcriptional activities of hPR-B and hPR-A in yeast. However, the A form of hPR was approximately 25–40% less active than the B form.

Effect of the *sin3* mutation on the activation function domains of hPR

hPR contains activation domains in both the N-terminus and the C-terminus. The N-terminal activation domain (AF1) is constitutively active, while the C-terminal activation domain (AF2) is hormone inducible. In order to study the effect of the *sin3* mutation on these activation domains, two receptor constructs, N-hPR (YEPEN-hPR) containing amino acids 1–638 and hPR-C (YEPEhPR-C) containing amino acids 537–934, were transformed into the wild-type and *sin3* mutant yeast strains (Fig. 2A). As shown in Fig. 2B, the transcriptional activity of the AF1 of hPR in the *sin3* mutant was three- to four fold higher than in the wild-type strain, and this activity was independent of hormone, as expected. However, the transcriptional activity of the AF2 in the *sin3* mutant was only slightly higher (range from no effect to two fold) when compared to that of the wild-type strain, and this activity was strictly hormone dependent (Fig. 2C). These results demonstrate that the SIN3 protein mainly acts as a negative regulator of the AF1 of hPR in yeast. Furthermore, expression of the SIN3 protein (Fig. 2D) reduces the transcriptional activity of the AF1 of hPR in the *sin3* mutant to a level comparable to that of the wild-type strain (Fig. 2D). The expression vector without the *SIN3* gene has no effect on the transcriptional activity (Fig. 2D control).

Complementation of the *sin3* mutation and the effect of the SIN3 PAH deletion mutants on the transcriptional activity of hPR

The SIN3 protein contains four copies of a PAH motif. To determine which PAH motif of the SIN3 protein is required for the negative regulation of hPR transcriptional activity, several SIN3 PAH deletion mutants (Fig. 3; Wang and Stillman 1993) were tested. These mutants, along with the full-length SIN3 expression vector, were transformed into a *sin3* mutant (DY984) strain containing an hPR expression plasmid and progesterone-responsive reporter plasmids. The transformants were selected by uracil, tryptophan and leucine prototrophy. As shown in Table 1, expression of the full-length SIN3 protein reduces hPR transcriptional activity to a level comparable to that of the wild-type strain. The expression vector without the *SIN3* gene has no effect on transcriptional activity (Table 1, see control). The data shown in Table 1 also suggest that the PAH1, PAH2, and PAH4 motifs are not required for *SIN3*-mediated repression of hPR transcriptional activity. However, the PAH3 deletion mutant was unable to reduce the transcriptional activity. Western blot analysis was performed to confirm that the loss in *SIN3* repressing activity was not due to low or lack of expression of the *SIN3* PAH3 deletion mutant. This analysis confirmed that all yeast cells carrying *SIN3* PAH deletion mutants had similar SIN3 protein levels (data not shown). These

