MOLECULAR AND CELLULAR BIOLOGY, Aug. 1997, p. 4852–4858 Vol. 17, No. 8
0270-7306/97/$04.00+0

A Large Protein Complex Containing the Yeast Sin3p and Rpd3p Transcriptional Regulators

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Received 3 December 1996/Returned for modification 11 March 1997/Accepted 6 May 1997

The *SIN3* gene is required for the transcriptional repression of diverse genes in *Saccharomyces cerevisiae*. Sin3p does not bind directly to DNA but is thought to be targeted to promoters by interacting with sequence-specific DNA-binding proteins. We show here that Sin3p is present in a large multiprotein complex with an apparent molecular mass, estimated by gel filtration chromatography, of greater than 2 million Da. Genetic studies have shown that the yeast *RPD3* gene has a function similar to that of *SIN3* in transcriptional regulation, as *SIN3* and *RPD3* negatively regulate the same set of genes. The *SIN3* and *RPD3* genes are conserved from yeasts to mammals, and recent work suggests that *RPD3* may encode a histone deacetylase. We show that Rpd3p is present in the Sin3p complex and that an *rpd3* mutation eliminates *SIN3*-dependent repression. Thus, Sin3p may function as a bridge to recruit the Rpd3p histone deacetylase to specific promoters.

The product of the *SIN3* gene (also known as *GAM3*, *RPD1*, and *UME4*) functions as a transcriptional repressor in *Saccharomyces cerevisiae*. Sin3p represses a variety of genes, including *HO*, which encodes a site-specific endonuclease that initiates mating type switching, *TRK2*, which encodes a potassium transporter, *IME2*, which encodes an inducer of meiotic genes, *INO1*, the inositol synthase gene, *SPO11* and *SPO13*, two sporulation genes, and *STA1*, a gene encoding an extracellular glucoamylase (3, 12, 30, 38, 39, 44). A LexA-Sin3p fusion protein represses transcription from promoters containing a LexA binding site, demonstrating that Sin3p functions as a transcriptional repressor (41). Nonetheless, the Sin3p protein itself does not directly bind to DNA (40), and it has been suggested that Sin3p may interact with sequence-specific DNA-binding proteins in order to target Sin3p to specific promoters for repression (41). A mouse *SIN3* protein homolog has been identified that interacts with the Mad family of DNA-binding proteins (1a), and we have recently shown that interaction between Mad1 and yeast Sin3p leads to transcriptional repression in yeast (15).

SIN3 encodes a 175-kDa protein that contains four paired amphipathic helix motifs. Each motif consists of two amphipathic helices separated by a short amino acid linker. Similar structural motifs have been identified in helix-loop-helix and tetratrico peptide repeat proteins, and each of these motifs has been proposed to mediate protein-protein interactions. The fact that Sin3p contains four proposed protein association domains implies that Sin3p may interact with multiple proteins and suggests that the Sin3p repressor may form a large regulatory complex.

The *RPD3* gene functions as a negative transcriptional regulator of the same promoters that are regulated by *SIN3* (3, 20, 29, 37). The phenotype of a *sin3 rpd3* double mutant is no more severe than that of either single mutant, in terms of transcriptional repression of the *HO*, *IME2*, *INO1*, and *SPO13* genes (28, 29). This suggests that *SIN3* and *RPD3* function in the same regulatory pathway (29). Recent work suggests that *RPD3* encodes a histone deacetylase (24, 32). As there is a strong genetic link between *SIN3* and *RPD3*, it has been suggested that Sin3p's interaction with specific DNA-binding proteins may function to target Rpd3p to specific promoters, leading to transcriptional repression of regions of chromatin (43). Recent work indicates that an *rpd3* mutation affects transcriptional silencing (7, 24, 36), but this may be an indirect effect.

The lysine residues near the N terminus of histones are subject to reversible modification by acetylation. It has been proposed that acetylation of histones stimulates transcription by weakening histone-DNA interactions, as transcriptionally silent regions of the genome are underacetylated (35, 43). It was recently shown that the *Tetrahymena* histone acetyltransferase A is homologous to the product of yeast *GCN5* (4). This result is consistent with the genetic data showing that *GCN5* encodes a transcriptional activator. Conversely, it makes sense that a negative regulator of transcription such as *RPD3* could encode a histone deacetylase, as an increase in histone acetylation at a specific promoter in an *rpd3* mutant could cause inappropriate gene activation.

In this report we demonstrate that Sin3p is present in a large protein complex, greater than 2 MDa in size. We show that Rpd3p is present in this complex, and that transcriptional repression by a LexA-Sin3p fusion protein is dependent upon Rpd3p. Thus, the recognition of DNA-binding proteins by Sin3p would also target Rpd3p, a putative histone deacetylase, to specific promoters.

