

Identification and Purification of a Protein That Binds DNA Cooperatively with the Yeast SWI5 Protein

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The *Saccharomyces cerevisiae* SWI5 gene encodes a zinc finger protein required for the expression of the *HO* gene. A protein fusion between glutathione *S*-transferase and SWI5 was expressed in *Escherichia coli* and purified. The GST-SWI5 fusion protein formed only a low-affinity complex in vitro with the *HO* promoter, which was inhibited by low concentrations of nonspecific DNA. This result was surprising, since genetic evidence demonstrated that SWI5 functions at the *HO* promoter via this site in vivo. A yeast factor, GRF10 (also known as PHO2 and BAS2), that promoted high-affinity binding of SWI5 in the presence of a large excess of nonspecific carrier DNA was purified. Final purification of the 83-kDa GRF10 protein was achieved by cooperative interaction-based DNA affinity chromatography. In vitro binding studies demonstrated that SWI5 and GRF10 bind DNA cooperatively. Methylation interference and missing-nucleoside studies demonstrated that the two proteins bind at adjacent sites, with each protein making unique DNA contacts. SWI5 and GRF10 interactions were not detected in the absence of DNA. The role of cooperative DNA binding in determining promoter specificity of eukaryotic transcription factors is discussed.

Proper control of gene expression often requires the interaction of multiple proteins with promoter elements. Although one might expect that sequence-specific DNA-binding proteins involved in gene regulation would have high affinities for binding sites, this expectation is not always true. For example, homeodomain proteins have affinities for specific binding sites only about 100-fold higher than that for nonspecific DNA in vitro (23). It has been suggested previously that additional protein factors participate in specific site recognition in vivo and that regulatory specificity is achieved through the combined action of multiple regulatory factors that bind to adjacent DNA sequences. Cooperative interactions between two DNA-binding proteins can provide the higher affinity and increased specificity required for promoter recognition. Additionally, specific interactions between different DNA-binding proteins allow different combinations of transcription factors to act at different genes, and thus a single transcription factor, depending on its partners, can be utilized in different contexts to regulate multiple target genes.

The *Saccharomyces cerevisiae* SWI5 gene was identified as a transcriptional activator of the yeast *HO* gene (42). *HO* encodes an endonuclease which initiates interconversion of the mating type loci (for reviews, see references 15 and 30). *HO* is expressed in only one of the two progeny cells produced by mitotic division, which leads to a specific pedigree in mating-type switching. It has been suggested previously that SWI5 plays a role in this asymmetric expression of *HO* (29, 31).

SWI5 encodes a zinc finger (TFIIIA-like) DNA-binding protein that binds in vitro to a site in the *HO* promoter that is known to be involved in transcriptional activation (43). In this report, we show that SWI5 protein purified from an *Escherichia coli* expression system binds to this site with a relatively low affinity and that another protein stimulates

high-affinity binding of SWI5 to this site. We show that the two proteins bind DNA cooperatively, and we take advantage of this cooperativeness to purify the stimulatory factor. In another report (5a), we demonstrate that this stimulatory factor is encoded by the *GRF10* gene, and therefore, we will refer to this factor that stimulates SWI5 binding as GRF10. The *GRF10* gene is also known as *PHO2* and *BAS2*. *PHO2* was identified as a transcriptional activator of *PHO5* (33), and *BAS2* is required for the basal transcriptional activation of the *HIS4* gene (3). The name *GRF10* (for general regulatory factor) was chosen as a new gene designation since *GRF10* plays a role in the transcriptional regulation of diverse genes (49). *GRF10* is also required for the full expression of the *HO* gene (5a).

MATERIALS AND METHODS

Yeast strains. Yeast strain DY411 (*MATa swi5::hisG ade2-1 can1-100 his3-11,15 leu2-3,112 trp1-1 ura3-52*) is a derivative of DY150 (9) which contains a *hisG* (2) disruption of *SWI5*. DY472 is strain DY411 which contains plasmid YCp50:pGAL::SWI5, which overexpresses SWI5 (43), and DY474 is strain DY411 which contains the YCp50 vector. Strain DY2299 (*MATa swi5::LEU2 pep4-3 prb1-1122 prc1-403 leu2 trp1Δ63 ura3-52*) was constructed from strain YPH420 (obtained from Phil Hieter) by transformation with an *swi5::LEU2* construct (43). Gene disruptions were verified by Southern analysis. Yeast cells were transformed by the method of Ito et al. (16).

Oligonucleotides. The following complementary oligonucleotides were synthesized, gel purified when necessary, and annealed. Actual *HO* promoter sequence is indicated by boldface type, and the mutated regions are underlined. The *HO* promoter sequence in the *HO*(46-mer) corresponds to nucleotides –1327 to –1282 upstream of the *HO* ATG start codon. The *HO* promoter sequence in the *HO*(31-mer) corresponds to nucleotides –1327 to –1297. The annealed forms of these oligonucleotides all have 4-base *SalI* overhangs. Sequences are as follows: *HO*(46-mer) top strand, 5'

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TCGACATCAATTTAAAAAACCAGCATGCTATA
 ATGCTGGAGCAAAG 3'; *HO*(46-mer) bottom strand, 5'
 TCGACTTTGCTCCAGCATTATAGCATGCTGGTTTT
 TTTTAAATTGATG 3'; *HO*(31-mer) top strand, 5' TCGA
 CATCAATTTAAAAAACCAGCATGCTAG 3'; *HO*
 (31-mer) bottom strand, 5' TCGACTAGCATGCTGGTTTT
 TTTTAAATTGATG 3'; *grf10* mutant *HO*(31-mer) top
 strand, 5' TCGACATACCGGGAAAAAACCAGCAT
 GCTAG 3'; *grf10* mutant *HO*(31-mer) bottom strand, 5' TCG
 ACTAGCATGCTGGTTTTTTTCCCGGTATG 3'.

Plasmid constructions. The GST-SWIS5 expression plasmid (M1202) was constructed in two steps. First, the *Bst*BI-*Sph*I fragment from plasmid YCp:SWIS5 (43) was cloned into *Eco*RI-digested pGEM3 (Promega) after the *Eco*RI and *Bst*BI ends were blunted with the Klenow fragment of DNA polymerase. This regenerated the *Eco*RI site, now at the 5' end of SWIS5. Next, the 4-kb *Eco*RI-*Pvu*II fragment containing SWIS5 was ligated into *Eco*RI-cleaved pGEX-3X (Pharmacia). The unligated *Eco*RI end was blunted with Klenow and religated with T4 DNA ligase, thus generating M1202. An *Eco*RI site is regenerated at the 3' end of the SWIS5 fragment in M1202. This cloning procedure led to the addition of three amino acids, SKK, between GST and the methionine codon of SWIS5.

The GST-SWIS5 Δ 275 expression plasmid (M1641) was constructed as follows. Plasmid M1202 was digested with *Pst*I, treated with S1 nuclease to blunt the *Pst*I end, and digested with *Eco*RI. The 1.6-kb *Pst*I-*Eco*RI fragment containing the C-terminal region of SWIS5 was isolated and ligated into pGEX-2T (Pharmacia) that had been cleaved with *Sma*I and *Eco*RI. This construct fuses amino acids 275 to 709 of SWIS5 to GST. The GST-SWIS5 fusion junction has the following sequence: GTT CCG CGT GGA TCC CCG AGA AAA AAC.

The GST-SWIS5 Δ 384 expression plasmid (M1642) was constructed as follows. Plasmid M1202 was digested with *Eco*RI to completion and then digested partially with *Dra*I. The 1.3-kb *Dra*I-*Eco*RI fragment containing the C-terminal region of SWIS5 was isolated and ligated into *Sma*I- and *Eco*RI-digested pGEX-2T. This construct fuses amino acids 384 to 709 of SWIS5 to GST. The GST-SWIS5 fusion junction has the following sequence: GTT CCG CGT GGA TCC CCA AAG AGT GCT.

The HIS-SWIS5 expression plasmid (M2024) was constructed in several steps as follows. First, the 2.4-kb *Bst*BI-*Pvu*II fragment of SWIS5 was purified, treated with Klenow, and blunt-end ligated into pIC19H (25), which had been digested with *Bam*HI and *Xho*I and blunted with Klenow. Plasmids with SWIS5 in pIC19H in both orientations were recovered. In one orientation (plasmid M550), the *Bam*HI site in the polylinker was at the 5' end of the SWIS5 fragment (just upstream of the ATG), and in the other orientation (plasmid M556), the *Bam*HI site was 3' to SWIS5. Next, a three-part ligation was performed with (i) the 0.4-kb *Bam*HI-*Sal*I fragment from M550 containing the N-terminal region of SWIS5 (the *Sal*I site is unique), (ii) the 2-kb *Sal*I-*Bam*HI fragment from M556 containing the C-terminal region of SWIS5, and (iii) *Bam*HI-digested pUC8 (46). This three-part ligation created plasmid M602, which contains the SWIS5 gene with *Bam*HI sites present at both the N and the C termini. Finally, the HIS-SWIS5 expression plasmid (M2024) was constructed by cloning the 2.4-kb *Bam*HI SWIS5 fragment from M602 into *Bam*HI-digested pET-16b (Novagen). The HIS-SWIS5 fusion junction has the following sequence: CTC GAG GAT CCG AAA AAG ATG.