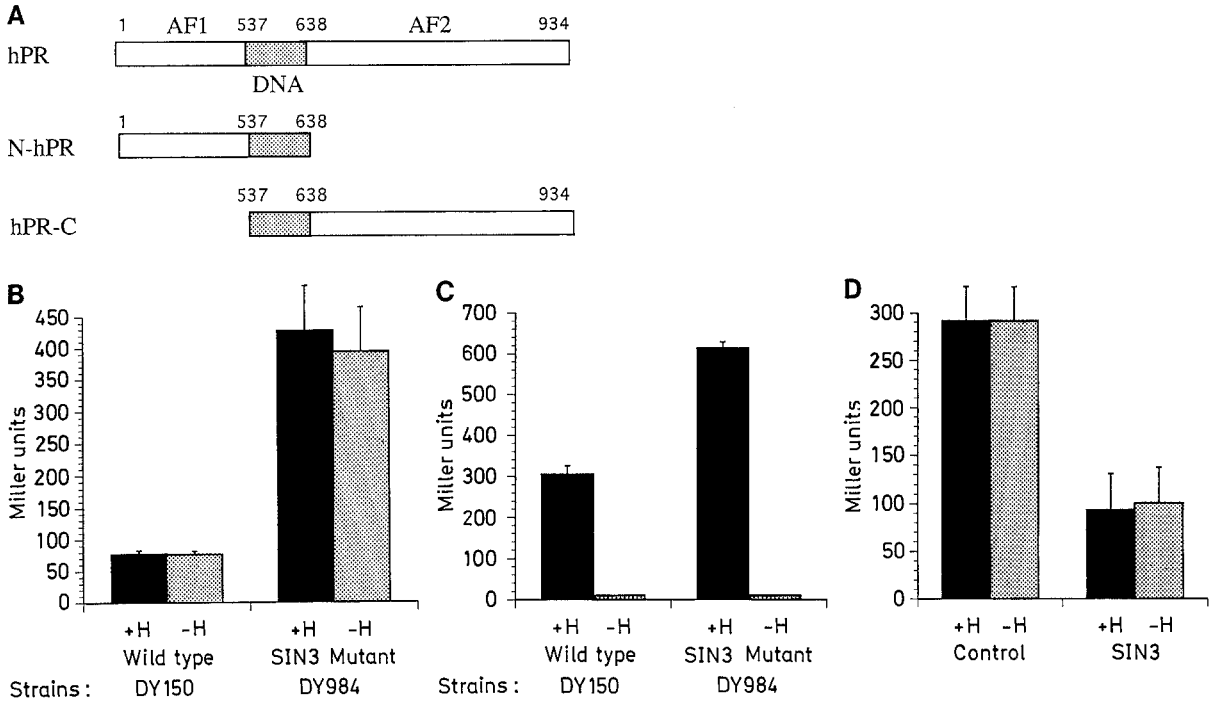

Fig. 2A–D Effect of *sin3* mutation on the activation function (AF) domain of hPR. **A** Schematic depiction of hPR. Numbers refer to amino acid end-points. The DNA-binding domain is indicated by a *filled box*. **B** The yeast plasmid containing amino acids 1–638 (YEPEN-hPR) and a progesterone-responsive reporter plasmid were transformed into wild-type and *sin3* mutant yeast strains. The transformants were grown in the presence (10^{-6} M) and absence of hormone and the induction of LacZ was measured. **C** The yeast expression plasmid, YEPEhPR-C, encoding amino acids 537–934, was transformed into the wild-type and *sin3* mutant yeast strains. The cells were grown in the presence and absence of 10^{-6} M progesterone. The β-galactosidase activities were measured. **D** The yeast expression vector containing the full-length *SIN3* gene and a control vector lacking the *SIN3* gene were transformed into a *sin3* mutant (DY984) strain containing expression plasmid YEPEN-hPR and a progesterone-responsive reporter plasmid. These cells were then grown in minimal medium in the presence and absence of hormone. The β-galactosidase activity (Miller units) was measured

Table 1 Influence of *sin3* paired amphipathic helix (PAH) deletion mutants on the transcriptional activities of various activators. The various PAH deletion mutants of SIN3 (see Fig. 3) were transformed into a *sin3* mutant (DY984) strain containing one of the activators and its respective reporter. The β-galactosidase activity (Miller units) was determined (*Control* an expression vector lacking the *SIN3* gene, *SIN3* an expression vector with the wild-type *SIN3* gene, *PAH1*, *PAH2*, *PAH3*, *PAH4* expression vectors with various SIN3 PAH deletions)

	β-Galactosidase activity		
	Human progesterone receptor	GAL4	HAP1
Control	3995±187	196±94	108±17
Full-length SIN3	1070±57	1816±192	1173±199
SIN3ΔPAH1	2025±126	821±51	1193±115
SIN3ΔPAH2	1780±119	1911±162	1252±124
SIN3ΔPAH3	4790±213	380±66	431±63
SIN3ΔPAH4	1900±175	1215±171	1054±143