MATERIALS AND METHODS

Strains and media. The isogenic yeast strains used in this study are in the W303 background (33). DY150 (wild type) and DY984 (*sin3*::*ADE2*) have been described previously (41). DY3425 (*pep4*::*URA3*) was provided by Rod Rothstein. Strain DY1539 (*rpd3*::*LEU2*) was made by Huaming Wang from DY150 by disrupting the *RPD3* gene with plasmid M1436; correct gene disruption was confirmed by Southern blotting. Strain DY4441 was constructed by integrating the *CYC1-lexA-HIS3* reporter from plasmid M3295 at the *LYS2* locus, and the isogenic strains DY4624 (*CYC1-lexA-HIS3*) and DY4625 (*CYC1-lexA-HIS3 rpd3*::*LEU2*) were isolated from a cross between DY4441 and DY1539. Isogenic strains DY4627 (*CYC1-lexA-LacZ RPD3*) and DY4629 (*CYC1-lexA-LacZ rpd3*) were isolated from crosses involving DY1539 and DY1609 (*CYC1-lexA-LacZ*) (41). Cells were grown at 30°C in synthetic complete medium (26) containing

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either 2% glucose or 2% galactose, as indicated, supplemented with adenine, uracil, and amino acids, as appropriate, but lacking essential components to select for plasmids. Medium containing galactose was used to induce expression from the *GAL1* promoter, and medium containing 20 mM 3-aminotriazole (3-AT) was used to analyze repression of the *CYC1-lexA-HIS3* reporter by LexA-Sin3p.

Plasmids. Plasmid p*GAL1*:GST-Sin3p (M2950; YCp with *URA3*) expresses a glutathione *S*-transferase (GST)-Sin3 fusion protein from the *GAL1* promoter and was constructed by first inserting a *Bam*HI-*Xho*I *SIN3* fragment from plasmid M1448 (41) into *Bam*HI-*Xho*I-digested pRD56 (provided by Ray Deshaies [21]) and then creating an in-frame fusion by sequential treatment with *Bam*HI, Klenow DNA polymerase, and DNA ligase. Plasmid p*ADH*:HA-Sin3p (M1155; YCp with *URA3*) expresses Sin3p with an N-terminal hemagglutinin (HA) epitope tag from the *ADH1* promoter and was constructed in several steps. First an *Xho*I site was engineered at the 5′ end of the *SIN3* open reading frame, and then an *Xho*I fragment with *SIN3* was cloned into *Sal*I-cleaved pAD5 (provided by Jeffrey Field [9]); finally, a *Bam*HI (partial)-*Sal*I fragment with p*ADH*:HA-Sin3p was cloned into *Bam*HI-*Sal*I-cleaved pRS316 (27) to make M1155. Plasmid p*SIN3*:Sin3p-HA (M1922; YCp with *HIS3*) expresses the native *SIN3* gene product, Sin3p, with two HA epitopes cloned onto its C terminus (at amino acid 1494) and was constructed by first cloning oligonucleotides encoding HA epitopes into the *Bam*HI site at the codon for amino acid 1494 of clone SD241 (derived by exonuclease III digestion [39]) and then cloning a *Not*I-*Bam*HI fragment with the *SIN3*-HA gene into *Not*I-*Bam*HI-cleaved pRS313 (27). p*GAL1*:GST-Sin3p, p*ADH*:HA-Sin3p, and p*SIN3*:Sin3p-HA all express functional proteins, as they complement a *sin3* defect.

Plasmid p*ADH*:LexA-Rpd3p (M1782; YEp with *HIS3*), which expresses a LexA-Rpd3p fusion protein from the *ADH1* promoter, was constructed in two steps. First, *Not*I linkers were ligated onto an *Eco*RV-*Afl*II (blunted) fragment containing *RPD3*, and the *Not*I fragment was cloned into *Not*I-cleaved Bluescript II KS+, constructing plasmid M1663. Then an *Eco*RI fragment with *RPD3* from M1663 was cloned into *Eco*RI-cleaved pSH2-1 (provided by Roger Brent [10]), constructing an in-frame LexA-Rpd3p fusion plasmid. Plasmid LexA-Sin3p (M1459; YCp with *TRP1*) expresses a LexA-Sin3p fusion protein from the *ADH1* promoter and was constructed by cloning a *Kpn*I fragment with *SIN3* from plasmid M1448 (41) into *Kpn*I-cleaved pJK152-1, provided by Roger Brent. Plasmids (YEp with *HIS3*) that express LexA (pSH2-1) or LexA-Sin3p (M1153) have been described previously (41). Plasmid M3295 (YIp with *LYS2*), which contains the *CYC1* promoter, driving expression of the *HIS3* open reading frame, and a LexA operator inserted between the *CYC1* UAS and TATA elements, was constructed in several steps. First, an *Xba*I-*Bam*HI fragment from plasmid pCK30 (41) with the *CYC1-lexA* promoter was cloned into *Xba*I-*Bam*HI-cleaved pTBH (provided by John Phillips), which has a *Bam*HI site engineered just upstream of the *HIS3* ATG codon, constructing M3287. Then a *Sma*I-*Eco*RV fragment from M3287 with the *CYC1-lexA-HIS3* reporter was cloned into the *Sma*I site of YDp-K (provided by Gilbert Berben [2]), constructing M3295. The *LYS2* gene in this YIp plasmid allows integration at the *LYS2* locus.

