Wild Type GAL4 Binds Cooperatively to the GAL1–10 UAS\(_G\) in Vitro\(^*\)

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Transcription of the genes required for utilization of galactose in Saccharomyces cerevisiae is controlled primarily by the transcriptional activator protein GAL4. The upstream activating sequences for galactose (UAS\(_G\)) of most GAL genes have multiple sites to which GAL4 can bind. In this report we compare the binding properties of wild type GAL4 and derivatives of GAL4 bearing the N-terminal DNA-binding domain to multiple DNA-binding sites in vitro. To produce wild type GAL4, we constructed a recombinant baculovirus containing a near-consensus 17-mer GAL4 DNA-binding site in electrophoretic mobility shift assays. Footprinting experiments revealed that wild type GAL4 binds cooperatively to the four GAL4 DNA-binding sites of the GAL1–10 UAS\(_G\); however, in contrast an N-terminal fragment of GAL4 containing only the DNA-binding/dimerization domains binds to each of these sites with slightly different affinity. With increasing concentrations of GAL4(1–147), the four sites become filled in the following order: site II, site IV, site I, and site III. In experiments with wild type GAL4, these four sites become fully occupied at approximately the same concentration of protein. In footprints of wild type GAL4 on the UAS\(_G\), enhancements and protections of DNase I-sensitive cleavages are detectable between sites III and IV, indicative of formation of a loop between these distantly spaced sites. Binding of wild type GAL4 to a strong near-consensus binding site assists binding to an adjacent mutant site in both electrophoretic mobility shift and footprinting assays. GAL4(1–147) and GAL4(1–147) fused to portions of GAL4’s activating region II were incapable of cooperative DNA binding in our assays. We conclude from these observations that wild type GAL4 has a cooperative DNA-binding function that is distinct from the DNA binding and dimerization or transcriptional activation functions, and likely plays and important role in precise regulation of GAL gene transcription.

The ability of the budding yeast Saccharomyces cerevisiae to utilize galactose or melibiose, a disaccharide consisting of α-linked galactose and glucose, is conferred by a group of genes whose transcription is activated by the GAL4 protein (for review, see Ref. 1). The products of these genes include those that comprise an apparatus for cleaving the melibiose disaccharide (α-galactosidase, MEL1), catalyzing entry of galactose into the cell (galactose permease, GAL2), and converting it into glucose 6-phosphate (galactokinase, GAL1; α-D-galactose-1-phosphate uridylyltransferase, GAL7; uridine diphosphoglucose 4-epimerase, GAL10; phosphoglucomutase, GAL5) for use in glycolysis. Transcription of these genes, apart from GAL5, is closely adjusted by the availability of galactose and glucose in the growth medium, and is dependent upon the transcriptional activator GAL4 which binds to its upstream activating sequences for galactose (UAS\(_G\)). When galactose is absent, transcriptional activation of these genes is prevented by the action of GAL80, a protein that binds to GAL4 and inhibits its transcriptional activation function (2–4). The presence of galactose induces an alteration in the DNA-bound GAL4-GAL80 complex such that GAL4’s transcriptional activating domains become functional, resulting in an approximately 1000-fold increase in transcription of GAL1, GAL10, GAL2, and GAL7, and a 2–100-fold increase in MEL1 and GAL80 (transcription (1)). Efficient induction of GAL4 activity requires GAL3, a protein with unknown function, but which is structurally similar to galactokinase (GAL1) (5, 6). This process of galactose-responsive induction is reversed, or prevented, by a battery of mechanisms in the presence of glucose, a carbon source that is consumed more easily than is galactose. Glucose causes inhibition of GAL4 transcription, inhibition of GAL4 activity, and stimulates the activity of a group of proteins that bind to upstream repression sequences for galactose (URS\(_G\)), whose function is to block activation of transcription by GAL4 (7–11). The combined effect of these glucose repression mechanisms causes rapid and complete repression of genes regulated by GAL4.