The GRF10-SWIS5-binding site plasmids were constructed as follows. The annealed *HO*(46-mer) oligonucleotide was

phosphorylated with polynucleotide kinase and ligated into *Sal*I-digested pIC20R (25). Recombinant plasmids with the oligonucleotide insert in both orientations were recovered. Plasmid CS169 contains the oligonucleotide with the top-strand sequence (shown above) 5' end flanked by the *Bam*HI site of the pIC20R polylinker and 3' end flanked by the *Xho*I site of the pIC20R polylinker. Plasmid CS170 contains the oligonucleotide in the opposite orientation.

Expression in *E. coli* and purification of GST-SWIS5 fusion proteins. The GST-SWIS5 fusion proteins were expressed in *E. coli* and purified essentially as described previously (4, 39). DH5 α *E. coli* cells (13) harboring a GST-SWIS5 fusion protein expression vector were grown in 50 ml of L broth containing 50 μ g of ampicillin (Sigma) per ml overnight at 37°C. This culture was diluted into 500 ml of L broth containing 50 μ g of ampicillin per ml and grown at 28°C until the optical density at 600 nm was approximately 0.8. Isopropyl- β -D-thiogalactopyranoside (IPTG) (Sigma) was added to a final concentration of 0.5 mM, and the incubation was continued for 2.5 h at 28°C. The following procedures were performed at 4°C with ice-cold buffers. Cells were harvested by centrifugation, and the cell pellet was washed with 20 ml of phosphate-buffered saline (PBS), pH 7.3 (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na₂PO₄ · 7H₂O, 1.4 mM KH₂PO₄). The cells were recentrifuged, the cell pellet was resuspended in 20 ml of PBS with protease inhibitors (174 μ g of phenylmethylsulfonyl fluoride [Sigma] per ml, 1.3 μ g of leupeptin [USB] per ml, 0.3 μ g of pepstatin A [USB] per ml), and the resuspended cells were lysed by sonication. Triton X-100 (Sigma) was added to the lysed cells to a final concentration of 1.0% (vol/vol), and the extract was clarified by centrifugation at 12,000 \times g for 30 min. Either the supernatant was used immediately in the protein purification or glycerol was added to a final concentration of 10% and frozen at -70°C for later use.

The GST-SWIS5 fusion proteins were purified in two steps, glutathione-Sepharose and DNA affinity chromatography. For the first step, the *E. coli* extract containing the GST-SWIS5 fusion protein was added to a 2-ml aliquot of glutathione-Sepharose beads (Sigma) equilibrated with PBS. The mixture was rotated at room temperature for 15 min and then centrifuged at 1,000 \times g for 5 min. After removal of the supernatant, the beads and extract were remixed and placed into a small column. The beads were then washed sequentially with 20 ml of PBS, 20 ml of PBS plus 500 mM NaCl, and 20 ml of PBS. The bound GST-SWIS5 fusion proteins were eluted with 10 mM reduced glutathione-50 mM Tris (pH 8.0). Fractions were collected and analyzed by sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE), and fractions containing GST-SWIS5 protein were pooled. The GST-SWIS5 protein pools were adjusted to 75 mM NaCl-0.5 mM dithiothreitol (DTT)-10% (vol/vol) glycerol and then chromatographed on an *HO* promoter DNA affinity column.

An *HO* promoter DNA affinity matrix was prepared by the methods of Kadonaga and Tjian (18) with a concatemered form of the double-stranded *HO*(46-mer) oligonucleotide described above. The following procedures were performed at 4°C. The glutathione-Sepharose-purified protein pool was applied to a 1-ml *HO*(46-mer) column equilibrated with AN75 buffer (AN0 contains 20 mM Tris [pH 8.0], 10% glycerol, 0.5 mM EDTA, and 0.5 mM DTT; AN75 also contains 75 mM NaCl). The column was washed with AN75 buffer, and the bound GST-SWIS5 protein was eluted with AN500 buffer (AN0 plus 500 mM NaCl). Fractions were collected and analyzed by SDS-PAGE, and fractions con-

taining GST-SWI5 were pooled. Bovine serum albumin (BSA) (Sigma) was added to a final concentration of 0.1 mg/ml, the pool was dialyzed into AN100 buffer (AN0 plus 100 mM NaCl) containing protease inhibitors, and aliquots were stored at -70°C .

Expression in *E. coli* and purification of HIS-SWI5 fusion proteins. The HIS-SWI5 fusion protein was expressed in *E. coli* essentially as described above for the GST-SWI5 fusion proteins, except for the following changes. BL21-pLysS cells (44) containing the HIS-SWI5 expression plasmid were used, and the L-broth cultures contained 50 μg of carbenicillin (Sigma) per ml and 34 μg of chloramphenicol (Sigma) per ml. The final concentration of IPTG used during induction was 1 mM, and the induction normally proceeded for 1 to 2 h. HIS binding buffer (500 mM NaCl, 5 mM imidazole, 20 mM Tris-HCl [pH 8.0]) was used for extract preparation, and Nonidet P-40 (Sigma) was added to a final concentration of 0.1% before sonication.

The HIS-SWI5 fusion protein was purified by procedures described previously (Novagen) (40). The following procedures were performed at 4°C or on ice. A 1-ml HiTrap Metal Chelating column (Pharmacia) was charged with NiSO_4 and then equilibrated with HIS binding buffer. The *E. coli* extract containing HIS-SWI5 protein was filtered through a 0.45- μm -pore-size syringe filter (Acrodisc; Gelman Sciences) and applied to the nickel column. The column was washed first with 10 ml of HIS binding buffer and then with 10 ml of 500 mM NaCl–60 mM imidazole–20 mM Tris-HCl (pH 8.0), and then the bound HIS-SWI5 protein was eluted with 500 mM NaCl–1 M imidazole–20 mM Tris-HCl (pH 8.0). Fractions were collected and analyzed by SDS-PAGE, and fractions containing HIS-SWI5 were pooled. The HIS-SWI5 pool was dialyzed into AN100 buffer containing protease inhibitors, and aliquots were stored at -70°C .

Yeast extract preparation. Yeast strain DY2299 was grown in 3 liters of concentrated YEPD medium (5.5% yeast extract, 11% peptone, 275 μg of adenine per ml, 10% glucose) in a high-density fermentor (Lab Line/S.M.S.) to midlog phase (optical density at 660 nm of approximately 20 to 30). Two fermentor runs were performed, and two S-100 extracts were prepared essentially as described previously (21), except that the solubilization buffer contained 200 mM Tris (pH 8.0), 10% glycerol, 10 mM MgCl_2 , 1 mM DTT, and protease inhibitors. The extracts were combined and dialyzed into AN100 plus protease inhibitors and stored at -70°C . The total yield of protein in the S-100 extract was 7 g (16 mg/ml, determined by the Bio-Rad protein assay with BSA as the standard).

Purification of GRF10. (i) Conventional chromatography. The following purification steps were performed on a fast protein liquid chromatograph (Pharmacia) at 4°C . A 125-ml heparin-agarose (Bethesda Research Laboratories, Inc.) column equilibrated with AN100 was loaded with 1 g of S-100 protein extract. The column was washed with AN100 until the A_{280} reached baseline, and then the bound proteins, including GRF10, were eluted with AN600 (AN0 plus 600 mM NaCl). The 600 mM protein fraction was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation (0.35-g/ml final concentration). The protein pellet was resuspended in MN0 buffer [50 mM 2-(*N*-morpholino)ethanesulfonic acid monohydrate (MES) (USB) (pH 6.0), 0.5 mM EDTA, 10% glycerol] plus protease inhibitors and dialyzed against MN100 (MN0 plus 100 mM NaCl) plus protease inhibitors. This heparin-agarose chromatography step was repeated six times with a total of 6 g of S-100 protein. The six HEP600 pools were combined to yield a total of 750 mg of protein. This HEP600 pool was applied

to a HiLoad 16/10 S Sepharose column (Pharmacia) equilibrated with MN100. The column was washed with MN100 until the A_{280} reached baseline, and the bound proteins, including GRF10, were eluted with a 330-ml 100 to 600 mM NaCl gradient. Fractions were dialyzed against AN100 plus protease inhibitors and assayed for SWI5 stimulatory activity (GRF10) by the gel retardation assay. Fractions from the S Sepharose column that contained maximal SWI5 stimulatory activity were pooled, and the pool contained 17 mg of protein. The S Sepharose protein pool was applied to an HR 5/5 Mono Q column (Pharmacia) equilibrated with AN100, and the column was washed with AN100 until the A_{280} reached baseline. The bound proteins, including GRF10, were eluted with a 30-ml 100 to 400 mM NaCl gradient. Fractions from the column were tested for (i) SWI5 stimulatory activity (GRF10) by the gel retardation assay, (ii) GRF10 DNA-binding activity by the gel retardation assay, and (iii) GRF10 DNA-binding activity by a Southwestern (DNA-protein) assay. Fractions 29 to 35 contained SWI5 stimulatory activity, and the peak of activity was present in fraction 32.