Chromatography. Cell extracts were prepared essentially as described previously (17) except that column buffer (350 mM NaCl, 10% glycerol, 20 mM Tris [pH 8.0], 0.1% Tween 20, 1 mM sodium azide, 0.5 mM dithiothreitol, and protease inhibitors [174-μg/ml phenylmethylsulfonyl fluoride, 1.3-μg/ml leupeptin, and 0.3-μg/ml pepstatin A]) was used for S-100 preparation. The protein concentrations of the extracts, as determined by Bradford assay (Bio-Rad), typically ranged between 20 and 45 mg/ml. Two milliliters of yeast extract (approximately 40 to 90 mg) was loaded onto a 1.6- by 40-cm Sephacryl S-400 HR (Sigma) column equilibrated in column buffer and connected to a fast protein liquid chromatography (FPLC) apparatus (Pharmacia). Fractions containing GST-Sin3p were pooled, and approximately 2.5 ml of the pool was mixed with 0.15 ml of glutathione-agarose (Sigma) resin, mixed by rotation for several hours, and washed six times with a solution containing 1 ml of 100 mM NaCl, 20 mM Tris (pH 8.0), 10% glycerol, and 0.5 mM EDTA. The bound proteins were eluted with a solution containing 0.1 ml of 100 mM NaCl, 50 mM Tris (pH 8.0), 10% glycerol, and 20 mM reduced glutathione. The elution profile of the Sephacryl S-400 HR column was determined with the following markers: Dextran Blue (2 MDa), thyroglobulin (669 kDa), ferritin (443 kDa), catalase (232 kDa), and aldolase (158 kDa). Additionally, anti-S4 antibody was used to probe yeast proteins eluting from the sizing column for the presence of the Yta5p subunit of the 26S proteosome.

Protein analysis. Proteins were detected directly on immunoblots incubated with either anti-LexA serum (obtained from Erica Golemis), anti-GST-Sin4p serum (absorbed with wild-type yeast extract), which recognizes the GST domain (13), 12CA5 monoclonal anti-HA epitope antibody, anti-Sin3p antibody (39), anti-Rpd3p antibody (24), or anti-S4 serum (obtained from Marty Rechsteiner), which recognizes the Yta5p subunit of the 26S proteosome, and the proteins were visualized with an enhanced chemiluminescence kit (Amersham or Pierce). Where indicated, proteins samples were concentrated approximately sevenfold by acetone precipitation before gel electrophoresis. Sodium dodecyl sulfate (SDS)-polyacrylamide gels were stained with silver as described previously (25), except that the gels were fixed in 40% methanol–5% formaldehyde and treated with 0.02% sodium thiosulfate (instead of dithiothreitol).

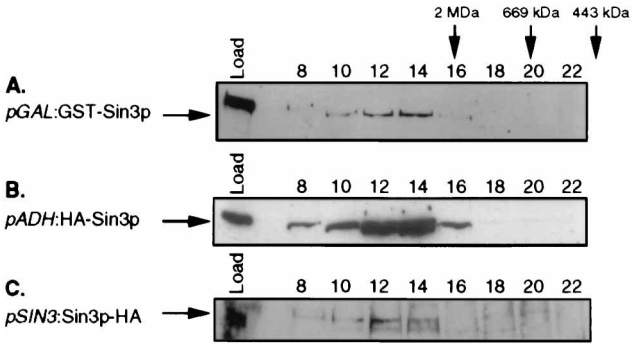


FIG. 1. The Sin3p complex is greater than 2 MDa in size. Yeast protein extracts were chromatographed on a Sephacryl S-400 HR FPLC sizing column, and samples were analyzed for Sin3p by immunoblotting. (A) Extracts were prepared from strain DY150 (wild type) expressing a GST-Sin3p fusion protein from the *GAL1* promoter. The blot was probed with antibody that recognizes GST. (B) Extracts were prepared from strain DY150 (wild type) expressing an N-terminal HA epitope-tagged Sin3p fusion protein from the *ADH1* promoter. The blot was probed with the 12CA5 monoclonal antibody, which recognizes the HA epitope. (C) Extracts were prepared from strain DY3425 (*pep4*::*URA3*) expressing a C-terminal HA epitope-tagged Sin3p fusion protein from the native *SIN3* promoter. The blot was probed with the 12CA5 monoclonal antibody, which recognizes the HA epitope. The protein samples in panels B and C were concentrated by acetone precipitation before gel electrophoresis. The arrows at the top indicate the peak elution fractions for the marker proteins: Yta5p in the 26S proteosome (2 MDa, fraction 16), thyroglobulin (669 kDa, fraction 20), and ferritin (443 kDa, fraction 23). The peak elution fractions for catalase (232 kDa, fraction 27) and aldolase (158 kDa, fraction 28) are shown in Fig. 3A.