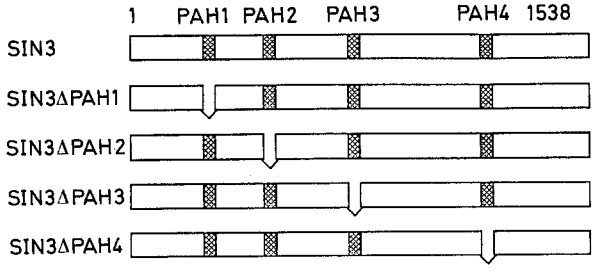

Fig. 3 Structures of the various paired amphipathic helix (PAH) deletion mutants of SIN3. Diagram of the various PAH deletion mutants of the *SIN3* used in this study (*SIN3* wild-type *SIN3* gene, *SIN3 ΔPAH1* deletion of the PAH1 motif in the *SIN3* gene, *SIN3 ΔPAH2* deletion of the PAH2 motif in the *SIN3* gene, *SIN3 ΔPAH3* deletion of the PAH3 motif in the *SIN3* gene, and *SIN3 ΔPAH4* deletion of the PAH4 motif in the *SIN3* gene

results suggest that the PAH3 motif is essential for the negative regulation of hPR.

Effect of the *sin3* mutation on the transcriptional activity of the human glucocorticoid receptor

To assess the effect of the *sin3* mutation on the transcriptional activity of hGR, the glucocorticoid receptor expression plasmid (YEPhGR) and glucocorticoid-responsive reporter plasmid (YEPEG2LEU2) were co-transformed into yeast DY150 (wild type) and DY984 (*sin3* mutant) strains, and the induction of LacZ was measured. As shown in Fig. 4A, the *sin3* mutation has no effect on the transcriptional activity of hGR. The level of hGR transactivation is the same both in the

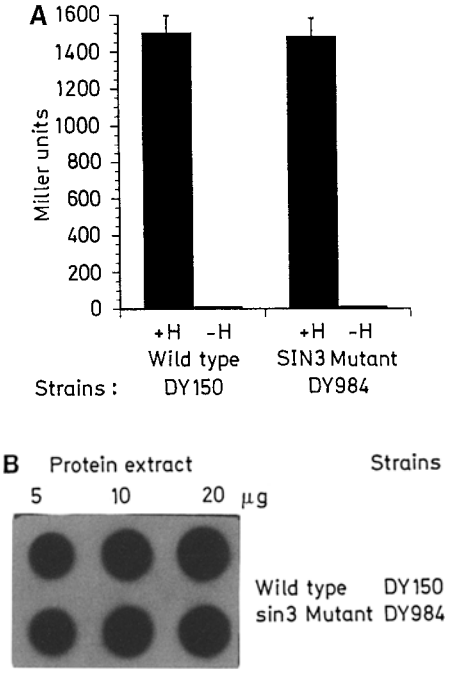

Fig. 4A, B Transcriptional activity of the human glucocorticoid receptor (hGR) in the wild-type strain and in the *sin3* mutant yeast strain. **A** The glucocorticoid receptor expression plasmid (YEPhGR) and glucocorticoid- responsive reporter plasmid (YEPEG2LEU2) were co-transformed into yeast DY150 (wild type) and DY984 (*sin3* mutant) strains. The transformants were grown overnight in minimal medium in the presence of 10^{-5} M triamcinolone acetonide, and the induction of LacZ was measured (Miller units). **B** Dot blot analysis showing hGR expression levels in DY150 and DY984. The extracts used for β-galactosidase assays (Fig. 3A) were immuno-assayed for hGR levels in wild-type and *sin3* mutant strains

