The Swi5 zinc-finger and Grf10 homeodomain proteins bind DNA cooperatively at the yeast HO promoter

ROBERT M. BRAZAS* AND DAVID J. STILLMAN†

Department of Cellular, Viral, and Molecular Biology, University of Utah Medical Center, Salt Lake City, UT 84132

Communicated by Mario R. Capecchi, August 20, 1993

ABSTRACT SWI5 encodes a zinc-finger protein required for expression of the yeast HO gene. Using Swi5 protein that was purified from a bacterial expression system, we previously isolated a yeast factor that stimulates binding of Swi5 to the HO promoter. N-terminal amino acid sequence analysis identified the Swi5 stimulatory factor as the product of the GRF10 gene, which encodes a yeast homeodomain protein. GRF10, also known as PHO2 and BAS2, is a transcriptional activator of the PHO5 acid phosphatase gene and the HIS4 histidine biosynthesis gene. Grf10 protein purified from a bacterial expression system binds DNA cooperatively with Swi5 in vitro. Analysis of dissociation rates indicates that the Grf10–Swi5–DNA complex has a longer half-life than protein–DNA complexes that contain only Swi5 or Grf10. Finally, we show that HO expression is reduced in yeast strains containing grf10 null mutations and that full expression of a heterologous promoter containing a SWI5-dependent HO upstream activation sequence element requires GRF10.

The control of gene expression is mediated by proteins binding to promoter and enhancer elements. Proper control of gene expression requires that these factors bind with sufficiently high affinities and specificities. However, many eukaryotic transcription factors do not appear to have the requisite affinities for specific DNA sequences to account for their specificities of action in gene regulation. In many cases, interactions between multiple proteins are needed to achieve the required affinity and DNA-binding specificity in DNA site recognition required for proper control of gene regulation. For example, the cooperative interactions of Oct-1, the C1 factor, and the viral VP16 gene product lead to specific recognition of promoter sites in herpes simplex virus (1). Similarly, interaction of the yeast Mcm1 protein with either the MATa1 or the MATa2 protein leads to specific patterns of transcriptional regulation at different promoters (2). In this report, we describe cooperative DNA binding between a zinc-finger protein and a homeodomain protein. The juxtaposition of these two DNA-binding motifs is interesting since many homeodomain and zinc-finger proteins have complex spatial and temporal patterns of expression during multicellular development.

The yeast SWI5 gene was identified as a transcriptional activator of the yeast HO gene (3). SWI5 encodes a Cys2-His2 zinc-finger DNA-binding protein that recognizes a regulatory site in the HO promoter (4). HO is expressed in only the mother cell, one of the two cells resulting from mitotic division, and SWI5 has been implicated in this pattern of asymmetric gene expression (5, 6). SWI5 is cell-cycle-regulated in two ways (5, 7). (i) SWI5 mRNA abundance is periodic in the cell cycle with maximal levels seen in the G2 phase of the cell cycle. (ii) The Swi5 protein accumulates in the cytoplasm during the G2 phase, and at the end of mitosis, the protein moves into the nucleus where it disappears rapidly. An additional level of control exerted on Swi5 is presented in this paper. We have identified a factor that binds to the HO promoter cooperatively with Swi5, and we believe this cooperative DNA binding determines the promoter specificity of Swi5.

METHODS

Saccharomyces cerevisiae Strains. Strains DY131, DY150, and DY999 have been described (8). DY411 is an isogenic derivative of DY150 that contains a swi5::HIS3 gene disruption. The grf10::LEU2 disruption was constructed by replacing the internal GRF10 Bgl II–Nhe I fragment with a BamHI–Xba I LEU2 fragment. The grf10::LEU2 disruptions in strains DY131, DY150, DY411, and DY999 were confirmed by Southern blot analysis.

In Vitro Binding Analysis. The gel-retardation assay was performed as described (9). The same binding conditions were used for the DNase I footprinting, except that 3 ng of labeled probe and poly(dI-dC)poly(dI-dC) at 16.5 μg/ml were used in a 40-μl binding reaction mixture.