(ii) Cooperative interaction-based DNA affinity chromatography (CIBDAC). A GRF10-SWI5 DNA affinity matrix was constructed with concatemericized *HO*(31-mer) oligonucleotides as described in reference 18. GST-SWI5 Δ 384 was purified on glutathione-Sepharose (4 ml; approximately 500 μg) and adjusted to 50 mM NaCl. A 1-ml aliquot (0.125 mg/ml) of the Mono Q pool (fractions 30 to 33) was combined with the 4-ml GST-SWI5 Δ 384 pool, and the NaCl concentration was adjusted to a final concentration of 75 mM. Poly(dI-dC) · poly(dI-dC) was added to a final concentration of 50 $\mu\text{g}/\text{ml}$, and DTT was added to a final concentration of 12.5 mM. Approximately 1.5 ml of the *HO*(31-mer) DNA affinity resin was added to the protein mixture, and the slurry was rotated at 4°C for 2 h. The mixture was placed into a column, and the flowthrough was collected. The column was washed with 3.5 ml of AN75, and then the bound protein was eluted sequentially with 0.5 ml of AN100-AN450 in 50 mM NaCl steps, 0.5 ml of AN500 (repeated four times), and AN1000 (repeated six times). Each 0.5-ml aliquot eluting from the column was collected, and 20- μl samples were analyzed by SDS-PAGE. Insulin (Sigma) was added at a final concentration of 0.1 mg/ml to the remaining 480 μl of each fraction, and these fractions were dialyzed against AN100. The presence of GST-SWI5 Δ 384 and SWI5 stimulatory factor (GRF10) in each fraction was assayed by gel retardation.

(iii) Glutathione-Sepharose separation of GST-SWI5 Δ 384 from GRF10. Fractions 450, 500-1,2,4, and 1000-1 (400 μl of each) were pooled and combined with 1 ml of glutathione-Sepharose equilibrated with AN100. The mixture was rotated at room temperature for 30 min. The glutathione-Sepharose beads were separated by centrifugation in a microcentrifuge, and the supernatant containing the nonadsorbed proteins was collected. The bound GST-SWI5 Δ 384 protein was eluted with 10 mM reduced glutathione–50 mM Tris (pH 8.0). Both fractions were analyzed by SDS-PAGE. The supernatant contained pure GRF10, and the eluted fraction contained GST-SWI5 Δ 384.

Preparation of protein fractions from SWI5 and *swi5* yeast strains. Extracts were prepared from yeast strains DY474, an *swi5* mutant, and DY472, a strain overexpressing SWI5 from the *GAL1* promoter. Six 1-liter cultures of each strain were grown at 30°C in synthetic complete medium (37), supplemented with amino acids, containing 2% raffinose as a carbon source. When the optical density at 660 nm reached

about 3.0, galactose was added to a final concentration of 2% and the incubation was continued for 4 h. The protein extracts for each strain were prepared as described previously (21) except for the following changes. The solubilization buffer also contained 500 mM NaCl, and the 0.4 M $(\text{NH}_4)_2\text{SO}_4$ extraction was omitted. Each extract yielded approximately 200 mg of protein.

The heparin-agarose chromatography step was performed on a fast protein liquid chromatograph (Pharmacia) at 4°C with a 25-ml column. The following procedure was performed in parallel, once with the *SWI5*⁺ extract and once with the *swi5* mutant extract. Protein (74 mg) was loaded onto the heparin-agarose column equilibrated with AN100, the column was washed with AN100, and the bound proteins were eluted with a 60-ml 100 to 1,000 mM NaCl gradient. Fractions were collected, dialyzed against AN100 plus protease inhibitors, and stored at -70°C.

Gel retardation assays. Binding reaction mixtures (20- μ l volume) contained 15 mM Tris (pH 8.0), 75 mM NaCl, 7.5% glycerol, 12.5 mM DTT, 0.375 mM EDTA, and 750 μ g of BSA per ml. The protein components and the amount of poly(dI-dC) · poly(dI-dC) (Pharmacia) used in each binding reaction mixture are indicated in the figure legends. Probes were all labelled with [γ -³²P]ATP (6,000 Ci/mmol; New England Nuclear) with polynucleotide kinase. Approximately 30,000 cpm of probe (about 1 ng) was used in each binding reaction mixture. Samples were incubated at 25°C for 40 to 60 min before being loaded onto a 4% polyacrylamide gel in 0.5 \times TBE (45 mM Tris [pH 8.3], 45 mM boric acid, 1.25 mM EDTA) at 15 V/cm. Gels were preelectrophoresed for 1 h before sample was loaded. Gels were normally run for 1.3 to 1.5 h, dried, and autoradiographed with an intensifying screen.

Immunoblotting with antibody to SWI5. A TrpE-SWI5 fusion protein was produced in *E. coli* from a plasmid containing a 1.6-kb *SalI-HindIII* fragment from *SWI5* cloned into the pATH2 vector (7). The fusion protein was purified by SDS-PAGE and used to immunize rabbits. The antiserum to SWI5 was affinity purified as described for antiserum to SIN3, by using an SWI5 protein affinity matrix (47). Immunoblotting was performed as described previously (47). The secondary antibody used was goat anti-rabbit immunoglobulin G alkaline phosphatase-conjugated antibody (Bethesda Research Laboratories, Inc.).

Methylation interference assay. The top-strand probe was prepared from the CS170 plasmid by digesting with *XhoI*, dephosphorylating with calf intestinal alkaline phosphatase, and labelling with polynucleotide kinase. The DNA was then digested with *EcoRI*, and the 110-bp 5'-end-labelled fragment was gel purified. The bottom-strand probe was prepared in a similar fashion with the CS169 plasmid. Approximately 80 ng of each probe was treated with dimethyl sulfate (Aldrich) as described previously (26). The methylated DNA was resuspended at 50,000 cpm/ μ l (about 1.7 ng/ μ l). Preliminary experiments were conducted to identify binding conditions such that approximately 50% of the probe was present in the bound fraction, and binding reactions were then conducted with individual components at the following concentrations: GST-SWI5-only reaction mixtures contained 6 μ l of GST-SWI5, 5 μ l of probe, and no poly(dI-dC) · poly(dI-dC) in a 100- μ l reaction mixture; GRF10-only reaction mixtures contained 12 μ l of Mono Q-purified GRF10, 10 μ l of probe, and 25 μ g of poly(dI-dC) · poly(dI-dC) per ml in a 200- μ l reaction mixture; and GST-SWI5 plus GRF10 reaction mixtures contained 10 μ l of GST-SWI5, 3 μ l of Mono Q-purified GRF10, 5 μ l of probe, and 25 μ g of

poly(dI-dC) · poly(dI-dC) per ml in a 100- μ l reaction mixture. Binding reaction incubations and gel retardation were performed as described above, except that the gel was autoradiographed wet. The bound and unbound bands were excised, and the DNA was purified by electrophoretic transfer to NA45 paper (Schleicher and Schuell) and elution with 1 M NaCl-0.1 mM EDTA-20 mM Tris (pH 8.0) at 55°C overnight. The eluted DNA was precipitated with ethanol, washed, and dried. The samples were incubated in 100 μ l of 20 mM sodium acetate (pH 7.0)-0.1 mM EDTA at 90°C for 15 min, and then 10 μ l of 10 M piperidine (Fisher) was added and the incubation at 90°C was continued for 30 min to generate G>A cleavage of the probe (26). Samples containing equal amounts of radioactive probe were electrophoresed in a 10% sequencing gel, and the gel was dried and autoradiographed with preflashed Cronex film (Dupont) with an intensifying screen at -70°C. The control lane contained methylated probe that was only cleaved.

Missing-nucleoside experiment. DNA probes were prepared as described for the methylation interference experiments. Approximately 180 ng of gapped DNA probe was prepared as described previously (8) with 1 mM ascorbate, 0.03% hydrogen peroxide, 10 μ M Fe(II), and 20 μ M EDTA reaction conditions. The gapped DNA molecules were resuspended at a concentration of 50,000 cpm/ μ l in water. The binding conditions, which were designed such that approximately 75 to 90% of the probe was in the bound fraction, contained 32 μ l of GST-SWI5, 16 μ l of Mono Q-purified GRF10, 8 μ l of probe, and 100 μ g of poly(dI-dC) · poly(dI-dC) per ml in a 150- μ l reaction mixture. Binding reaction incubations and gel retardation were performed as described above, except that the gel was autoradiographed wet. The DNA in the bound and unbound bands was eluted from gel slices, precipitated, washed, dried, and electrophoresed in a 10% sequencing gel. Dimethyl sulfate-methylated and cleaved probes were also electrophoresed as markers. The gel was dried and autoradiographed with preflashed Cronex film and an intensifying screen at -70°C. The autoradiograph was scanned with a Bio-Rad densitometer, and the bands' intensities were determined by using 1-D Analyst Macintosh Software (Bio-Rad).