RESULTS

Sin3p is present in a large complex. As many transcriptional components are present in large complexes, it was of interest to determine the size of the native Sin3p complex. In preliminary experiments, we used glycerol gradient centrifugation to determine that Sin3p is present in a complex sedimenting at 23S (data not shown). This sedimentation rate suggests that Sin3p is present in a very large protein complex. To examine the possible interaction of other proteins with Sin3p, we decided to tag Sin3p with GST. GST binds with high affinity to glutathione, and GST fusion proteins can be purified by chromatography on a glutathione-agarose affinity matrix. A plasmid was constructed which expresses in yeast a GST-Sin3p fusion protein from the inducible *GAL1* promoter. We demonstrated that this GST-Sin3p fusion plasmid complemented a *sin3* defect when cells were grown on galactose-containing medium (data not shown).

Whole-cell extracts were prepared from strains expressing the GST-Sin3p fusion and applied to an FPLC Sephacryl S-400 HR gel filtration column. Antibody against GST detected a 200-kDa GST-Sin3p fusion protein on immunoblots. This 200-kDa protein, which was also detected by antisera to Sin3p, was absent from immunoblots of extracts prepared from cells lacking the GST-Sin3p fusion plasmid (data not shown). The GST-Sin3p peak elutes from the gel filtration column in fractions 12 to 14 (Fig. 1A), which indicates an apparent molecular mass of greater than 2 MDa. Thus, Sin3p is present in a very large protein complex.

To verify that the profile of Sin3p elution from the Sephacryl S-400 column was not an artifact from expression of a GST-Sin3p fusion protein, we examined the chromatography of extracts prepared from strains with two different epitope-tagged *SIN3* constructs. Plasmid p*ADH*:HA-Sin3p expresses an N-terminally epitope-tagged Sin3p protein from the strong *ADH1* promoter. As shown in Fig. 1B, the HA-Sin3p peak elutes from the gel filtration column in fractions 12 to 14, as observed for

GST-Sin3p. Finally, we used plasmid p*SIN3*:Sin3p-HA, which expresses a C-terminally tagged Sin3p protein; in this case the fusion protein is expressed from the native *SIN3* promoter. Again, the gel filtration shows that the Sin3p complex elutes from the column at a volume expected for a protein complex with a molecular mass greater than 2 MDa. The *SIN3* gene encodes a protein of 175 kDa (or 200 kDa for GST-Sin3p), and if this protein is present as a monomer, uncomplexed with other proteins, it should elute from a sizing column between the positions of the ferritin (443 kDa) and aldolase (158 kDa) markers. However, we have never detected any Sin3p eluting after the ferritin marker, even when overproduced from the *ADH1* or *GAL1* promoter. This suggests that all of the Sin3p protein is present in large complexes and that the other proteins that are required to form these complexes are not limiting. In the first two experiments Sin3p was overproduced from the strong *ADH1* or *GAL1* promoters, but this last experiment with p*SIN3*:Sin3p-HA shows that the large size is not an artifact caused by overproduction.

Sin3p is part of a multiprotein complex. Since Sin3p contains four paired amphipathic helix motifs that are proposed to be protein-binding domains, we examined whether other proteins are present in the Sin3p complex. The high affinity of GST for glutathione allows one to purify a GST fusion protein, along with any associated proteins, on a glutathione-agarose resin. Protein extracts from a strain expressing GST-Sin3p were first sized by gel filtration chromatography, and then the 2-MDa pool containing GST-Sin3p was fractionated by glutathione affinity chromatography. After extensive washing, the proteins specifically bound to the column were eluted with glutathione; less than 1% of the proteins present in the load are present in the eluate. Aliquots of the load and eluate fractions from the glutathione column were electrophoresed on 6% polyacrylamide gels containing SDS, and the proteins were visualized by silver staining. We also analyzed the last wash fraction to demonstrate that no proteins were coming off the column when the eluate buffer was applied. As a control, we also sought to identify proteins that bind to the GST portion of GST-Sin3p. An extract expressing GST only was also size fractionated, and the 100-kDa and 2-MDa pools were also fractionated on glutathione-agarose.

Approximately 10 protein bands with sizes ranging from 80 to 225 kDa are found to be specifically associated with GST-Sin3p (Fig. 2). The estimated sizes for the most prominent proteins are as follows: four proteins between 162 and 172 kDa, one at 135 kDa, and one at 80 kDa. Fainter bands are estimated to be proteins of 225, 155, 150, and 100 kDa. These proteins are not degradation products of the 200-kDa GST-Sin3p or the native Sin3p (175 kDa), as they do not react with anti-Sin3p antibody on immunoblots (data not shown). Proteins in the complex smaller than 43 kDa would not be detected on the 6% gel used here. However, analysis on a 7.5% gel did not reveal any specific protein bands between 29 and 43 kDa (data not shown). Proteins smaller than 29 kDa have not been examined. It is possible that the Sin3p complex contains specific proteins smaller than 80 kDa that are not visible in the silver-stained gel due to lack of sensitivity. There is a very prominent band at 75 kDa, and several fainter bands (e.g., 215 kDa) are seen in the glutathione eluate of the 100-kDa fraction from the GST-only strain, suggesting that these proteins associate with the GST portion of the GST-Sin3p fusion protein. Thus, the 75- and 215-kDa proteins do not specifically associate with Sin3p. This experiment shows that there are a number of proteins that are stably associated with Sin3p.