The transcriptional activator GAL4, which in combination with the accessory activator protein GAL11 (12, 13), appears to be solely responsible for transcriptional activation of the GAL genes. Molecular dissection of the GAL4 protein has revealed distinct functional domains that confer specific DNA binding, transcriptional activation, interaction with GAL80, and repression by glucose (see schematic diagram, Fig. 2A), GAL4 binds as a dimer to a 17-base pair sequence with partial dyad symmetry (Fig. 2B). Specific binding to the “17-mer” GAL4 DNA-binding site is conferred by a zinc-requiring DNA-binding motif contained within the N-terminal 65 residues (14). Protein fragments containing this portion of GAL4 and residues 65–94, that mediate the formation of dimers (15), bind specifically to this 17-base pair sequence, but are incapable of activating transcription in vitro or in vivo (16, 17). Transcriptional activation is conferred by two major activating domains, termed activating region I (residues 148–238) and activating region II (residues 767–881) (16, 18). Residues

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94-106 also contain sequences that contribute to transcriptional activation by GAL4 derivatives in
vitro (activating region III, not indicated in Fig. 2A) (17). Repression by GAL80 requires the presence of the C-terminal 30 residues of GAL4, contained within activating region II (residues 848-881) (4). GAL80 has been shown to bind directly to this C-terminal region (2, 3). The central portion of GAL4, between activating regions I and II may contribute several functions, including inhibition of activity in the presence of glucose, and the ability to activate transcription from distant sites (19). Direct inhibition of GAL4 by glucose requires the presence of the central region (residues 238-767). Fusion of this portion of GAL4 to a heterologous transcriptional activator that is not otherwise affected by glucose confers inhibition by glucose. We have identified a group of inhibitory domains within this central portion, and a region that is required for activity of GAL4 in the absence of glucose, called the glucose response domain.1

Most genes regulated by GAL4 have multiple DNA-binding sites for GAL4 in their upstream activating sequences for galactose; the divergent GAL1-10 promoter has four sites, while the promoters for GAL7 and GAL2 each have two sites (1). Experiments performed in vivo suggest that multiple DNA-binding sites contribute synergistically to transcriptional activity (11, 20). In vivo footprinting experiments have demonstrated that binding of GAL4 to a single non-consensus DNA-binding site is assisted by the presence of an adjacent binding site (20). Furthermore, transcriptional activation of reporter genes bearing multiple GAL4 DNA-binding sites was found to be much greater than the sum of transcriptional activation of genes containing individual GAL4 DNA-binding sites (20). However, this cooperative effect on transcriptional activation cannot solely be attributed to DNA binding since it was later demonstrated that many transcriptional activator proteins, including GAL4, can interact synergistically with their target(s) for activation of transcription. The central portion of GAL4, between activating regions I and II, may contribute sequences that are required for synergistic interaction of GAL4 with its target(s) for activation of transcription and DNA-binding/dimerization functions.

**EXPERIMENTAL PROCEDURES**

**Construction of Recombinant Baculovirus**—The transfer vector for production of the recombinant GAL4 baculovirus consisted of a HindIII fragment encoding wild type GAL4 from pMA210 (16) cloned upstream of the BarHI site into the polyhedron promoter. The transfer vector was cotransfected with wild type Autographa californica nuclear polyhedrosis virus (AcNPV) DNA into Spodoptera frugiperda SF9 cells by calcium phosphate coprecipitation. Plaque assays were performed on fresh 10-cm diameter lawns of SF9 cells with culture supernatants from transfections. Agar overlays from plates with 200–500 plaques were lifted, and viral DNA was absorbed onto nitrocellulose filters. Plaques containing GAL4 DNA were identified by probing the filters with 32P-labeled GAL4 DNA. Recombinant virus was recovered from the agar overlays and plaque-purified. Secondary and tertiary plaque screening were performed by western blotting of infected cultures with antibodies against GALA(1-147) (23). SF9 cells were maintained at 27 °C in TC100 culture medium ( Gibco) containing 10% fetal bovine serum.