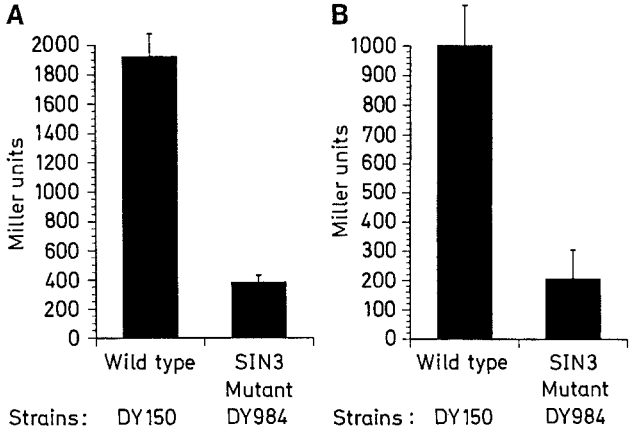

Fig. 5A, B Effect of *sin3* mutation on the transcriptional activities of various yeast activators. **A** The GAL4 expression vector (YEPGAL4) and the GAL4-responsive reporter plasmid (PLGSD5LEU2) were transformed into wild-type and *sin3* mutant strains. The cells were grown overnight in minimal medium containing 2% glucose. The β-galactosidase activities (Miller units) were determined as a measure of GAL4 transactivation. **B** The yeast reporter plasmid, containing a HAP1 binding site fused to the *CYC1-lacZ* gene (pLGΔAlu*Xho*LEU2), was transformed into yeast DY150 (wild type) and DY984 (*sin3* mutant) strains. The cells were grown overnight in minimal medium containing 2% glucose and induction of LacZ was measured (Miller units)

wild-type and *sin3* mutant strains even at lower concentrations of agonist (data not shown). These results demonstrate that hPR and hGR use different mechanisms to activate gene transcription in yeast cells. The expression levels of hGR in the *sin3* mutant and in the wild-type strains were similar, as determined by quantitative immuno-dot blot analysis (Fig. 4B).

Effect of the *sin3* mutation on the transcriptional activities of various yeast activators

In this study we used the GAL4, HAP1, and GCN4 proteins as our models to examine the effect of the *sin3* mutation on the transactivation functions of yeast transcriptional activators. To determine the effect of the *sin3* mutation on GAL4 transcriptional activity, the GAL4 expression vector (YEPGAL4) and the GAL4-responsive reporter plasmid (pLGSD5LEU2) were transformed into wild-type and *sin3* mutant strains. The cells were grown overnight in minimal medium containing 2% glucose and the transcriptional activity of GAL4 was assessed by measuring the β-galactosidase activities of the transformants. As shown in Fig. 5A, the GAL4 activity in the *sin3* mutant strain (DY984) was reduced approximately five fold when compared to that of the wild-type strain (DY150). Western blot analysis of GAL4 confirmed that the difference in the transcriptional activity shown in Fig. 5A was not due to the different levels of GAL4 in the wild-type and *sin3* mutant strains (data not shown). However, when the cells were grown in medium containing 2% galactose as a carbon source, the *sin3* mutation had no significant effect on GAL4 activity. The GAL4 transcriptional activity was the same in both the wild-type and the *sin3* mutant strains under galactose conditions even when the GAL4 protein was overexpressed (data not shown). It is possible that the *sin3* mutation affects the expression or the activity of GAL80 under glucose conditions, and that this leads to decreased GAL4 activation function that can be seen when GAL4 is overexpressed, as in this experiment. In contrast to GAL4, the activity of hPR in the *sin3* mutant was identical when glucose or galactose was used as a carbon source (data not shown).