The identity of these Sin3p-associated proteins, and their role in transcriptional regulation, is clearly an interesting question. The *CCR4* (6a, 8), *NOT* (6), and *SWI/SNF* (5, 22) transcriptional regulators are all present in large protein complexes. However, apparently none of these gene products correspond to the sizes of the proteins present in the Sin3p complex. We have conducted a two-hybrid screen and identified five gene products that interact with Sin3p, and two of these proteins from this screen, Stb1p and Stb2p, can be detected in the GST-Sin3p complex purified by glutathione affinity chromatography (16).

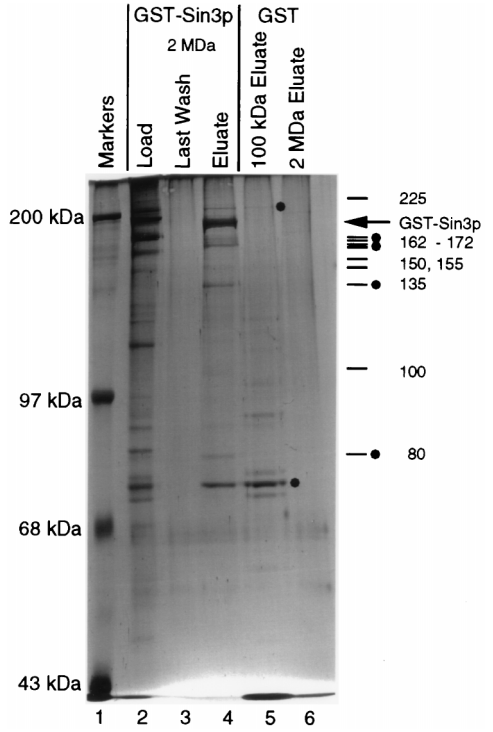


FIG. 2. Sin3p associates with a number of other proteins. Extracts prepared from strain DY150 (wild type) expressing either GST-Sin3p or GST from the *GAL1* promoter were fractionated by size, subjected to glutathione affinity chromatography, electrophoresed on a 6% polyacrylamide gel with SDS, and stained with silver. Approximately 1% of the protein loaded on the column was recovered in the eluate fraction. For GST-Sin3p, proteins with an approximate molecular mass of 2 MDa (including GST-Sin3p) were chromatographed on the glutathione resin, and proteins present in the glutathione load (lane 2), last wash (lane 3), and eluate (lane 4) fractions were examined by electrophoresis. For the GST-only experiment, the proteins approximately 2 MDa in size (where GST-Sin3p elutes) and the proteins approximately 100 kDa in size (where GST elutes) were examined (lanes 5 and 6). Lane 1 contains protein size standards. The following amounts of the protein fractions are shown on the gel: 0.4% of the load sample (10 μl/3 ml) and 40% of the last wash and eluate samples (60 μl/150 μl). The GST protein (26 kDa) has run off the gel. The arrow on the right indicates GST-Sin3p. The short lines depict the migration of the 10 proteins that specifically coelute with GST-Sin3p, with the more prominent bands being designated with a circular mark. The 215- and 75-kDa proteins that are nonspecific, as they associate with GST alone, are indicated by a mark adjacent to lane 5.

The relative amounts of the different proteins in the Sin3p complex detected by silver staining raises some questions about stoichiometry, as the associated proteins do not appear to be present in stoichiometric amounts with Sin3p. This observation might be explained by the fact that there is variability in the staining of individual protein species by silver (34). The 200-kDa GST-Sin3p band appears to be much more abundant than the other Sin3p-associated proteins. There are several ways to explain this observation. First, there could be several other 200-kDa proteins that migrate at the same position as GST-Sin3p, and their staining with silver gives the false im-

pression of abundance. Second, it is possible that each protein complex contains several Sin3p molecules, along with one molecule of each of the associated proteins. Third, some of the proteins may be only weakly associated with the Sin3p complex, and thus the apparent lack of stoichiometry could reflect a loss of proteins during glutathione affinity chromatography. Finally, the apparent lack of stoichiometry may be due to the fact that GST-Sin3p is produced at high levels from the *GAL1* promoter. Nonetheless, we emphasize that Sin3p elutes from a sizing column at an approximate molecular mass of 2 MDa, whether Sin3p is expressed from its native promoter or from a strong promoter.

Rpd3p copurifies with Sin3p. Yeast strains with an *rpd3* mutation have many of the same phenotypes as *sin3* mutants (3, 20, 37). Additionally, genetic analysis suggests that *SIN3* and *RPD3* function in the same pathway (29). We were therefore interested in determining whether Rpd3p is present in the Sin3p complex. In the first series of experiments protein extracts were prepared from a yeast strain expressing both GST-Sin3p and LexA-Rpd3p and chromatographed on the Sephacryl S-400 gel filtration column. An antibody to LexA (generously provided by E. Golemis) allowed us to detect the LexA-Rpd3p fusion protein. The LexA-Rpd3p peak elutes from the gel filtration column in fraction 13 (Fig. 3A), the fraction in which the Sin3p peak elutes. There is some additional LexA-Rpd3p eluting in fractions 28 to 31, which correspond to sizes of less than 200 kDa. LexA-Rpd3p is 75 kDa in size, and the elution profile suggests that this LexA-Rpd3p protein, in fractions 28 to 31, is not complexed with Sin3p and may be monomeric. In this experiment LexA-Rpd3p is overproduced from the *ADH1* promoter, and thus there could be an excess of LexA-Rpd3p compared to other proteins in the 2-MDa complex.