Protein Purification. The HIS–Swi5 and Grf10–HIS proteins were purified from Escherichia coli as described (9). The HIS–Swi5 construct has been described (9). The plasmid construct used for overexpression of Grf10–HIS was constructed by cloning the 1479-bp Bcl I fragment of GRF10 into the BamHI site of pET-21b (Novagen). This construct expresses a fusion protein that contains aa 35–528 (of 559 aa total) of Grf10 fused to 6 His residues at the Grf10 C terminus.

S-100 protein extracts were prepared and fractionated as described (9) from cultures of DY411 (swi5−) and DY1938 (swi5−, grf10−). Each extract was fractionated in parallel, first on heparin-agarose and then on Q-Sepharose columns. Fractions eluting from the heparin column at 0.6 M NaCl (HEP600) and from the Q-Sepharose column at 0.3 M NaCl (Q300) were used for in vitro binding studies. The S-100 fractions were adjusted to 6.1 mg/ml, and the HEP600 and Q300 fractions were adjusted to 0.67 mg/ml.

RESULTS

Swi5 Requires an Additional Protein for High-Affinity Binding. We have purified the Swi5 protein from an E. coli expression system and demonstrated that Swi5 recognizes HO promoter sequences weakly in vitro (9). This result is surprising because genetic evidence indicates that Swi5 functions as a transcriptional activator of HO in vivo. We have identified a factor that stimulates binding of Swi5 to the HO promoter in a gel-retardation assay and used this assay to purify an 83-kDa protein from yeast extracts. Methylation-interference and missing-nucleoside experiments identified DNA contact sites for both the 83-kDa protein and Swi5.

Abbreviation: UAS, upstream activation sequence.

*Present address: Department of Microbiology and Immunology, University of California, San Francisco, CA 94143.

†To whom reprint requests should be addressed.
Each protein binds DNA in the absence of the other, but formation of a 83-kDa protein–Swi5–DNA ternary complex is cooperative. However, the two proteins do not interact detectably in the absence of DNA.

We have now sequenced the N-terminal end of the purified 83-kDa protein that stimulates Swi5 binding. The sequence of the first 20 residues is MEIFSYPYDHFNTHFTATLD. This sequence was compared to protein databases, and a perfect match to the predicted product of the *GRF10* gene (also known as *PHO2* or *BAS2*) was found. Since *GRF10* plays a role in the transcriptional activation of diverse genes, it was agreed to replace the *PHO2* and *BAS2* gene designations with *GRF10* (for general regulatory factor) (10). *PHO2* was first identified as a transcriptional activator of the *PHO5* gene, which encodes acid phosphatase (11); a *pho2* mutant is unable to grow in the absence of exogenous inorganic phosphate. *BAS2* is required for the basal expression of the *HIS4* gene, which is required for histidine biosynthesis (12). Recently, it has been shown that *GRF10* is required for the expression of *TRP4* (13) and several adenine biosynthesis genes (14).

The sequence of the *GRF10* gene reveals that it is a homeodomain protein (15). In previous studies, the *Grf10* protein synthesized in *E. coli* has been used for DNA-binding experiments (13, 14, 16, 17). The *Grf10* protein bound to sites in the *PHO5*, *HIS4*, *TRP4*, *ADE2*, and *ADE5* promoters. However, other than a TAAT sequence, little homology between these binding sites is discernible. The sequence TAAT is a component of most homeodomain binding sites (18, 19).

The identity of the Swi5 stimulatory factor as the product of the *GRF10* gene was verified in two ways. First, a yeast strain was constructed with a disruption of the *GRF10* gene. Extracts were prepared from two isogenic strains, *GRF10*+ and *grf10Δ::LEU2*, and used for *in vitro* DNA-binding experiments. As shown in Fig. 1, lane 2, Swi5 bound DNA weakly in the presence of a nonspecific DNA such as poly(dl-dC)-poly(dl-dC). Addition of the *GRF10*+ yeast S-100 extract led to the appearance of a slower-migrating protein–DNA complex (lane 3); this complex contains both Swi5 and Grf10 (9). This complex was not observed when the extract was prepared from a *grf10* mutant (lane 4). The two extracts were chromatographed on identical heparin-agarose columns. The protein fraction eluted with 600 mM NaCl from the *GRF10*+ column stimulated Swi5 DNA binding, whereas the same fraction from the *grf10* column did not stimulate Swi5 DNA binding (lanes 5 and 6). The eluted proteins from each heparin-agarose column were then fractionated on identical Q-Sepharose columns. Again, Swi5 stimulatory activity was recovered from only the *GRF10*+ column (lanes 7 and 8). When each fraction was assayed in the absence of added Swi5, Grf10 DNA-binding activity was detected in only the column fractions prepared from the *GRF10*+ strain (Fig. 1, lanes 9–14). The enrichment of Grf10 DNA-binding activity paralleled the enrichment of the stimulatory activity, as shown previously (9). These experiments demonstrated that *grf10* mutants lack the factor that stimulates Swi5 binding to DNA.