Southwestern blot. The Southwestern blotting method was adapted from Staudt et al. (41) and Hager and Burgess (12). The Southwestern experiment was performed in parallel with one of two probes, either the wild-type *HO(31-mer)* probe or the *grf10* mutant *HO(31-mer)* probe. The probes were prepared by concatemerizing the wild-type *HO(31-mer)* and mutant *HO(31-mer)* with T4 DNA ligase. The concatemerized probes were labelled by using the Nick Translation System (Bethesda Research Laboratories, Inc.) with [α -³²P]dATP (3,000 Ci/mmol; Amersham). Samples from fractions 29 to 35 (20 μ l) from the Mono Q column were electrophoresed in an 8% SDS-polyacrylamide gel. The separated proteins were transferred to nitrocellulose in the absence of methanol (48). The blot was incubated for 1 h at room temperature in blocking buffer (50 mM Tris [pH 8.0], 10% glycerol, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 5% nonfat dry milk). The blocking buffer was discarded and the protein blot was denatured by a 30-min incubation at room temperature in 20 ml of denaturation buffer (50 mM Tris [pH 8.0], 10% glycerol, 100 mM NaCl, 50 mM DTT, 0.1 mM EDTA, 0.25% nonfat dry milk, 6 M guanidine-HCl). A 17.5-ml volume of the denaturation buffer was removed and replaced with 125 ml of renaturation buffer (50 mM Tris [pH 8.0], 10% glycerol, 100 mM NaCl, 1 mM DTT, 0.1 mM EDTA, 0.25% nonfat dry milk). Renaturation was allowed to

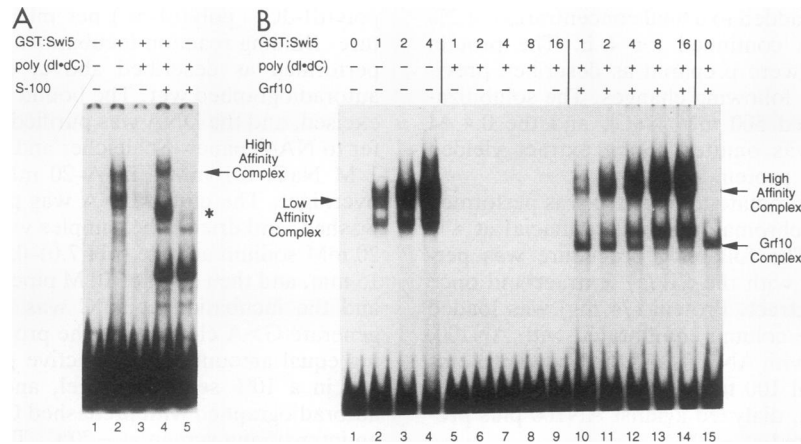


FIG. 1. Identification of an activity that stimulates SWI5 DNA binding. (A) Gel retardation assay for stimulatory activity in an S-100 fraction. A gel retardation assay was performed with the radiolabelled *HO* promoter probe from plasmid CS169. The binding reaction mixtures contained, where indicated, the following variable components: GST-SWI5 (1 μ l in lane 2 and 8 μ l in lanes 3 and 4), 12.2 μ g of S-100 extract, and 100 μ g of poly(dI-dC) \cdot poly(dI-dC) per ml. The S-100 fraction was prepared from strain DY411 (*swi5 Δ ::LEU2*). The asterisk indicates a band present in lane 4 which is dependent upon both GST-SWI5 and GRF10; this band probably results from proteolytic cleavage of GST-SWI5 (data not shown). (B) Gel retardation with partially purified stimulatory activity termed GRF10. The binding reaction mixtures contained, where indicated, the following variable components: GST-SWI5 (1 U = 0.2 μ l), 100 μ g of poly(dI-dC) \cdot poly(dI-dC) per ml, and 2 μ l of GRF10. The GRF10 protein was purified partially by heparin-agarose, S Sepharose, and Mono Q chromatography. Low Affinity Complex, SWI5-dependent complex that is inhibited by poly(dI-dC) \cdot poly(dI-dC); High Affinity Complex, SWI5-dependent complex that persists in the presence of poly(dI-dC) \cdot poly(dI-dC) competitor; Grf10 Complex, protein-DNA complex containing only GRF10.

proceed overnight at 4°C. The blot was rinsed with 20 ml of binding buffer (15 mM Tris [pH 8.0], 7.5% glycerol, 50 mM NaCl, 0.375 mM DTT, 0.375 mM EDTA, 0.25% nonfat dry milk), and the blot was equilibrated in 50 ml of binding buffer for 30 min at room temperature. The blot was then incubated for 30 min at room temperature with 12 ml of binding buffer containing 10 μ g of poly(dI-dC) \cdot poly(dI-dC) per ml and the DNA probe (10^6 to 10^7 cpm/ml), either the wild type or the *grf10* mutant. The binding buffer was removed, and the blot was washed three times at room temperature with 25 ml of binding buffer (5 to 10 min each wash). The blots were allowed to air dry and then autoradiographed with XAR5 film (Kodak) with an intensifying screen at -70°C .

RESULTS

DNA-binding properties of GST-SWI5 protein purified from *E. coli*. It has been demonstrated previously that the yeast SWI5 protein binds to the *HO* promoter at a site approximately 1,300 nucleotides upstream of the *HO* ATG start codon (43). In order to analyze the DNA-binding characteristics of SWI5 in more detail, an SWI5 fusion protein was purified from an *E. coli* expression system. The SWI5 open reading frame was cloned into the pGEX-3X (Pharmacia) plasmid, which directs expression of a fusion protein between glutathione *S*-transferase and SWI5 under the control of the *tac* promoter. The GST domain permits rapid purification of the fusion protein on a glutathione-Sepharose matrix (39). DNA affinity chromatography (18), utilizing the SWI5 binding site from the *HO* promoter, was used as a second step to achieve purification of GST-SWI5 to near homogeneity. Because the GST domain was located at the N terminus of this fusion protein and the SWI5 zinc finger DNA-binding domain was located at the C terminus, this double-affinity purification strategy eliminated any fusion protein that was not full length. Additionally, the DNA affinity step ensured that all GST-SWI5 molecules were capable of binding to DNA.

The DNA-binding characteristics of the purified GST-SWI5 protein were analyzed in a gel retardation assay with a probe from the *HO* promoter (Fig. 1A). As expected, GST-SWI5 purified from *E. coli* bound to the *HO* probe (lane 2). However, the addition of the nonspecific competitor DNA poly(dI-dC) \cdot poly(dI-dC) (38) eliminated the *in vitro* DNA-binding activity of GST-SWI5 (lane 3). Specific binding by GST-SWI5 was abolished by very small quantities of poly(dI-dC) \cdot poly(dI-dC), as little as 2.5 μ g/ml. Other nonspecific competitors, such as calf thymus or salmon sperm DNA, were also effective competitors (data not shown). These results suggested that GST-SWI5 interacts with the *HO* promoter fragment with either low affinity or poor specificity.

A factor that promotes high-affinity binding by GST-SWI5. This apparent weak affinity of GST-SWI5 for the *HO* promoter was surprising, since SWI5 has been shown by previous genetic studies to be a transcriptional activator of *HO* expression acting through this binding site at position -1300 (43). Therefore, we decided to look for a factor that stimulated binding of GST-SWI5 to the *HO* binding site in an S-100 extract prepared from a strain with an *swi5* null mutation. A gel retardation assay was used to look for a high-affinity protein-DNA complex containing GST-SWI5, with poly(dI-dC) \cdot poly(dI-dC) added to inhibit formation of the low-affinity GST-SWI5-DNA complex. The addition of the S-100 extract stimulated formation of a specific protein-DNA complex (Fig. 1A, lane 4). This complex was not seen with either the yeast extract (lane 5) or GST-SWI5 (lane 2) alone, suggesting that the complex contained GST-SWI5 and another factor.

This factor stimulated GST-SWI5 DNA-binding activity in the presence of excess poly(dI-dC) \cdot poly(dI-dC) in a gel retardation experiment, and this activity was used as an assay to purify the stimulatory factor. The SWI5 stimulatory activity of GRF10 was purified by heparin-agarose, S Sepharose, and Mono Q chromatography, as described in Materials and Methods. Figure 1B shows a gel retardation

assay performed with GST-SWI5 and the GRF10 fraction after the Mono Q purification step. Binding by GST-SWI5 (lanes 2 to 4) was blocked readily by the addition of poly(dI-dC) · poly(dI-dC) (lanes 5 to 9). The addition of the GRF10 fraction led to the appearance of a more slowly migrating high-affinity complex (lanes 10 to 14). As GRF10 was purified by S Sepharose and Mono Q chromatography, we observed a specific DNA-binding activity in the absence of added GST-SWI5 (lane 15). This DNA-binding activity cofractionated with the SWI5 stimulatory activity during purification (see below). This cofractionation in Mono Q fractions was demonstrated by gel retardation (see Fig. 6; also see below). The copurification of these two activities suggested that the GST-SWI5-independent DNA-binding activity and the SWI5 stimulatory activity were one and the same (see below). GST-SWI5 was unable to bind DNA under these conditions (lane 9), while the addition of GRF10 stimulated binding by GST-SWI5 dramatically (lane 14).