The gel filtration experiment shows only that GST-Sin3p and LexA-Rpd3p are present in complexes of similar size. To demonstrate that GST-Sin3p and LexA-Rpd3p are present in the same complex, we used glutathione affinity chromatography. The fractions with proteins approximately 2 MDa in size, containing GST-Sin3p and LexA-Rpd3p, were pooled and applied to glutathione-agarose. The load, the final wash, and the eluate fractions from the glutathione affinity chromatography column were analyzed on immunoblots probed with antibodies recognizing either GST or LexA. Both the GST-Sin3p and LexA-Rpd3p proteins are present in the eluate from the glutathione column, demonstrating that Rpd3p associates with Sin3p (Fig. 3B, lanes 1 to 3). Additionally, a 2-MDa size-fractionated protein sample containing Sin3p and Rpd3p was chromatographed on either MonoS or MonoQ resins, and Sin3p and Rpd3p cofractionated on both resins (data not shown).

To demonstrate the specificity of this interaction, extracts were prepared from several control strains and analyzed. An extract prepared from a strain expressing GST-Sin3p and LexA (instead of LexA-Rpd3p) shows that the LexA DNA-binding domain does not interact with GST-Sin3p (data not shown). To demonstrate that LexA-Rpd3p does not interact with the GST domain, an extract was prepared from a strain expressing GST (instead of GST-Sin3p) and LexA-Rpd3p and subjected to gel filtration chromatography. Two protein pools from this size-fractionated GST–LexA-Rpd3p extract, one at 2 MDa (the size of the GST-Sin3p complex) and one at 100 kDa (the native size of tetrameric GST), were then fractionated by glutathione affinity chromatography. The GST protein is clearly present in the glutathione column eluate loaded with the 100-kDa pool (Fig. 3B, lane 5). Importantly, LexA-Rpd3p did not associate with GST (Fig. 3B, lanes 4 and 5). We conclude that the interaction between Sin3p and Rpd3p is specific.

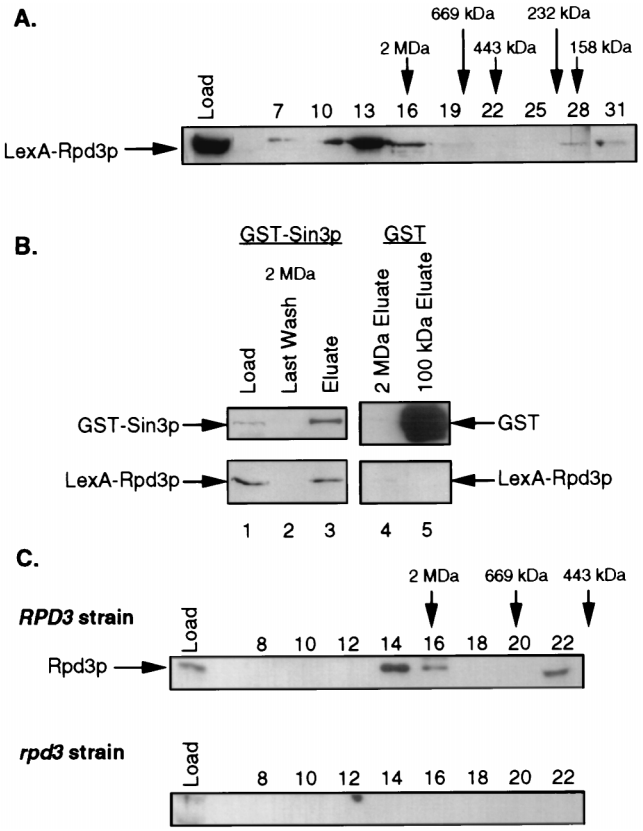
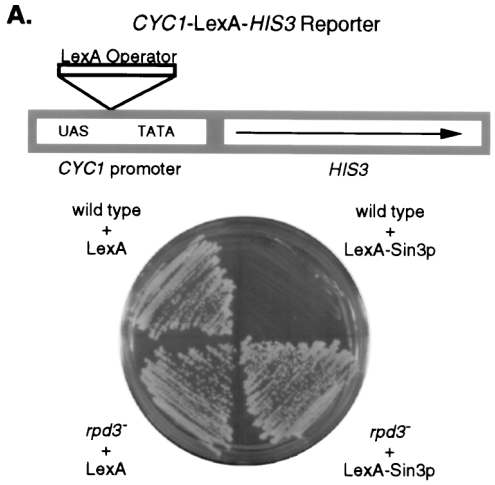