**Plasmids for Expression of GAL4 in Escherichia coli—**Plasmids for expression of GAL4 derivatives in E. coli were derived from pTMCI, a vector that expresses GALA(1-74) fused to the C terminals of the T7 phage repressor from the T7 promoter (15). pTMCI and pTMCI (17) for expression of GALA(1-147) and GALA(1-147+ activating region II) were constructed by exchanging the Xhol/HindIII fragment of pTMCI with Xhol/HindIII inserts from pMA241 and pMA256 (16), respectively. pTMCI and pTMCI (17) for expression of GALA(1-147+ activating region IIa) was derived from pTMCI by digestion with MluI, which cleaves between activating region IIa and IIb, and inserting an XbaI oligonucleotide adapter containing an in-frame translational termination corder.

**Preparation of Lysates from Baculovirus-infected SF9 Cells—**SF9 cells were grown in 200 ml suspension cultures to a density of 2 × 109 cells/ml prior to infection with recombinant GAL4 baculovirus at a multiplicity of infection of 3. Infected cells were cultured for an additional 72 h prior to harvesting by centrifugation at 2,000 × g for 5 min. The cells were washed once with Tris-buffered saline (20 mM Tris-HCl, pH 7.4, 150 mM NaCl, and resuspended in 5 ml of extraction buffer (20 mM Tris, pH 8.0, 150 mM NaCl, 5 mM EDTA, 1% (v/v) polyethylenimine, 2 μg/ml chymostatin, 100 μg/ml L-1-p-tosylamino-2-phenylethyl chloromethyl ketone, 1 mM phenylmethylsulfonyl fluoride). The suspensions were homogenized with 29 strokes in a Dounce homogenizer and the lysate clarified by centrifugation at 10,000 × g for 20 min. The supernatants contained approximately 50% of expressed wild type GAL4 protein and were fractionated by chromatography on phosphocellulose or diluted with an equal volume of saline. Excess extraction buffer containing 20% glycerol and stored frozen in aliquots at −70 °C.

**Purification of GAL4 and GAL4 Derivative Proteins—**Wild type GAL4 was partially purified by chromatography on phosphocellulose. Solubilized extracts containing wild type GAL4 were loaded onto phosphocellulose in buffer A (20 mM Tris-HCl, pH 8.0, 150 mM NaCl, 1 mM DTT, 1 mM EDTA, 1 mM NiCl2, 0.6 M NaCl). The column was washed with four volumes of buffer A and then developed with a gradient of 50 mM to 2 M NaCl in buffer A. GAL4 was eluted from the column at approximately 1 M NaCl. GAL4 was found to be approximately 85% pure in these fractions. GALA(1-147), GALA(1-147+II), and GALA(1-147+IIa) were purified from E. coli as described by Carey et al. (15). XA90 E. coli cells harboring GAL4 expression plasmids were grown to O.D. A595 = 0.6 at 25 °C, and the T7 promoter induced by the addition of isopropyl-b-D-thiogalactopyranoside to 10 mM. The cultures were grown for a further 3 h and harvested by centrifugation. Cells from a 2-liter culture were washed in buffer B (20 mM HEPES, pH 7.0, 20 mM ZnSO4, 1 mM DTT, 1 mM phenylmethylsulfonyl fluoride, 200 mM NaCl) and resuspended in 50 ml of the same. The cells were disrupted by sonication and the insoluble material removed by centrifugation at 12,000 × g for 20 min. The supernatants were loaded onto phosphocellulose to the initial concentration of 0.5% (v/v) and the solution stirred for 15 min on ice. Insoluble material was removed by centrifugation at 12,000 × g for 20 min. To the supernatant, solid ammonium sulfate was added to a final concentration of 45%, proteins were allowed to precipitate with stirring on ice for 1 h. The precipitated material was redissolved by centrifugation and dissolved in 8 ml of buffer B. The protein solution was then dialyzed against buffer B containing 0.2 M NaCl

1 G. Stone and I. Sadowski, manuscript in preparation.

2 The abbreviation used is: DTT, diithiothreitol.
protein bands in Coomassie Blue-stained gels to standards of bovine homogeneous preparations (approximately 90%). Concentration of contains a 365-nucleotide DdeIISau3AI fragment bearing the four GAL4 derivatives was determined by comparing the intensity of For footprinting of the erase I. Labeled DNA was reprecipitated, digested with labeling with [cY-~'P]~ATP and the Klenow fragment of DNA polymerase I.