DNA-binding properties of SWI5 protein from yeast cells. It was possible that the stimulation of GST-SWI5 binding by a second factor was an artifact resulting from overexpression and purification of GST-SWI5 from *E. coli*. The protein could have been misfolded or have lacked proper cysteine interactions. There are reports of DNA-binding proteins that do not effectively bind DNA when purified from bacterial expression systems (1, 27, 34). In order to eliminate this possibility, we analyzed the DNA-binding properties of SWI5 protein prepared from yeast cells. Yeast extracts were prepared from two isogenic strains. The first strain contained an *swi5* null mutation, and the second strain overexpressed SWI5 from the inducible *GAL10* promoter. Each extract was fractionated in parallel over a heparin-agarose column, and the SWI5 DNA-binding activity in each fraction was assayed by a gel retardation assay. The SWI5⁺ fractions showed DNA-binding activity in fractions 33 to 61, with the strongest activity present in fractions 41 to 59 (Fig. 2A). This activity was absent in column fractions from the *swi5* mutant extract (data not shown). The peak of SWI5 DNA-binding activity, however, did not coincide with the maximal amount of SWI5 protein (Fig. 2B). An immunoblot performed with SWI5-specific antisera showed that the maximal amount of SWI5 protein was actually present in fraction 59; fractions 63 to 67 contained SWI5 protein but lacked any SWI5-dependent DNA-binding activity. The heparin column fractions from the *swi5* mutant extract were assayed for their ability to stimulate GST-SWI5 DNA binding (Fig. 2C). The stimulatory activity overlapped with the SWI5-dependent DNA-binding activity seen in Fig. 2A.

It appears that only the fractions from the SWI5⁺ column that contained both SWI5 protein and the stimulatory activity showed SWI5 DNA-binding activity in vitro. This hypothesis was tested by combining fraction 53 from the *swi5* mutant column, which contained the peak of stimulatory activity, with fractions 59 through 73 from the SWI5⁺ column, which contained SWI5 protein (by immunoblot) but showed little DNA-binding activity, and assaying for the recovery of SWI5 DNA-binding activity in these fractions by gel retardation (Fig. 2D). The addition of fraction 53, which contained the SWI5 stimulatory activity of GRF10, to these fractions stimulated SWI5 DNA binding (Fig. 2D), and the relative abundance of SWI5 DNA-binding activity in each fraction paralleled the amount of SWI5 protein present, as determined by immunoblotting (Fig. 2B). We have also produced a complete SWI5 protein (not a fusion protein) in baculovirus-infected insect cells. This baculovirus-produced SWI5 protein also required GRF10 for high-affinity binding

in vitro (data not shown). These results indicate that the weak binding and stimulation of GST-SWI5 binding are not artifacts of *E. coli* protein production and more importantly that SWI5 produced in yeast cells also requires GRF10 for maximal in vitro DNA binding in the presence of poly(dI-dC) · poly(dI-dC).

Characterization of DNA binding by SWI5 and GRF10. GRF10 could promote stronger DNA binding by GST-SWI5 by several distinct mechanisms. It is possible that GRF10 posttranslationally modifies SWI5 or promotes dimer formation by SWI5. Alternatively, GRF10 could bind cooperatively with SWI5 to the SWI5 binding site in the *HO* promoter. The relative mobilities of GST-SWI5, GST-SWI5 plus GRF10, and GRF10 complexed with DNA in a gel retardation assay can be seen in Fig. 1. One might expect that the formation of a ternary complex containing GST-SWI5, GRF10, and DNA would show a large difference in mobility when compared with the binary GST-SWI5-DNA complex. Because the mobility of the GST-SWI5-GRF10-DNA complex is just slightly slower than that of the GST-SWI5-DNA complex, we cannot distinguish between these models. However, the GST-SWI5 fusion protein is large (106 kDa), and the addition of the GRF10 protein to the GST-SWI5-DNA complex might show only a subtle change in electrophoretic mobility compared with the GST-SWI5-DNA complex.

In order to enhance possible gel mobility differences between the different complexes, we constructed a different *E. coli* expression plasmid that directed synthesis of an 83-kDa SWI5 fusion protein. This plasmid fuses 10 histidine residues to the N terminus of the SWI5 open reading frame, which allowed for rapid purification of the HIS-SWI5 fusion protein by nickel column chromatography (40). The HIS-SWI5-DNA complex formed in the presence of GRF10 showed a dramatic decrease in gel mobility compared with the HIS-SWI5-DNA complex (Fig. 3). This result favors a model in which both SWI5 and GRF10 proteins are present in the high-affinity protein-DNA complex.

Methylation interference experiments were performed on protein-DNA complexes in order to identify residues important for binding by GST-SWI5 and GRF10 (Fig. 4A). Probes were partially reacted with dimethyl sulfate to modify guanines and adenines, incubated with protein, and electrophoresed to separate the protein-DNA complexes from the free DNA. Each DNA fraction was localized by autoradiography, eluted from the gel, cleaved at modified residues, and electrophoresed on a denaturing polyacrylamide gel. Because the region of the *HO* promoter recognized by SWI5 is A-T rich, we used conditions which allowed cleavage at methylated adenine as well as methylated guanine residues. The GRF10 protein used in these experiments was partially purified with heparin-agarose, S Sepharose, and Mono Q resins, and it bound to the *HO* promoter in the absence of SWI5 (Fig. 3, lane 3). The results, summarized in Fig. 4C, demonstrate that SWI5 and GRF10 each recognizes distinct but adjacent sites in the *HO* promoter, with each protein displaying specific DNA contacts. Dimethyl sulfate methylates guanine residues at the N-7 position in the major groove and adenine residues predominantly at the N-3 position in the minor groove. Methylation of both adenine and guanine residues interferes with DNA binding by GRF10, suggesting that GRF10 makes DNA contacts in both the major and the minor grooves. The methylation interference data indicate that SWI5 also contacts DNA in both the major and the minor grooves.