To examine the effect of the *sin3* mutation on the transcriptional activities of the HAP1 activator and GCN4, the reporter plasmid containing a HAP1 binding site fused to the *E. coli lacZ* gene (pLGΔAlu*Xho*LEU2), and the reporter plasmid containing a GCN4 binding site (14 × 2) were transformed into yeast DY150 (wild type) and DY984 (*sin3* mutant) strains. Figure 5B shows that, like GAL4, HAP1 activity in the *sin3* mutant strain is also reduced approximately five fold when compared to that of the wild-type strain. The reporter plasmid which we used, pLGΔAlu*Xho*LEU2, contains only intact UAS1 se-

quences (the UAS2 region of the *CYC1* promoter is deleted). It appears that the *sin3* mutation only affects transcription through UAS1 sequences since the *sin3* mutation has no effect on the intact *CYC1* promoter (D.J.S., unpublished observations). The *sin3* mutation has no effect on the transcriptional activity of GCN4. The level of GCN4 transactivation is the same both in wild-type and *sin3* mutated strains (data not shown). These results suggest that the SIN3 protein positively regulates the transcriptional activities of GAL4 and the HAP1 activator and has no effect on the transcriptional activity of GCN4.

Effect of the *SIN3* deletion mutants on the transcriptional activities of GAL4 and the HAP1 activator

In order to identify the regions of the SIN3 protein that are required for the positive regulation of GAL4 and the HAP1 activator, the *SIN3* PAH deletion mutants (shown in Fig. 3) were transformed into the *sin3* mutant strain (DY984). As shown in Table 1, the vector directing the expression of full-length SIN3 restores GAL4 and HAP1 activities in the *sin3* mutant strains to a level comparable to that of the wild-type strain, while the vector lacking the *SIN3* gene was unable to restore the activities of GAL4 and the HAP1 activator (see controls). Data shown in Table 1 also demonstrate that the deletion of the PAH2 motif of the SIN3 protein has no significant effect on GAL4 activity, while the deletions of the PAH4 and PAH1 motifs have a moderate effect. However, deletion of the PAH3 motif has a more drastic effect on GAL4 activity and, furthermore, GAL4 activity in the presence of this deletion is comparable to that of cells which do not express SIN3. Data shown in Table 1 also suggest that, similar to hPR, the deletion of the PAH1, PAH2 or PAH4 motifs has no significant effect on the transcriptional activity of HAP1. However, the PAH3 deletion mutant was unable to restore HAP1 activity. Expression levels of all the deletion mutants (PAH1–PAH4) are comparable when judged by western blot analysis (data not shown). These results suggest that the PAH1 and PAH3 motifs of the SIN3 protein are required for GAL4 transcriptional activity (when GAL4 is expressed under glucose conditions), whereas only the PAH3 motif is essential for maximum HAP1 transcriptional activity.

The data presented in this paper demonstrate that the SIN3 protein differentially influences the transcriptional activities of various diverse transcriptional activators. It negatively regulates the transcriptional activity of human progesterone receptor and positively regulates the transcriptional activities of GAL4 and the HAP1 activator. However, it has no effect on the transcriptional activities of the human glucocorticoid receptor and GCN4.

Discussion

The discovery that steroid hormone receptors and other mammalian transcription factors function in yeast has opened up a powerful new avenue for dissecting the transcriptional regulatory mechanisms of steroid hormone receptors and various other transactivators (Giguere et al. 1986; Mak et al. 1989; McDonnell et al. 1991; Metzger et al. 1988; Nawaz et al. 1992; Vegeto et al. 1992; Yoshinaga et al. 1992). It has been shown that the transactivation functions of DNA-binding factors can be regulated by non-DNA-binding regulatory proteins (Berger et al. 1992; Pugh and Tjian 1991; Wang et al. 1990). This study has focused on examining the mechanisms by which a non-DNA-binding protein (SIN3) regulates the transactivation functions of the various mammalian and yeast DNA-binding transcription factors.