365-nucleotide UASc fragment purified by agarose gel electrophoresis. Templates containing the single MH103-binding site were derived from plasmids pMH103 and pMH100/103(B/K/H), respectively (24). Both of these templates were labeled at the EcoRI site flanking the MH103-binding site on pMH103, and flanking the MH100-binding site on pMH100/103(B/K/H); 583 nucleotide and 643 nucleotide template fragments, respectively, were released by digestion with AsaII. Binding reactions contained 20 mM HEPES, pH 7.5, 5 mM MgCl2, 100 μg/ml bovine serum albumin, 10% (v/v) glycerol, 10 μM ZnSO4, 50 mM NaCl, 2 μg/ml poly(dI-dC), and 10 μg/ml sheared salmon sperm DNA and were performed at room temperature for 10 min. The reactions were treated with DNase I at a final concentration of 6 units/ml (Promega) for 1 min prior to the addition of an equal volume of stop buffer (8 M NH4 OAc, 50 mM EDTA). The digestion products were precipitated, redissolved in sample buffer, and resolved on 6% sequencing gels. The position of GAL4 DNA-binding sites on the templates was determined by Maxam and Gilbert sequencing.

The probe containing a single near consensus MH100-binding site for gel mobility shift assays of GAL4 derivatives consisted of two annealed oligonucleotides: 5′-T-C-G-A-G-C-G-A-G-T-A-C-G-G-3′ and 5′-T-C-G-A-G-C-G-A-G-T-A-C-G-G-3′. The probe containing a strong MH100-binding site adjacent to the mutant MH103-binding site was generated by digestion of plasmid pMH100/103(B/K/H) with BamHI/HindIII. DNA-binding site probes were labeled with [α-32P]dATP and the Klenow fragment of DNA polymerase I. Binding reactions were performed on ice for 45 min in the same buffer as for DNase I footprinting (see above). DNA-protein complexes were resolved on 4.5% non-denaturing gels at 150 V for 3 h.

RESULTS

Production of Intact Wild Type GAL4 in SF9 Insect Cells—To enable efficient production of wild type GAL4 protein, we isolated a recombinant baculovirus from a cotransfection of SF9 cells with wild type baculovirus DNA and the transfer vector pVL841 containing the GAL4 coding sequence inserted immediately downstream of the polyhedron promoter. Recombinants containing GAL4 DNA were initially identified by DNA hybridization of plaque lifts; positive plaques were purified and examined for production of GAL4 protein by Western blotting of infected cells. A recombinant viral clone producing the largest amount of GAL4 in a 72-h infection was used for subsequent experiments. GAL4 protein can be detected 24 h following infection of SF9 cells with this recombinant virus; production increases over the following 48 h until GAL4 becomes the primary protein in crude extracts of infected cells (Fig. 1, lane f). Western blotting of extracts prepared from cells infected for 72 h demonstrates that most GAL4 protein in infected lysates migrates on SDS-polyacrylamide gel electrophoresis as a doublet of approximately 100 and 105 kDa. Wild type GAL4 expressed in yeast exists as at least three species with different electrophoretic mobilities (23, 25, 26). These multiple forms, resulting from phosphorylation, can be converted into a single unmodified form of approximately 100 kDa by treatment with phosphatase in vitro. Consistent with these observations, we find that the 105-kDa GAL4 form detectable in SF9 cells can be converted into a single 100-kDa form with phosphatase (not shown), suggesting that GAL4 protein is phosphorylated in insect cells as well. GAL4 protein seems to be highly stable when expressed in insect cells, since unmodified wild type GAL4 expressed in yeast migrates identically as GAL4 produced in SF9 cells (not shown).