The high-affinity complex containing both SWI5 and

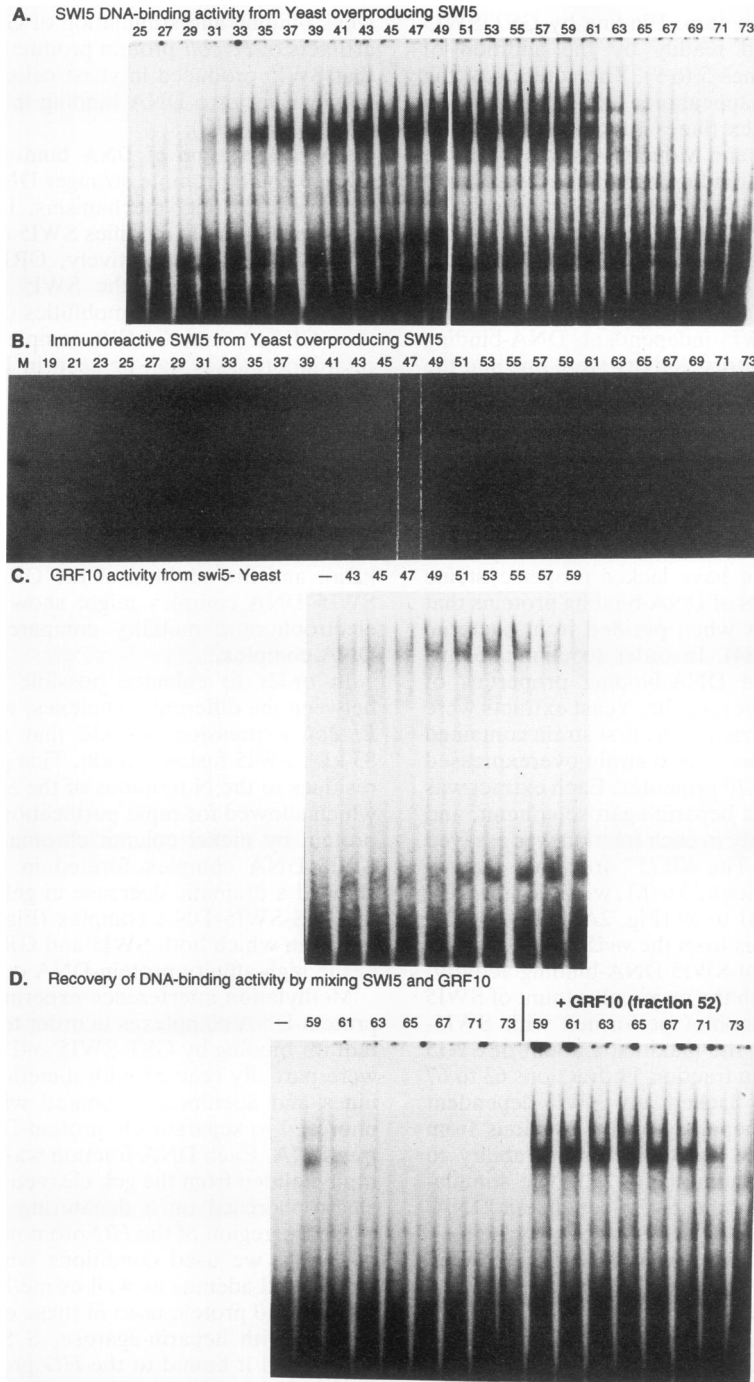


FIG. 2. DNA-binding activity of SWI5 protein from yeast cells can be stimulated by GRF10. Protein extracts prepared from strains DY472 (overproducing SWI5 from the *GAL1* promoter) and strain DY474 (*swi5::LEU2*) were fractionated on identical heparin-agarose columns. (A) Gel retardation assay for SWI5-dependent DNA-binding activity. Binding reaction mixtures contained 10 μ l of each *SWI5*⁺ heparin column fraction, 100 μ g of poly(dI-dC) · poly(dI-dC) per ml, and the *HO*(46-mer) probe. DNA binding was assayed by a gel retardation assay. (B) Immunoblot analysis of SWI5 protein. Samples (10 μ l) of each *SWI5*⁺ heparin column fraction were electrophoresed on SDS-polyacrylamide gels, blotted to nitrocellulose, and probed with affinity-purified antisera to SWI5. (C) Assay for GRF10 activity in *swi5* mutant heparin column fractions. Binding reaction mixtures contained 5 μ l of each *swi5* mutant heparin column fraction, 3 μ l of GST-SWI5, 150 μ g of poly(dI-dC) · poly(dI-dC) per ml, and the *HO*(46-mer) probe. The presence of GRF10 in each heparin column fraction was determined by its ability to promote SWI5 DNA-binding activity in a gel retardation assay. (D) GRF10 will stimulate binding of yeast SWI5. Binding reaction mixtures contained 5 μ l of fractions 59 to 73 from the *SWI5*⁺ heparin column either alone (left) or in combination with added GRF10 protein (right). Fraction 53 (5 μ l) from the *swi5* mutant heparin column was used as a source of GRF10 protein. Binding reaction mixtures also contained 100 μ g of poly(dI-dC) · poly(dI-dC) per ml and the *HO*(46-mer) probe. A gel retardation assay was used to measure the ability of GRF10 to stimulate SWI5 DNA binding.

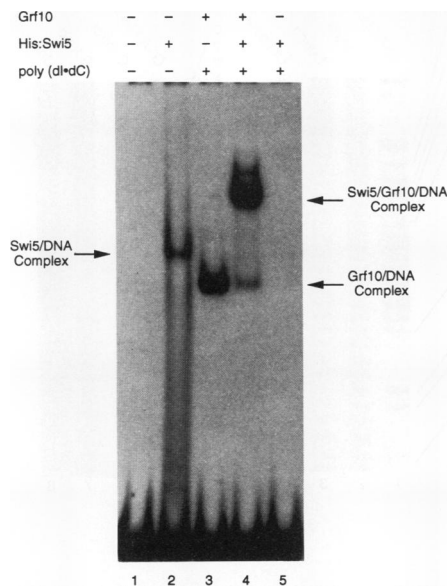


FIG. 3. GRF10-SWI5-DNA ternary complex has slower mobility. A gel retardation assay was performed with the radiolabelled *HO* promoter probe from plasmid CS169. The binding reaction mixtures contained, where indicated, the following variable components: HIS-SWI5 (0.04 μ l in lane 2 and 4 μ l in lanes 3 and 4), 1 μ l of GRF10, and 100 μ g of poly(dI-dC) · poly(dI-dC) per ml. The HIS-SWI5 fusion protein (83 kDa) contains 10 histidine residues fused to the N terminus of the entire SWI5 protein. The GRF10 protein was partially purified by heparin-agarose, S Sepharose, and Mono Q chromatography.

GRF10 showed DNA contacts that were a combination of those seen with the single proteins. Subtle differences between the interference patterns of binary and ternary complexes could be seen. The methylation of a guanine residue on the lower strand inhibited binding by GRF10, but methylation of this residue did not interfere with GST-SWI5 plus GRF10 binding. Methylation of two adenine residues on the top strand interferes with GST-SWI5 binding but did not affect binding by GST-SWI5 plus GRF10 (see Discussion). These results support the ternary complex model in which SWI5 and GRF10 bound simultaneously to the SWI5 binding site in the *HO* promoter.

A missing-nucleoside experiment (14) was performed with the GST-SWI5 plus GRF10 protein-DNA complex in order to define specific bases that were important for binding in the ternary complex. This technique is similar to methylation interference, except that the DNA probe is first exposed to hydroxyl radicals, which remove individual nucleosides from the DNA backbone. A gel retardation assay with the modified DNA is used to fractionate the bound and free DNA molecules, allowing the identification of nucleosides that are important for protein-DNA complex formation. The missing-nucleoside experiment identified two regions that are important for the formation of the GST-SWI5-Grf10-DNA ternary complex (Fig. 4B). One region overlaps the methylation interference pattern of GST-SWI5, and the other region overlaps the methylation interference pattern of GRF10 (Fig. 4C).

Identification of the GRF10 polypeptide by Southwestern blotting. The data from the methylation interference and missing-nucleoside experiments were used to design a *grf10* mutant oligonucleotide that would not bind GRF10. Trans-

version substitutions were made at the 6 nucleotides at the center of the GRF10 binding site, while the bases recognized by SWI5 were not altered. The wild-type and *grf10* mutant oligonucleotides were tested for in vitro DNA binding by GST-SWI5, GRF10, and GST-SWI5 plus GRF10 in a gel retardation assay. GST-SWI5 bound equally well to both probes (low-affinity complex), while GRF10 and GST-SWI5 plus GRF10 (high-affinity) complexes did not form on the mutant probe (Fig. 5).

A Southwestern blotting experiment was performed to identify the polypeptide (GRF10) that would bind specifically to the wild-type *HO*(31-mer) oligonucleotide but not to the *grf10* mutant oligonucleotide (Fig. 6). Protein fractions eluted from a Mono Q column containing GRF10 activity were electrophoresed in two parallel SDS-polyacrylamide gels and transferred to nitrocellulose filters. The blots were then blocked, denatured, renatured, and probed with either the wild-type or the *grf10* mutant oligonucleotide. A protein with an approximate molecular mass of 83 kDa bound specifically to only the wild-type probe, and this Southwestern DNA-binding activity paralleled both the GRF10 in vitro DNA-binding activity and the SWI5 stimulatory activity of GRF10 present in these same fractions. The correlation between the Southwestern DNA-binding activity and the GRF10 DNA-binding and stimulatory activities, as well as the lack of binding to the mutant binding site probe, suggested strongly that the 83-kDa protein represents GRF10.

Purification of GRF10 by CIBDAC. Since GRF10 binds to a site in the *HO* promoter, we attempted to purify GRF10 by DNA affinity chromatography (18) using a column containing the SWI5 and GRF10 binding sites from the *HO* promoter. A pool of GRF10, purified on heparin-agarose, S Sepharose, and Mono Q columns, was applied to the GRF10-SWI5 binding site column. The bound GRF10 was eluted from the column with a NaCl step gradient. The two activities, SWI5 stimulatory activity and direct DNA binding by GRF10 alone, coeluted from this column (data not shown). However, this method did not give sufficient enrichment of GRF10 to achieve purification, presumably because of the relatively low affinity of GRF10 for the binding site from the *HO* promoter.

We exploited the cooperative interactions between SWI5 and GRF10 to enhance purification of GRF10 by a DNA affinity method. We call the method CIBDAC, and it is similar to a method developed independently by Ranish and Hahn (36). The pool of partially purified GRF10 was mixed with GST-SWI5 Δ 384 and loaded onto the DNA affinity column in the presence of a large excess of nonspecific DNA, poly(dI-dC) · poly(dI-dC). (The 63-kDa deletion derivative of SWI5, GST-SWI5 Δ 384, which still interacts with GRF10, was used in the purification for the following reason. Our goal was SDS-gel purification of the 83-kDa species for protein sequencing. If we used the larger GST-SWI5 protein, it was possible that the 83-kDa band would be contaminated by proteolytic products of GST-SWI5. However, the smaller GST-SWI5 Δ 384 protein could not contaminate the 83-kDa band.) The column was washed with loading buffer, and the bound proteins were step eluted with increasing salt concentrations. The majority of the GRF10 protein, assayed by stimulation of GST-SWI5 Δ 384 binding, was detectable in the 450 to 1,000 mM NaCl eluates (Fig. 7A, lanes 10 to 16). The proteins present in these fractions were visualized by Coomassie blue staining of an SDS-polyacrylamide gel. Only two proteins, GST-SWI5 Δ 384 and GRF10, were present in the fractions that contained high-affinity DNA-binding activity (Fig. 7B). It is noteworthy that GRF10 eluted from the