Swi5 and Grf10 Bind DNA Cooperatively *in Vitro*. In our second approach to proving that Grf10 is the Swi5 stimulatory factor, we used Grf10 protein synthesized in *E. coli* for *in vitro* DNA-binding studies. Purified Grf10 and Swi5 proteins were used in a gel-retardation assay with a labeled DNA probe containing sequences from the *HO* promoter (Fig. 2). Many features of these assays are identical to our previous assays with Swi5 stimulatory factor isolated from yeast. Grf10 and Swi5 each bound *HO* DNA in the absence of the other protein (lanes 2–6 and 14–18). Incubation of both Grf10 and Swi5 with *HO* DNA results in a slower-migrating protein–DNA complex (lanes 8–12 and 20–24). Based on our previous methylation interference experiments, we predict that both Grf10 and Swi5 are present in this complex.

The combination of Grf10 and Swi5 binds DNA with a greater apparent affinity than either protein alone (Fig. 2), which was also true using Grf10 purified from yeast (9). Comparison of Swi5 binding in the absence (lanes 2–6) and presence (lanes 8–12) of Grf10 demonstrated that Grf10 increases the apparent affinity of Swi5 for this DNA. Grf10 addition caused a decrease in the number of binary Swi5–DNA complexes as the number of ternary complexes increased (compare lanes 3 and 9). The reduced amount of free DNA probe (lanes 11 and 12 vs. lanes 5 and 6) reveals the increase in protein–DNA complexes in the presence of the two proteins. Similarly, Grf10 binds DNA weakly (lanes 13–18) and addition of Swi5 stimulates DNA binding by Grf10 (lanes 20–24). We have calculated the ω factor (20) as a measure of the free energy of the Swi5–Grf10 interaction. The ω factor is 136, indicating a very high degree of cooperativity. We conclude that the Grf10 gene encodes a factor that stimulates Swi5 DNA binding and vice versa.

DNase I footprinting experiments were conducted with the Swi5 and Grf10 proteins (Fig. 3). Swi5 (lanes 1–5) and Grf10 (lanes 8–13) protected adjacent regions of the *HO* promoter from DNase I digestion. Simultaneous incubation of both proteins (lane 7) with DNA resulted in a footprint that was the sum of the two single footprints. The DNase I protection experiments also confirmed the cooperative binding between Grf10 and Swi5 (lanes 4, 7, and 8). Two units of Swi5 (lane 4) and 1 unit of Grf10 (lane 8) provide little protection from DNase I digestion. However, complete protection was achieved by the simultaneous addition of these amounts of Swi5 and Grf10 in a single binding reaction mixture (lane 7). We conclude that Grf10 and Swi5 bind DNA in a mutually cooperative fashion.

It is interesting to note that the Swi5 binding site in the *HO* promoter was first identified using a combined DNase I and gel-retardation protocol with extracts prepared from cells overproducing Swi5 (4). In this experiment, a 38-nt region of the *HO* promoter was protected from DNase I digestion. In contrast, the DNase I footprint obtained using a Swi5 polypeptide consisting of only the Swi5 zinc-finger region purified.
from an *E. coli* expression system was much smaller (20 nt), leading to the suggestion that other regions of Swi5 were responsible for the extended footprint (21). However, the footprint of the Swi5 zinc-finger polypeptide corresponds very well to our footprint using the full-length Swi5 protein shown in Fig. 3. We believe that the extended footprint obtained by Stillman et al. (4) using S-100 extracts was the result of both Swi5 and Grf10 being present in the footprinted complex.