SIN3 was originally identified as a negative regulator of the *HO* gene (Nasmyth 1993; Vidal et al. 1991). It also negatively regulates other diverse yeast genes such as *SPO11* (required for sporulation), *PHO5* (*enco*ding acid phosphatase), and *FUS1* (required for mating) (Vidal et al. 1991). It also has been reported that the SIN3 protein functions as a positive transcriptional activator. It positively regulates the transcriptional activity of the *STE2*, *STE3*, and *STE6* genes (genes required for mating) (Vidal et al. 1991). The activation function of the SIN3 protein may be indirect. The data presented in this study also suggest that the SIN3 protein negatively regulates the transcriptional activity of hPR and positively regulates the transactivation functions of GAL4 (under glucose conditions) and HAP1 by an indirect mechanism. Our data also suggest that the SIN3 protein is not a general regulatory protein since it specifically affects the transactivation functions of hPR, GAL4, and HAP1 but not those of hGR and GCN4. The SIN3 protein has a number of features in common with the SSN6 protein. Both are non-DNA-binding proteins and have similar repeated motifs, the PAH repeat in SIN3 and the TPR repeat in SSN6. Recently, it has been demonstrated that LexA-SSN6 and LexA-SIN3 fusion proteins can repress transcription of promoters containing lexA operators suggesting that *SIN3* and *SSN6* interact with DNA by a similar mechanism (Keleher et al. 1992; Wang and Stillman 1993). Previously, work from our laboratory has shown that the SSN6 protein affects the function of the AF1 activator of the human estrogen receptor without affecting the AF2 activator, suggesting specificity of SSN6 (McDonnell et al. 1992). Results presented in this study also suggest that the SIN3 protein, like SSN6, has a regulatory preference for the AF1 of hPR. This observation suggests that the SIN3 and SSN6 proteins regulate the transactivation functions of hPR by similar mechanisms.

hPR and hGR are very closely related proteins and both interact with the same *cis*-acting DNA promoter elements (Chalepakis et al. 1988; Greene and Chambon 1986), yet the *sin3* mutation only affects the activity of

hPR. The fact that the same reporter plasmid was used in our experiments to measure the transcriptional activation functions of hPR and hGR allows us to conclude that the *sin3* effect is not mediated through the promoter itself. Several possible mechanisms could account for this differential gene regulation. One possibility is that the SIN3 protein could interact differentially with various receptor proteins. However, we were unable to detect direct interaction of the SIN3 protein with the receptors. Alternatively, the differential effect of the *sin3* mutation on the transcriptional activity of hPR and hGR could be indirect.

In this study, we also show that in *sin3* mutant strains the transcriptional activities of GAL4 and the HAP1 activator are reduced. This observation is in agreement with that of Vidal et al. (1991), who showed that the transcriptional activities of the *STE2*, *STE3*, and *STE6* genes were reduced in *sin3* mutant strains. Recently, it has been shown that in *sin3* mutant strains the expression of the *STA1* (glucoamylase), *STE*, and *SWI1* (*ADR6*, *GAM3*) genes is decreased and, therefore, it has been argued that the SIN3 protein functions as an activator (Yoshimoto et al. 1992). However, our results show that the SIN3 protein has no effect on the expression levels of GAL4 and other transcriptional activators used in this study. Therefore, the SIN3 protein affects the functions of GAL4 and the HAP1 activator by a mechanism not related to the expression levels. It is possible that SIN3 functions primarily as a transcriptional repressor and that transcriptional activation defects in *sin3* mutants are indirect. This possibility is consistent with the fact that we were unable to detect an interaction of GAL4 with the SIN3 protein. Moreover, the LexA-SIN3 fusion protein fails to function as a transcriptional activator of lexA operator-containing promoters (Wang and Stillman 1993).