Approximately half of the baculovirus produced GAL4 protein could be obtained in the soluble fraction of lysates prepared by homogenization in buffer containing 1% Nonidet P-40, whereas lysis in the absence of detergent invariably resulted in recovery of all GAL4 protein in the insoluble fraction. Solubilized GAL4 prepared by this technique was found to be the major protein in the extract, and bound with similar efficiency as a GAL4 DNA-binding domain fragment (GAL4(1-147), see Fig. 2A) to an oligonucleotide containing the MH100 near-consensus GAL4 DNA-binding site (see Fig. 2B), as assayed by electrophoretic mobility shift assays (Fig. 3). We have purified wild type GAL4 protein to approximately 85% homogeneity from these detergent-solubilized extracts by phosphocellulose chromatography. However, we found that
of sites I and IV. In stark contrast, we find that even at very high concentrations of GAL4, we first observe significant protection of site I1. X-ray diffraction studies (28) also supported by the appearance of enhancement and protection of DNase I-sensitive cleavages between the distantly spaced sites I11 and IV. Cooperative binding mediated by multiple sites of the UASc is particularly striking when one compares occupancy of site III. In footprints of wild type GAL4 site III becomes completely protected at the same concentration of protein as site IV. In stark contrast, we find that even at very high concentrations of GAL4(1-147) site III is only partially occupied (Fig. 4, lane f), despite the fact that sites II and IV become occupied at 4-fold lower protein concentration. These observations suggest that binding of wild type GAL4 to the GAL1-10 UASc is cooperative: binding to the stronger sites assists binding to adjacent weaker sites. This conclusion is also supported by the appearance of enhancement and protection of DNase I-sensitive cleavages between the distantly spaced sites III and IV. Cooperative binding mediated by interaction between GAL4 dimers would promote formation of a loop between these two sites (28), which are separated by approximately six helical turns. DNase I cleavage sites on the outside of the loop would become more sensitive to cleavage, resulting in an enhancement (Fig. 4, lane l, closed circles), whereas sites on the inside of the loop would become more resistant to DNase I cleavage, resulting in protection (open circles) of DNase I-sensitive cleavages are indicated with arrows.

**FIG. 3.** Binding of wild type GAL4 and GAL4(1-147) to an oligonucleotide containing the MH100 near-consensus binding site. Wild type GAL4, or GAL4(1-147) was incubated with a labeled oligonucleotide probe containing the MH100-binding site; protein-DNA complexes were resolved by electrophoresis on 4.5% native polyacrylamide gels. Binding reactions contained no protein (lanes a and f), GAL4(1-147) (60 nM, lane b; 120 nM, lane c; 240 nM, lane d; 480 nM, lane e), or wild type GAL4 (3.2 nM, lane g; 6.4 nM, lane h; 12.8 nM, lane i; 25 nM, lane j; 50 nM, lane k). Migration of free probe, GAL4(1-147) and wild type GAL4 protein-DNA complexes are indicated.

The differences in DNA binding properties of GAL4(1-147) and wild type GAL4 are particularly striking when one compares occupancy of site III. In footprints of wild type GAL4 site III becomes completely occupied at the same concentration of protein as site IV. In stark contrast, we find that even at very high concentrations of GAL4(1-147) site III is only partially occupied (Fig. 4, lane f), despite the fact that sites II and IV become occupied at 4-fold lower protein concentration. These observations suggest that binding of wild type GAL4 to the GAL1-10 UASc is cooperative: binding to the stronger sites assists binding to adjacent weaker sites. This conclusion is also supported by the appearance of enhancement and protection of DNase I-sensitive cleavages between the distantly spaced sites III and IV. Cooperative binding mediated by interaction between GAL4 dimers would promote formation of a loop between these two sites (28), which are separated by approximately six helical turns. DNase I cleavage sites on the outside of the loop would become more sensitive to cleavage, resulting in an enhancement (Fig. 4, lane l, closed circles), whereas sites on the inside of the loop would become more resistant to DNase I cleavage, resulting in protection (open circles) of DNase I-sensitive cleavages are indicated with arrows.