**The Grf10–Swi5–DNA Complex Is More Stable.** The apparent enhancement of affinities of both Grf10 and Swi5 in the Grf10–Swi5–DNA ternary complex could be due to an increase in association rate or a decrease in the dissociation rate of the ternary complexes relative to the binary complexes. Gel-retardation assays were performed to determine the $t_{1/2}$ of the various protein–DNA complexes. In the assay shown in Fig. 4, protein and probe DNA were incubated until equilibrium was reached, and a 600-fold molar excess of unlabeled binding site DNA was then added. After various times, the mixtures were loaded onto a gel to determine the amount of each protein–probe complex still present. This assay allowed us to measure the $t_{1/2}$ of protein–DNA complexes (Fig. 4). The Swi5–DNA complex was relatively unstable, and the Grf10–DNA complex was very unstable. However, the Grf10–Swi5–DNA ternary complex was quite stable. The actual $t_{1/2}$ of the Grf10–DNA complex was $<15$ sec. The Swi5–DNA complex had a $t_{1/2}$ of $\approx2$ min, and the Grf10–Swi5–DNA ternary complex had a $t_{1/2}$ of $\approx20$ min. We conclude that the Grf10–Swi5–DNA ternary complex is much more stable than either of the protein–DNA complexes containing a single protein. These kinetic studies indicate that the mutual enhancement of binding affinities of Grf10 and Swi5 is due in part to the increased stability of the ternary complex.

**A grf10 Mutation Reduces HO Expression.** The in vitro DNA-binding assays suggest that Grf10 is a coactivator of *HO* gene expression. To test this hypothesis, experiments were performed to determine the effect of a grf10A::LEU2 null mutation on expression of HO in vivo. A chromosomal

Table 1. *HO* expression is reduced by a grf10 null mutation

<table>
<thead>
<tr>
<th>Strain</th>
<th>Relevant genotype</th>
<th>LacZ activity</th>
<th>% parental activity or fold stimulation</th>
</tr>
</thead>
<tbody>
<tr>
<td>DYY131</td>
<td>HO–lacZ</td>
<td>SWI+</td>
<td>236.3 ± 26.0</td>
</tr>
<tr>
<td>DYY999</td>
<td>HO–lacZ</td>
<td>swi5Δ</td>
<td>3.4 ± 0.2</td>
</tr>
<tr>
<td>DYY1910</td>
<td>HO–lacZ</td>
<td>SWI+</td>
<td>61.0 ± 0.4</td>
</tr>
<tr>
<td>DYY1946</td>
<td>HO–lacZ</td>
<td>swi5Δ</td>
<td>3.0 ± 1.6</td>
</tr>
<tr>
<td>DYY150</td>
<td>(no UAS)–CYC1–lacZ</td>
<td>SWI+</td>
<td>8.8 ± 0.4</td>
</tr>
<tr>
<td>DYY150</td>
<td>HO(46)–CYC1–lacZ</td>
<td>SWI+</td>
<td>130 ± 15</td>
</tr>
<tr>
<td>DYY150</td>
<td>HO(46)–CYC1–lacZ</td>
<td>SWI+</td>
<td>130 ± 15</td>
</tr>
<tr>
<td>DYY1411</td>
<td>HO(46)–CYC1–lacZ</td>
<td>swi5Δ</td>
<td>18 ± 1</td>
</tr>
<tr>
<td>DYY1921</td>
<td>HO(46)–CYC1–lacZ</td>
<td>SWI+</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>DYY1938</td>
<td>HO(46)–CYC1–lacZ</td>
<td>swi5Δ</td>
<td>13 ± 0.3</td>
</tr>
</tbody>
</table>

Isogenic strains were created by disruption of the *GRF10* gene. Quantitative $\beta$-galactosidase activity assays were performed as described (22). The integrated *HO–lacZ* reporter has been described (22). Plasmid pLGa-178, which is designated in Table 1 as (no UAS)–CYC1–lacZ, contains the *lacZ* gene under the control of the *CYCI* promoter. In this construct, *lacZ* is not expressed because the promoter lacks a UAS. A 46-nt oligonucleotide derived from the *HO* promoter (9) containing the Grf10/Swi5 binding site was inserted into the pLGa-178 vector to create the reporter designated *HO(46)–CYC1–lacZ*. LacZ activity is normalized to protein concentration, as described (22).
FIG. 3. Grf10 and Swi5 bind cooperatively to the HO promoter. Relative amounts (indicated in the figure) of Grf10-HIS and HIS-Swi5 fusion proteins were incubated either alone or in combination with a 5'-end-labeled HO DNA probe for 60 min at 25°C before treatment with DNase I. The top strand of the Grf10/Swi5 binding site in the CS169 probe (9) was 5'-end-labeled in this experiment. The relative protein composition of each reaction mixture is indicated in the figure, where 1 unit = 1.3 μl of HIS-Swi5 and 1 unit = 0.5 μl of Grf10-HIS (−, not added).