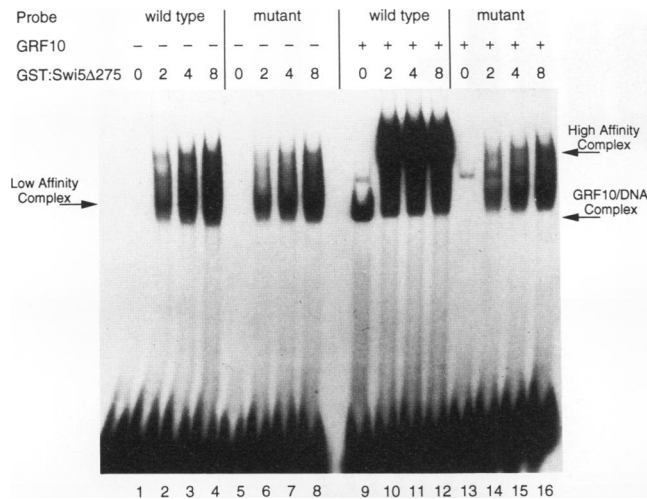


FIG. 5. Binding site mutation blocks GRF10 binding. A gel retardation assay was performed with either the wild-type *HO*(31-mer) oligonucleotide (lanes 1 to 4 and 9 to 12) or the *grf10* mutant *HO*(31-mer) oligonucleotide (lanes 5 to 8 and 13 to 16) with substitutions in the GRF10 binding site. GST-SWI5Δ275 (a truncated version containing amino acids 275 to 709 of SWI5) was added to some samples, with volumes (in microliters) indicated in the figure. Partially purified GRF10 (0.1 μl) from the Mono Q column was added to the samples in lanes 9 to 16. Poly(dI-dC) · poly(dI-dC) was also added to a final concentration of 25 μg/ml. Low Affinity Complex, SWI5-DNA complex; High Affinity Complex, GRF10-SWI5-DNA. In the mutant oligonucleotide, the GRF10 binding site CATCAATTTAAA region is changed to CATAACGGGAAA.

DNA affinity column under CIBDAC conditions at a much higher salt concentration than when it was chromatographed separately on the same DNA affinity column (data not shown). This result was presumably due to the higher affinity of GRF10 for DNA in the presence of SWI5. To determine whether SWI5 and GRF10 interacted in the absence of DNA, we loaded the pool of GST-SWI5Δ384 and GRF10 onto a glutathione-Sepharose column. The GST-SWI5Δ384 protein bound to the column, because of the GST moiety, while the GRF10 protein flowed through the column (Fig. 7C). This result suggested that GRF10 does not interact with SWI5 in the absence of DNA.

DISCUSSION

Using a GST-SWI5 fusion protein purified from an *E. coli* expression system, we have shown that the SWI5 protein binds with low affinity or low specificity to the *HO* promoter. This result is surprising, since it has been previously shown that SWI5 can activate transcription from this site in vivo (43). In order to explain the low affinity of SWI5 for the *HO* promoter, we suggested that an additional factor may be needed for high-affinity binding. We devised an assay for such a factor and have identified an 83-kDa protein, GRF10, which binds DNA cooperatively with SWI5.

In addition to interacting cooperatively with SWI5, the GRF10 protein binds to the *HO* promoter on its own, albeit with a low affinity. GRF10 recognizes a region of DNA adjacent to the SWI5 DNA-binding site, and thus, SWI5 and GRF10 bind DNA individually at adjacent sites in the *HO* promoter. The methylation interference pattern of the GRF10 plus SWI5-DNA complex is nearly identical to the sum of the patterns seen when GRF10 and SWI5 bind DNA

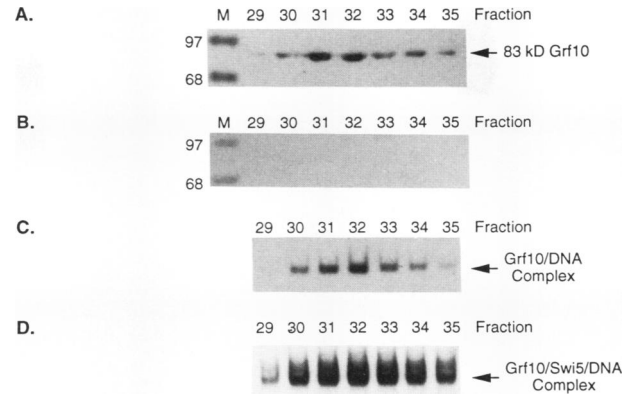


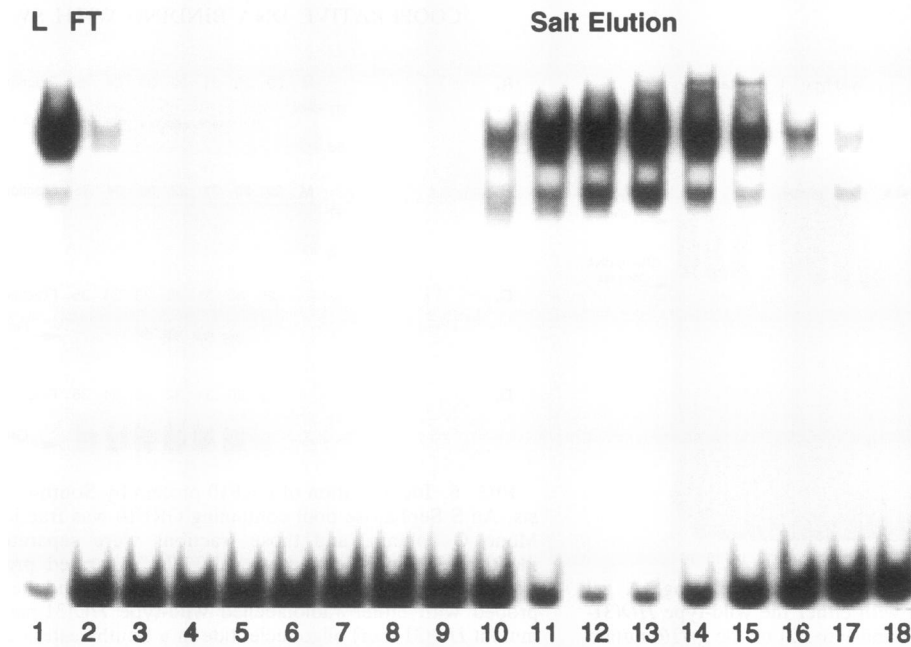
FIG. 6. Identification of GRF10 protein by Southwestern analysis. An S Sepharose pool containing GRF10 was fractionated on a Mono Q column, and those fractions were separated on two identical SDS-polyacrylamide gels. The separated proteins were transferred from the gels to nitrocellulose filters, and the filters were probed with either radiolabelled wild-type *HO*(31-mer) or *grf10* mutant *HO*(31-mer) oligonucleotide in a Southwestern experiment. The same fractions were also assayed for GRF10 DNA-binding activity and SWI5 stimulatory activity by gel retardation assay. (A) Southwestern experiment using wild-type probe. Lane M, molecular mass markers. (B) Southwestern experiment with GRF10 mutant probe. Lane M, molecular mass markers. (C) Gel retardation assay for GRF10 DNA-binding activity using wild-type probe. Binding reaction mixtures contained 0.5 μl of each Mono Q fraction, 100 μg of poly(dI-dC) · poly(dI-dC) per ml, and the CS169 probe. (D) Gel retardation assay for GRF10 stimulation of SWI5 DNA-binding activity using wild-type probe. Binding reaction mixtures contained 0.5 μl of each Mono Q fraction, 3 μl of GST-SWI5, 100 μg of poly(dI-dC) · poly(dI-dC) per ml, and the CS169 probe.

individually (Fig. 4C). This result strongly supports the idea that both GRF10 and SWI5 contact DNA in a ternary complex. As described earlier, there are subtle methylation interference pattern differences between the GRF10-plus-SWI5 ternary complex and the single-protein binary complexes. The methylation of a particular residue may interfere with protein binding either by disturbing a specific contact made by a protein or by changing the conformation of the DNA molecule. Some of the methylation events that affect only protein binding in the binary complexes may not interfere with protein binding in the ternary complex, because protein-protein contacts may provide sufficient energy to overcome their interfering effect.

The crystal structure of the ZIF268 protein demonstrated that the zinc fingers make contacts with DNA only in the major groove and that contacts are made to only one strand of DNA (35). Our methylation interference experiments (Fig. 4C) suggest differences in how the SWI5 zinc fingers recognize DNA. First, the interference studies identify essential DNA residues contacted by SWI5 located on both DNA strands. Second, there are adenine residues whose methylation interferes with binding by SWI5. This result suggests that SWI5 makes DNA contacts in the minor groove because adenine is methylated at positions located in the minor groove. However, it is also possible that methylation of these adenine residues could change the conformation of the DNA molecule, and it is this change in conformation that actually interferes with SWI5 binding. Further experiments will be needed to address these different possibilities.

The nuclear magnetic resonance-based solution structure

A. DNA-Affinity Column Fractions assayed for DNA-Binding Activity

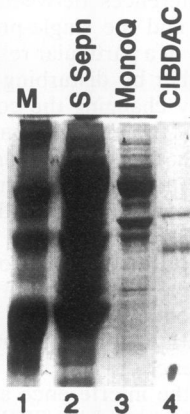


B.