**FIG. 4.** Footprinting of wild type GAL4 and GAL4(1-147) on the GAL1-10 UASc. Labeled GAL1-10 UASc template DNA was incubated with wild type GAL4 or GAL4(1-147) protein and the DNA-protein complexes subjected to limited DNase I cleavage. Digestion products were resolved on 6% sequencing gels. Reactions contained no protein (lanes a and g), GAL4(1-147) (6 nM, lane b; 12 nM, lane c; 24 nM, lane d; 48 nM, lane e; 96 nM, lane f), or wild type GAL4 (0.32 nM, lane h; 0.64 nM, lane i; 1.3 nM, lane j; 2.5 nM, lane k; 5 nM, lane l). The position of GAL4 DNA-binding sites I-IV is as determined by chemical sequencing, are indicated. Sites of enhancements (closed circles) and protections (open circles) of DNase I-sensitive cleavages are indicated with arrows.
circles). Similar enhancements and protections are not observed in footprints of GAL4(1-147). We conclude from these observations that wild type GAL4 binds cooperatively to DNA. Since GAL4(1-147) does not bind cooperatively, this property of GAL4 must be conferred by regions of the protein other than the DNA-binding/dimerization domain.

### Binding of Wild Type GAL4 to a Strong Consensus Site Assists Binding to an Adjacent Weak Site

The binding sites for GAL4 within the GAL1–10 UAS differ in affinity for the GAL4 DNA-binding domain by only approximately 4-8-fold. To assay wild type GAL4's cooperative DNA-binding function under more stringent conditions, we asked whether binding to a strong consensus site could assist binding to an adjacent mutant site. For this purpose, we assayed binding to a DNA template containing the strong near-consensus MH100-binding site spaced approximately three helical turns adjacent to a weak mutant MH103-binding site (see Fig. 2B). The mutant MH103-binding site has substitutions of both outside “C” residues of the consensus GAL4 17-mer changed to “G” residues; these substitutions result in an approximately 100-fold lower binding affinity for GAL4 DNA-binding domain fragments (24, and data not shown). In electrophoretic mobility shift assays using this template as probe for binding of GAL4(1-147), we observe only a single protein-DNA complex corresponding to binding of GAL4(1-147) to the strong MH100 site (GAL4(1-147), Fig. 5). A faster migrating protein-DNA complex is usually present in assays of GAL4(1-147), which likely results from protein degradation (Figs. 3 and 5). Additionally, at the highest concentrations of GAL4(1-147), we often observe an additional slower migrating complex (Fig. 5, lane d, indicated with arrow). This latter complex likely does not represent binding to both sites of the template, as a similar complex is observed in assays using a single oligonucleotide-binding sites as probe (Fig. 3, lanes d and e). This complex may result from aggregation of the GAL4(1-147) protein. In assays of wild type GAL4, using the template containing the MH100 and mutant MH103-binding sites as probe, we observe an additional protein-DNA complex indicative of binding to both sites on the template (Fig. 5, lanes g-i). Note that such a complex is not observed in assays of wild type GAL4 using a single MH100 oligonucleotide as probe (Fig. 3, lane h). These results suggest that binding of wild type GAL4 to a weak mutant site can be assisted by binding to an adjacent strong near-consensus binding site.

To confirm these results, we compared binding of wild type GAL4 to the weak MH103-binding site alone, or to this weak site when adjacent to the strong MH100 site, by DNase I footprinting. In these experiments, we found that we could not detect significant protection of the template containing only the MH103-binding site (Fig. 6, MH103), even at very high concentrations of protein (lane e). However, the MH103-binding site, when spaced approximately three helical turns on the same DNA template as the strong MH100 site, became occupied at only 2-fold higher protein concentration than did the MH100 site (Fig. 6, MH103/MH100). Thus, wild type GAL4 can bind much more efficiently to the mutant MH103 site when bound to an adjacent strong MH100 site. This result conclusively demonstrates that binding of wild type GAL4 is cooperative in vitro.