HO-lacZ reporter gene (22) was used to quantitate HO promoter activity in four strains differing only at the SWI5 and GRF10 loci. As shown in Table 1, a swi5 mutation reduced HO-lacZ levels by a factor of 100, and the grf10 mutation reduced HO-lacZ levels by a factor of ~4. We conclude that the GRF10 gene is required for full activity of the HO promoter.

Stillman et al. (4) have shown that the Grf10/Swi5 binding site present in the HO promoter =1300 nt upstream of the HO ATG behaves as a SWI5-dependent upstream activation sequence (UAS) in a heterologous promoter. To determine the role of GRF10 in activating this UAS, we placed the Grf10/Swi5 binding site of HO into a heterologous promoter that lacks a UAS and tested the effect of a grf10 mutation on transcription of this reporter. One copy of a 46-bp oligonucleotide derived from the HO promoter containing the Grf10 and Swi5 binding sites was inserted into the vector pGAD-178, thus creating HO(46)-CYCl-lacZ. This HO promoter fragment provided UAS activity, and a swi5 mutation caused a decrease in promoter activity by a factor of 7 (Table 1). Importantly, the grf10 mutation also caused a reduction in activity of this reporter by a factor of 7. We can rule out the possibility that the grf10 mutation reduces transcription of all genes nonspecifically, since expression of a number of genes is not affected by the grf10 null mutation (ref. 14 and data not shown). The failure to find epistasis in this in vivo experiment is consistent with the in vitro cooperative DNA binding of Grf10 and Swi5. This result demonstrates that transcriptional activity from the Grf10/Swi5 binding site of the HO promoter requires the Grf10 and Swi5 transcription factors.

GRF10 appears to make different contributions to the transcriptional activity of the complete HO promoter and the HO(46)-CYCl-lacZ plasmid reporter (Table 1). Expression of HO-lacZ was reduced dramatically by a swi5 mutation, but a grf10 mutation had only a modest effect. In contrast, expression of the HO(46)-CYCl-lacZ reporter is reduced equally by either mutation. The failure of the grf10 mutation to reduce HO-lacZ expression to the same degree as the swi5 mutation could be explained by proposing that an additional SWI5-dependent UAS exists in the HO promoter and that binding of Swi5 to this UAS does not require Grf10. This hypothesis is supported by the identification of a second Swi5 binding site in the HO promoter (23). This second binding site may provide the residual SWI5-dependent HO-lacZ transcription in the absence of GRF10. This model leads to the prediction that binding of Swi5 to this site will not require Grf10, and this second site will function as a SWI5-dependent UAS that acts independently of GRF10.

DISCUSSION

We have demonstrated that the Swi5 and Grf10 proteins each bind to the HO promoter with low affinity and that the two proteins bind DNA cooperatively. Moreover, the Swi5-Grf10-DNA ternary complex is more stable than either of the binary complexes containing a single protein. DNase I footprint analysis demonstrates that the two proteins recognize adjacent regions on the DNA. We have also shown that both Swi5 and Grf10 are required for full expression of HO. Swi5 is a DNA-binding protein with three Cys2-His2 zinc fingers, and Grf10 (also known as Pho2 or Bas2) is a homeodomain protein.
A large number of genes encoding zinc-finger and homeodomain proteins have been identified (19, 24, 25). Complex spatial and temporal patterns of expression during development have been observed for many of these genes. These observations, along with genetic studies in Drosophila, have led to the suggestion that they play critical roles in development in multicellular organisms. In vitro DNA-binding studies with homeodomain proteins have led to a conceptual difficulty. Many homeodomains recognize very similar sequences in vitro yet regulate different genes in vivo (19). This leads to the view that regulatory specificity is achieved through the cooperative interactions between multiple proteins, each binding DNA with a relatively low specificity (26–28). Moreover, such models of combinatorial control allow a relatively small number of factors to produce complex patterns of gene regulation. It is possible that, like Grf10 and Swi5, some Drosophila and mammalian homeodomain proteins may bind DNA cooperatively with zinc-finger proteins to achieve proper gene regulation.