DNA-Affinity Column Fractions on SDS Gel



C. CIBDAC Purification



D. Grf10 does not Bind to Swi5 in the Absence of DNA

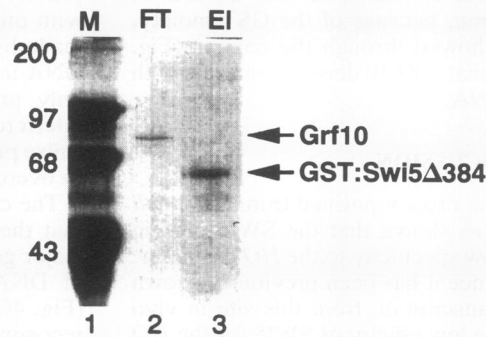


FIG. 7. CIBDAC. GRF10 protein, purified by heparin-agarose, S Sepharose, and Mono Q chromatography, was mixed with GST-SWI5Δ384 (a truncated version containing amino acids 384 to 709 of SWI5) and applied to an *HO*(31-mer) DNA affinity column, which contains the GRF10 and SWI5 binding sites from the *HO* promoter. The column was washed, and the flowthrough fraction was collected. Bound proteins were eluted with increasing concentrations of NaCl. (A) Gel retardation assay for DNA-binding activity. DNA binding reaction mixtures contained 1 μ l of each CIBDAC fraction, 100 μ g of poly(dI-dC) · poly(dI-dC) per ml, and the CS169 probe. L, load; FT, flowthrough. Lanes: 3 to 10, 100 to 450 mM NaCl fractions; 11 to 14, 500 mM NaCl fractions; 15 to 18, 1,000 mM NaCl fractions. (B) SDS gel of proteins in CIBDAC fractions. A sample (20 μ l) of each CIBDAC fraction was electrophoresed in a 7.5% SDS-polyacrylamide gel, and the gel was stained with Coomassie blue. Lane M, prestained molecular weight markers (in thousands). The sample in the second lane contains the GST-SWI5Δ384 protein as a marker. (C) Purification by CIBDAC. Samples were electrophoresed in a 7.5% SDS-polyacrylamide gel, and the gel was stained with Coomassie blue. Lanes: 1, molecular weight markers; 2, S Sepharose column pool; 3, Mono Q column pool; 4, CIBDAC column pool. (D) Glutathione-Sepharose chromatography. Fractions 10 to 12 and 14 and 15 from the CIBDAC column, containing GRF10 and GST-SWI5Δ384, were dialyzed, pooled, and loaded onto a glutathione-Sepharose column. The flowthrough fraction was collected, and proteins adhering to the column were eluted with glutathione. M, prestained molecular weight markers (in thousands); FT, flowthrough; EI, eluate.

of the SWI5 zinc fingers has been determined previously (32), and it shows a significant difference from the structures of other zinc finger proteins (22, 24, 35). Structural analyses have shown that each zinc finger contains two β strands and one alpha helix. In the first zinc finger of SWI5, an additional β strand is present at the N termini of the first two β strands. The region containing this additional β strand is necessary for complete DNA-binding activity of the SWI5 zinc finger protein (28). The DNA-binding properties of SWI5 derivatives containing either two or three zinc fingers have been examined by Nakaseko et al. (28). Their data allow us to orient SWI5 on the DNA molecule, placing the N-terminal three- β -stranded region at the 5' end of the binding site. These results lead to the suggestion that the region of SWI5 containing this third strand of β sheet may be involved in contacting the minor groove of DNA. For the *Drosophila* Tramtrak and yeast ADR1 zinc finger proteins, it has been shown previously that a similar region, just N terminal to the first zinc finger, is essential for full DNA-binding activity in vitro (10, 45).

The DNA-binding properties of SWI5 protein prepared from yeast cells were analyzed in order to eliminate the possibility that the in vitro stimulation of DNA binding was an artifact resulting from *E. coli* overexpression. There are reports of DNA-binding proteins that do not bind DNA effectively when purified from bacterial expression systems. The AP-1 DNA-binding activity is composed of subunits encoded by the Fos and Jun genes (20). When Fos and Jun proteins are purified from bacterial expression systems, efficient DNA binding requires an additional activity present in nuclear extracts (1). It was shown subsequently that DNA-binding activity of the Fos-Jun heterodimer is modulated by reduction-oxidation of conserved cysteine residues in Fos and Jun. In vitro DNA binding of highly purified GAL4 protein in a gel retardation assay also requires an additional factor (34). Similarly, purified human estrogen receptor binds DNA weakly in vitro, but the in vitro DNA-binding activity can be restored by the addition of single-stranded DNA-binding protein (27). We have shown that GRF10 stimulates DNA binding of SWI5 protein produced in yeast cells, and thus, the interaction is not an artifact resulting from *E. coli* overexpression of SWI5.

Since GRF10 can bind to the *HO* promoter alone, we attempted to purify GRF10 using DNA affinity chromatography (18). This approach was not successful, presumably because GRF10 binds to the *HO* promoter sequences with a relatively low affinity. Instead, we took advantage of the cooperative DNA binding and devised a purification method that we call CIBDAC. GRF10 was combined with SWI5 and loaded onto a DNA affinity column containing the *HO* promoter binding site in the presence of a large excess of nonspecific DNA. The column was eluted with increasing salt concentrations, and the SWI5 and GRF10 binding activities coeluted at about 0.5 M NaCl. Only two major bands, GST-SWI5 Δ 384 and an 83-kDa species, were seen on a protein gel stained with Coomassie blue. The evidence suggests that the 83-kDa protein is GRF10, as discussed below. The purity of SWI5 and GRF10 after the CIBDAC step is remarkable, suggesting that this method may be useful for the purification of other proteins that interact cooperatively.

SWI5 and GRF10 do not interact detectably in the absence of DNA. When the mixture of GRF10 and GST-SWI5 Δ 384 was applied to a glutathione-Sepharose column, the GST-SWI5 Δ 384 protein remained on the column because of the

interaction between GST and glutathione, but the GRF10 protein flowed through.

Several observations support the hypothesis that the 83-kDa protein that eluted from the CIBDAC column is indeed the factor that stimulates SWI5 DNA binding. First, the purified 83-kDa protein had a DNA-binding activity that recognized a region of DNA adjacent to the SWI5 DNA-binding site and was also capable of forming a ternary complex with SWI5 and DNA. Second, when we destroyed the binding site for this factor, this factor would no longer bind alone, and the high-affinity ternary complex would not form. Third, the Southwestern experiment identified an 83-kDa protein that would recognize this binding site in the absence of SWI5 but would not bind the mutant oligonucleotide binding site. Fourth, when the Mono Q pool containing the stimulatory activity was fractionated in a protein gel, the proteins that were eluted and renatured from the 83-kDa region contained an activity that would stimulate DNA binding of GST-SWI5 in the presence of excess poly(dI-dC) · poly(dI-dC) (data not shown). Finally, we have identified the gene encoding GRF10 and have used it to demonstrate that the DNA-binding and ternary complex activities are absent from *grf10* null mutants and that these same activities are present in the GRF10 protein expressed in *E. coli* (5a).

The term combinatorial control is often used to describe regulation by particular combinations of transcription factors. Cooperative binding of two DNA-binding proteins may be required for the recognition of a specific promoter element. Interactions with multiple regulatory proteins allow a particular protein to function at diverse promoters. We suggest that, on the basis of our results with SWI5, many zinc finger proteins may bind DNA cooperatively with additional factors. For the ADR1 zinc finger transcription factor, comparison of in vitro binding data with in vivo experiments led to the proposal for an additional regulatory component (45).

Transcriptional control by the yeast mating type (MAT) loci provides an elegant example of combinatorial control of gene expression (17). The yeast MATa1 and MATa2 proteins bind DNA cooperatively and function in α/α diploids to repress transcription of haploid-specific genes (11). In α haploids, expression of α -specific genes is blocked by MATa2 binding cooperatively with a different protein, MCM1 (19). In contrast, transcriptional activation of α -specific genes in a haploid cells requires the cooperative binding of MCM1 with the MATa1 protein (5). Thus, the combination of a limited number of different transcription factors can regulate many different target genes through combinatorial controls.

The cooperative DNA binding exhibited by SWI5 and GRF10 may play an important role in controlling the promoter specificity of SWI5 in vivo. SWI5 and ACE2 are DNA-binding proteins with 83% identical DNA-binding domains, and the proteins show similar patterns of cell cycle regulation (6, 9). SWI5 and ACE2 regulate different genes in vivo, despite the fact that SWI5 and ACE2 bind to the same DNA sequences in vitro (8a, 9). We believe that it is the ability of SWI5 to interact with GRF10 that allows it to bind to the *HO* promoter with sufficient affinity to activate *HO* transcription. The inability of ACE2 to bind DNA cooperatively with GRF10 (unpublished observations) prevents it from functioning as an activator of *HO* transcription in vivo. This represents a system in which cooperative interactions between DNA-binding proteins determine promoter specificity in vivo.

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