### The GAL4 DNA-binding Domain Fused to Portions of Activating Region II Do Not Bind Cooperatively to DNA

Our results suggest that wild type GAL4 has a cooperativity function that is not a property of the N-terminal GAL4 DNA-binding/dimerization domain. To examine whether GAL4's transcriptional activating domains contribute to cooperative DNA-binding function, we examined binding of GAL4(1-147) fused to portions of activating region II. The C-terminal 30 amino acid residues of activating region II contain a high proportion of negatively charged residues, while the remaining

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**Fig. 5.** Binding of wild type GAL4 and GAL4(1-147) to a template containing one strong MH100-binding site and one weak MH103-binding site in electrophoretic mobility shift assays. Wild type GAL4 or GAL4(1-147) were incubated with a labeled DNA fragment containing the near consensus MH100-binding site spaced three helical turns adjacent to the mutant MH103-binding site. Protein-DNA complexes were resolved by electrophoresis on 4.5% native polyacrylamide gels. Binding reactions contained no protein (lane e), GAL4(1-147) (30 nM, lane a; 60 nM, lane b; 120 nM, lane c; 240 nM, lane d), or wild type GAL4 (3.2 nM, lane f; 6.4 nM, lane g; 12.8 nM, lane h; 25 nM, lane i). Migration of free probe and GAL4(1-147) and wild type GAL4 protein-DNA complexes are indicated.

**Fig. 6.** Binding of wild type GAL4 to the strong MH100-binding site assists binding to an adjacent weak MH103-binding site. Wild type GAL4 protein was footprinted on labeled template bearing a single mutant MH103-binding site (MH103, left) or near consensus MH100-binding site spaced three helical turns adjacent to an MH103-binding site (MH103/MH100, right). Reactions contained no protein (lanes a and f), or wild type GAL4 (0.8 nM, lanes b and g; 1.6 nM, lanes c and h; 3.2 nM, lanes d and i; 6.4 nM, lanes e and j). The position of the MH103 and MH100 GAL4 DNA-binding sites on both templates, as determined by chemical sequencing, are indicated.
Cooperative GAL4 DNA Binding

The results we present in this paper convincingly demonstrate that wild type GAL4 binds cooperatively to multiple adjacent DNA-binding sites in vitro. In footprinting experiments with the GAL1–10 UASG, wild type GAL4 fully occupies all four of its binding sites at approximately the same protein concentration, despite the fact that these multiple sites have different affinities for a GAL4 DNA-binding domain fragment. Furthermore, we show that binding of wild type GAL4 to a mutant binding site is greatly assisted by the presence of an adjacent strong near-consensus binding site. We find that an N-terminal fragment of GAL4 containing the DNA-binding and dimerization domains binds as efficiently, if not better, to individual DNA-binding sites as does wild type GAL4. However, the DNA-binding fragment is incapable of binding cooperatively to multiple sites, implying that this function must be conferred by another region of the GAL4 protein. Cooperative DNA binding is also distinct from GAL4’s transcriptional activation function, as fusion of activating region II to the DNA-binding domain does not confer cooperativity; additional sequences between residues 148–767 are required. Cooperative DNA binding of wild type GAL4 is likely to be mediated by the formation of contacts between GAL4-dimers, interactions which must occur within the C-terminal portion of GAL4. We consistently find that wild type GAL4 produced by expression in insect cells is easily precipitated out of solution, and this tendency to aggregate may reflect its cooperative DNA-binding function.

Transcription of the GAL genes in S. cerevisiae are tightly regulated by a combination of mechanisms that ensure galactose is utilized when available, but that glucose is used preferentially (1). These regulatory mechanisms are directed at controlling synthesis and activity of the transcriptional activator GAL4; small changes in either can have profound effects on transcription of the GAL genes. The results we present in this paper add to an increasingly complex network of interactions that control GAL4 function. The effectiveness of GAL4 appears to be modified at several levels. When galactose is absent, GAL4 is inhibited by the negative regulator GAL80; this inhibitory effect is relieved upon addition of galactose. Glucose has a precisely opposite effect on GAL4 activity, but this effect is exerted through several different mechanisms. Transcription of GAL4 is reduced in the presence of glucose (11), and this effect eventually translates into loss of GAL4 protein from the UASG (27). Transcriptional activation by DNA-bound GAL4 appears to be blocked in the presence of glucose by a group of glucose-activated repressors bound to cis-acting URSG (7–10). In addition, we have recently shown that GAL4 activity is directly inhibited by the presence of glucose.1 This additional effect requires the presence of the central portion of GAL4.