The yeast mating type (MAT) loci control the expression of many unlinked genes. This combinatorial control of gene expression is achieved through cooperative interactions between DNA-binding proteins. In α/α diploid cells, the MATα2 and MATα1 homeodomain proteins bind DNA cooperatively and function in α/α diploids to repress transcription of haploid-specific genes (29). In haploid α cells, expression of α-specific genes is blocked by MATα2 binding cooperatively with a different protein, Mcm1 (30). The MATα2 protein is, therefore, capable of repressing transcription of different target genes based on its interaction with various protein partners. In addition, the Mcm1 protein or its mammalian homologue, serum response factor, is able to interact with many other DNA-binding proteins (31–35).

Grf10, also known as Pho2 or Bas2, functions as a transcriptional activator of the yeast Pho5, His4, Trp4, and Ade1, Ade2, Ade5/7, and Ade8 genes (11–14). Interestingly, at each of these genes, Grf10-dependent transcriptional activation requires a second DNA-binding transcription factor, either Pho4 or Bas1. No evidence has been presented thus far suggesting that the Grf10 protein binds DNA cooperatively with either Pho4 or Bas1. Moreover, there is no indication that Swi5 activates any of these other Grf10-regulated genes. Thus, regulation by Grf10 is an example of combinatorial control of gene expression since Grf10 requires distinct partners at different promoters.

Swi5 and Ace2 are transcriptional regulators with many similar characteristics. Ace2 was identified as a high-copy suppressor that allowed Cup1 gene expression in the absence of the Ace1 activator (36). Ace2 encodes a protein that is very similar to Swi5. The DNA-binding domains of Swi5 and Ace2 are 83% identical and 95% similar (including conservative substitutions), and Swi5 and Ace2 can bind to the same DNA sites in vitro (P. R. Doehrmann, R.M.B., and D.J.S., unpublished observations). Moreover, it has been demonstrated that Ace2 undergoes the same patterns of cell-cycle-regulated transcription and cell-cycle-regulated nuclear localization as Swi5 (8).

Although Swi5 and Ace2 show many parallels, they regulate different genes in vivo (8). Swi5 is an activator of HO, whereas Ace2 cannot activate HO unless Ace2 is overexpressed. Similarly, Ace2 can function as an activator of the chitinase gene CTS1, but Swi5 cannot. The fact that Swi5 and Ace2 can both bind to the HO and CTS1 promoters (unpublished observations) leads to the question of what prevents the cross regulation of CTS1 by Swi5 and HO by Ace2.

We believe that the Grf10 protein may play a critical role in determining the promoter specificity of Swi5. Grf10 may interact only with Swi5 at the HO promoter, thus providing the specification for Swi5 action at HO. This is supported by experimental observations indicating that Grf10 cannot bind cooperatively with Ace2 to the HO promoter and cannot bind to all at the CTS1 promoter (P. R. Doehrmann, R.M.B., and D.J.S., unpublished observations). Another factor may also be present in cells that interacts specifically with Ace2 in binding to the CTS1 promoter, and this protein may provide the specificity for Ace2 regulation of CTS1. Therefore, the cooperative interaction of the zinc-finger DNA-binding domain protein, Swi5, and the homeodomain DNA-binding protein, Grf10, may provide the specificity required for the proper transcriptional regulation of the HO gene.

We thank B. Schackmann for determining the N-terminal sequence of Grf10, K. Arndt for a plasmid containing the Grf10 gene, and Y. W. Jiang for constructing the grf10::LEU2 disruption. We also thank B. Graves, G. Herrick, C. Hull, and S. Sakonju for comments on the manuscript. Oligonucleotides were synthesized and proteins sequenced at the University of Utah Protein/DNA Core Facility supported in part by National Cancer Institute Grant 5 P30 CA42014. R.M.B. was a predoctoral trainee supported by National Institute of Health Genetics Training Grant 5 T32 GM07464. This work was supported by a grant from the National Institutes of Health awarded to D.J.S.