FIG. 3. Rpd3p copurifies with Sin3p. (A) Yeast protein extracts prepared from strain DY150 (wild type) expressing LexA-Rpd3p fusion protein expressed from the *ADH1* promoter were chromatographed on a Sephacryl S-400 HR FPLC sizing column, and samples were analyzed for LexA-Rpd3p by probing an immunoblot with antibody that recognizes LexA. The small arrows indicate the peak elution fractions for the marker proteins described in the legend to Fig. 1. (B) Extracts prepared from strain DY984 (*sin3*::*ADE2*) containing a plasmid expressing LexA-Rpd3p and a second plasmid expressing either GST-Sin3p or GST only were first fractionated by size and then subjected to glutathione affinity chromatography. For the GST-Sin3p experiments, proteins with an approximate molecular mass of 2 MDa (including GST-Sin3p) were chromatographed on the glutathione resin, and samples from the glutathione load (lane 1), last wash (lane 2), and eluate (lane 3) fractions were analyzed on immunoblots with antisera recognizing either GST or LexA. For the GST-only experiments, proteins with an approximate molecular mass of 2 MDa (lane 4), where GST-Sin3p elutes, and with an approximate molecular mass of 100 kDa (lane 5), where GST elutes, were chromatographed on glutathione, and the eluate fractions were analyzed. Proteins migrating at 200, 68, and 26 kDa were probed for GST-Sin3p, LexA-Rpd3p, and GST, respectively. (C) Yeast protein extracts prepared from strain DY150 (*RPD3*) and strain DY1539 (*rpd3*) expressing LexA-Rpd3p (from the *ADH1* promoter) and Sin3p-HA (from the *SIN3* promoter) were chromatographed on a Sephacryl S-400 HR FPLC sizing column, and samples were analyzed for Rpd3p by probing an immunoblot with anti-Rpd3p antibody (23). The arrows indicate the peak elution fractions for marker proteins described in the legend to Fig. 1.

In the next experiment we examined native Rpd3p expressed from its own promoter, in contrast to the LexA-Rpd3p fusion protein expressed at high levels from the *ADH1* promoter. *RPD3* and *rpd3* strains were transformed with the p*SIN3*: Sin3p-HA plasmid that expresses epitope-tagged Sin3p-HA from the native *SIN3* promoter. Protein extracts were chromatographed on the Sephacryl S-400 gel filtration column, and fractions were probed with antisera to Rpd3p (24), generously provided by Michael Grunstein. Rpd3p elutes from the sizing column at the fractions that contain Sin3p-HA, with an apparent molecular mass of greater than 2 MDa (Fig. 3C). A portion of the Rpd3p protein elutes from the column with an apparent

molecular mass of approximately 500 kDa. Importantly, the antisera did not recognize any 49-kDa protein species in the protein fractions prepared from the *rpd3* null-mutant strain. We used anti-Rpd3p antibody to detect Rpd3p in the glutathione affinity-purified GST-Sin3p complex, but only a weak signal was seen (data not shown). We explain the result of this experiment by noting that GST-Sin3p is overproduced from the *GAL1* promoter while Rpd3p is expressed at low levels from its own promoter.

***RPD3* is required for *SIN3*-dependent repression.** Sin3p does not bind DNA, but the fact that Sin3p binds to certain DNA-binding proteins (14, 15) provides a mechanism for targeting Sin3p to specific promoters. We have previously used a LexA-Sin3p fusion protein to demonstrate that Sin3p functions as a transcriptional repressor (41). The LexA DNA-binding domain targets the fusion protein to specific promoters containing a LexA operator, and the Sin3p portion of the fusion protein causes a 50- to 300-fold repression of a LacZ reporter gene (41). The experiment whose results are shown in Fig. 4A used yeast strains containing an integrated *CYC1-lexA-HIS3* reporter. In this reporter, expression of the *HIS3* gene is under the control of the *CYC1* promoter, with a LexA operator between the *CYC1* UAS and TATA elements. In this experiment we assayed the ability of the promoter to provide sufficient *HIS3* gene product for histidine prototrophy in the presence of 20 mM 3-AT (a competitive inhibitor of the *HIS3* gene product, imidazole glycerol phosphate dehydratase). LexA-Sin3p represses transcription of *CYC1-lexA-HIS3* to an extent that the strain is unable to grow in the absence of histidine, while expression of LexA alone allows growth. Importantly, disruption of the *RPD3* gene allows the LexA-Sin3p strain to grow in the absence of exogenous histidine. Thus, repression of the *CYC1-lexA-HIS3* reporter by LexA-Sin3p is lost in the *rpd3* mutant strain. An integrated *CYC1-lexA-LacZ* reporter allowed us to quantitate the extent of LexA-Sin3p repression simply by measuring β-galactosidase levels. As shown in Fig. 4B, LexA-Sin3p causes a nearly 250-fold repression of expression compared to the expression of *CYC1-lexA-LacZ* in the strain expressing LexA alone. The repression by LexA-Sin3p is largely reduced in an *rpd3* mutant. Finally, repression by LexA-Sin3p occurs when the LexA operator is placed upstream of the UAS element, suggesting that the decrease in transcription is not simply steric hindrance (data not shown and reference 41). Immunoblot analysis shows that the *rpd3* mutation does not affect the accumulation of the LexA-Sin3p fusion protein (data not shown), ruling out the possibility that the *rpd3* mutation has an indirect effect by altering the stability of LexA-Sin3p. These experiments with both the *CYC1-lexA-HIS3* and *CYC1-lexA-lacZ* reporters demonstrate that Rpd3p is required for transcriptional repression by Sin3p.