On the basis of the results presented in this study as well as those from other studies, several models can account for the observed effects of the *sin3* mutation on transcriptional regulation. In the first model, *SIN3* regulates the chromatin structure. It has been suggested that chromatin structure has an influence on gene regulation. The observation that not all genes are regulated by *SIN3* could be explained by local differences in chromatin structure. *SIN3*-dependent global alterations in chromatin structure may enhance the activity of DNA-binding proteins and thus enhance transcriptional regulation. Studies analyzing the regulation of the *PHO5* gene by *PHO4* and *PHO2* (Straka and Horz 1991), the regulation of *GAL1* by *GAL4* (Axelrod et al. 1993), the regulation of the MMTV promoter by the human glucocorticoid receptor (Pina et al. 1990), and the function of the α2 repressor in *S. cerevisiae* (Roth et al. 1990) have established that transcriptional regulators are able to alter nucleosomes in order to modulate gene transcription. In addition to studies which show the inhibitory effect of chromatin structure on gene transcription, there are a few studies showing that formation of a nucleosome provides a scaffolding that results in transcriptional enhancement of *trans*-acting factors (Croston and Kadonaga 1993; Eglin 1988; Kamakaka et al. 1993; Schild et al. 1993). Therefore, it is possible that *SIN3* can regulate gene transcription in both directions by modulating nucleosome stability. Our data do not support this model since SIN3 only regulates the activity of hPR and not hGR, even though the same reporter plasmid was used to measure the activities of both receptors.

In the second model, the effect of the *sin3* mutation on the transactivation functions of the *trans*-acting factors could be indirect. Based on our data we favor this model. It is possible that the SIN3 protein modulates the activities of the transcription factors by affecting post-translational modification (glycosylation, phosphorylation, etc.) of these proteins. For example, the *sin3* mutation leads to increased expression of a protein phosphatase, and the altered phosphatase activity may lead to a change in both the phosphorylation state and transcriptional activity of a factor such as hPR and GAL4. This possibility is consistent with our previously published results that okadaic acid, an inhibitor of protein phosphatases I and 2A, activates transcription of PR without affecting the basal transcription (Denner et al. 1990). This model is further supported by the work of Wang et al. (1994) who demonstrated that the *sin3* mutation alters *STE6* expression by affecting the phosphorylation state of the STE12 transcriptional activator. Alternatively, the SIN3 protein may affect the DNA-binding activity of these *trans*-acting factors, thereby modulating their transactivation functions.

In the third model, the SIN3 protein alters the activities of the transcription factors through protein-protein interactions. However, we were unable to detect direct interaction of the SIN3 protein with hPR or GAL4.

The results presented in this study suggest that the PAH1, PAH2, and PAH4 motifs of the SIN3 protein are not essential for *SIN3*-mediated gene regulation of hPR and the HAP1 activator. However, the PAH3 motif is absolutely essential for *SIN3*-mediated gene regulation of these *trans* factors. This observation is somewhat puzzling in that the same motif (PAH3) is required for *SIN3*-mediated gene repression and activation. It is possible that the PAH3 motif of the SIN3 protein is essential for post-translational modification of the transcription factors. Alternatively, it is possible that the PAH3 motif exerts its effect on gene regulation by maintaining the tertiary structure of the SIN3 protein. In constrast, the PAH1, PAH3, and PAH4 motifs of the SIN3 protein are required for *SIN3*-mediated gene regulation of GAL4. Based on these observations, we speculate that the *SIN3* PAH domains have distinct regulatory functions for the regulation of hPR, GAL4, and HAP1 transcriptional activities.

Acknowledgements We thank Dr. Sophia Tsai, and all other members of the laboratory for critically reading the manuscript. We also thank Dr. G. Greene from the University of Chicago for providing JZB39 antibodies; Dr. Wade Harper from Baylor College of Medicine for the GAL4 antibodies; Dr. Leonard Guarente for the 14 × 2, pLGSD5LEU2, and pLGΔAlu*Xho*LEU2 plas-

mids; Jutta Koenigsfeld from the laboratory of Dr. Rainer Renkawitz for plasmid PCL2; and Paula Howard for skilled technical assistance and proof reading. T.P.B. was supported by a postdoctoral fellowship from the National Institutes of Health (HD-07738). This work was supported by ACS (BE-63) funding.

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