	LexA	LexA-Sin3p	Fold Repression
wild type	7600	25	300 x
rpd3 mutant	2300	360	6 x

FIG. 4. *RPD3* is required for repression by LexA-Sin3p. (A) The diagram shows the structure of the integrated *CYC1-lexA-HIS3* reporter, which has *HIS3* expression under the control of the *CYC1* promoter but with a LexA operator inserted between the *CYC1* UAS and TATA elements. The wild type (DY4624) and the *rpd3* mutant (DY4625) were transformed with TRP1 plasmids expressing either LexA (pJK152-1) or LexA-Sin3p (M1459), and the ability of these cells to grow on selective medium lacking tryptophan and histidine (containing 20 mM 3-AT) was determined. LexA-Sin3p represses expression of the *CYC1-lexA-HIS3* reporter to an extent that the strain is a histidine auxotroph, but an *rpd3* mutation eliminates this repression. (B) The diagram shows the structure of the integrated *CYC1-lexA-LacZ* reporter, which has *lacZ* expression under the control of the *CYC1* promoter but with a LexA operator inserted between the *CYC1* UAS and TATA elements. Strains DY4627 (*RPD3*) and DY4629 (*rpd3*) were transformed with plasmids that express either LexA (pSH2-1) or LexA-Sin3p (M1153). β-Galactosidase levels were measured from three independent transformants grown under selection to maintain the plasmids. The standard errors for β-galactosidase measurements were <20%.

DISCUSSION

The Sin3p transcriptional repressor does not bind DNA. Sin3p does interact with sequence-specific DNA-binding proteins (14, 15), leading to the idea that Sin3p is targeted to promoter sites by recognizing DNA-binding proteins. We show that Sin3p is present in a large protein complex whose profile of elution from a sizing column suggests a molecular mass exceeding 2 MDa and that the Rpd3p protein is present in this complex. Kadosh and Struhl (14) have also reported an interaction between yeast Sin3p and Rpd3p, using an immunoprecipitation assay, and similar results have been recently described for the mammalian homologs of Sin3p and Rpd3p (1, 11, 11a, 19, 20a, 45). The *RPD3* gene encodes a transcriptional repressor in the same pathway as Sin3p (29), and recent work suggests that *RPD3* encodes a histone deacetylase (24, 32). A LexA-Sin3p fusion protein represses transcription of reporter genes containing LexA operators, but this repression is lost in an *rpd3* mutant. Thus, the presence of Rpd3p in the Sin3p complex suggests the following mechanism for transcriptional repression. The Sin3p complex recognizes DNA-binding proteins and brings Rpd3p to a specific promoter. Rpd3p then functions to deacetylate histones in the vicinity, leading to transcriptional repression of that promoter.

The apparent molecular mass of the Sin3p complex, determined from the Sephacryl S-400 gel filtration column, is 2 MDa. How accurate is such a size estimate? Size determinations from gel filtration columns are based upon an assumption that the protein complex is globular. The fact that the Sin3p complex sediments at 23S in a glycerol gradient is consistent with the large size measured by size chromatography. A molecular mass of 2 MDa was determined for the Swi-Snf complex by gel filtration on a Superose 6 column (22). However,

the 2-MDa apparent molecular mass for the Swi-Snf complex is much greater than the 870-kDa total mass of the subunits (5, 34), and it has been suggested that the complex is somewhat elongated rather than globular (22). Although our size estimate is not precise, we can nonetheless conclude that the Sin3p complex is quite large.

It is intriguing that so many of the proteins involved in transcriptional regulation are present in large, multiprotein complexes. The *SSN6* (*CYC8*) and *TUP1* gene products, which repress the transcription of many genes, are present in a complex having an apparent size of 1.2 MDa (42). The Spt5p and Spt6p proteins physically interact, and genetic data suggest that the Spt4p protein may also be part of the complex (31). The Ccr4p transcriptional regulator is in a complex with several other proteins (8), and the size of the complex is greater than 2 MDa (6a). The products of *NOT* genes, which negatively regulate transcription, are part of a 500-kDa complex (6). The Swi-Snf multiprotein complex, which is believed to activate transcription by modulating chromatin structure, is 2 MDa in size (5, 22). Finally, RNA polymerase II can be isolated in a holoenzyme complex greater than 2 MDa in size (18). An obvious question remaining is how do all these large protein complexes interact to precisely control transcription?

ACKNOWLEDGMENTS

We acknowledge Erica Golemis, Michael Grunstein, Yi Wei Jiang, and Marty Rechsteiner for antibodies, Rod Rothstein and Huaming Wang for strains, and Gilbert Berben, Roger Brent, Ray Deshaies, Jeffrey Field, and John Phillips for plasmids. We also thank Clyde Denis and David Virshup for helpful advice and Yaxin Yu and Wendy Du for excellent technical assistance.

This work was supported by a grant from the National Institutes of Health awarded to D.J.S.

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