Cooperative binding of GAL4 to DNA can modify the effect of several of these regulatory mechanisms, in particular those imposed by the presence of glucose. Transcription of GAL4-regulated genes containing multiple sites to which GAL4 can bind will be very sensitive to GAL4 concentration. Small changes in GAL4 concentration can translate into very large effects on transcriptional activation, this effect is particularly noticeable for transcription of GAL1. Production of GAL4 is reduced by approximately 5-fold in cultures grown in glucose (11). This small change in GAL4 expression is accompanied by an approximately 100-fold decrease in transcriptional activation from the GAL1–10 UASG (11). In contrast, transcription of GAL80, which has only one binding site for GAL4, is not appreciably reduced in glucose (29). These observations are consistent with our in vitro results which indicate that...
recently been shown that insertion of a "protein spacer" to sites distantly spaced from the GAL1 TATA-box (19). Wild central portion of GAL4 may be required for other functions of GAL4 as well. For example, it was shown that transcriptional activation from distantly placed sites. It has rendering their DNA-binding function more sensitive to the of DNA-binding by glucose may be offset by cooperativity.

Cooperative binding of wild type GAL4 to DNA may prevail on wild type GAL4 is directly inhibited by the presence of glucose in the C terminus (residues 148-881) is required for some of the inhibitory effects of glucose, even when galactose is present (7, 9, 10). Perhaps GAL80 mediates its effect in glucose by preventing cooperative binding of GAL4; this effect, in combination with decreased expression of GAL4 would severely reduce occupancy of the sites for GAL4 on DNA. Experiments examining the effect of GAL80 on wild type GAL4's DNA-binding function are in progress.

We have also recently demonstrated that activity of wild type GAL4 is directly inhibited by the presence of glucose in the absence of other repression mechanisms. This inhibition requires the presence of the large central portion of GAL4 between residues 238–767. The central region of GAL4 has at least three inhibitory domains between residues 236-500 that can inhibit transcriptional activation by activators derived from GAL4 and LexA DNA-binding domains and GAL4 and VP16 activating regions. In wild type GAL4, the function of these inhibitory domains appears to be suppressed by a region of GAL4 between residues 600 and 767, termed the glucose response domain. The inhibitory effect of the central region on wild type GAL4 is most apparent shortly after addition of glucose. Curiously, direct inhibition by glucose can easily be overcome by overexpression of GAL4. GAL4 expressed from the ADH1 promoter is not inhibited by the presence of glucose, but inhibition of GAL4 activity can be detected when GAL4 is expressed at low levels from the PH05 promoter. In contrast, we find that a series of GAL4 mutants deleted of various portions of the C terminus, and fusion proteins consisting of LexA-VP16 fused to the GAL4 central region, are inhibited by glucose regardless of their expression levels.

We have preliminary evidence suggesting that the central region may inhibit GAL4's DNA-binding function in the presence of glucose. If this should indeed prove to be the case, inhibition of DNA-binding by glucose may be offset by cooperativity. Cooperative binding of wild type GAL4 to DNA may prevail when it is overexpressed. Our results suggest that a region of GAL4 in the C terminus (residues 148–881) is required for cooperative DNA-binding. Deletions of this portion of GAL4 may eliminate the cooperative DNA-binding function, thus rendering their DNA-binding function more sensitive to the presence of glucose.

In addition to controlling GAL4 function in response to glucose, our results suggest that at least a portion of the GAL4 central region is required for cooperative DNA-binding. It is possible that these two functions are mutually exclusive. The central portion of GAL4 may be required for other functions of GAL4 as well. For example, it was shown that transcriptional activation by derivatives of GAL4 lacking the central region are inefficient at activating transcription when bound to sites distantly spaced from the GAL1 TATA-box (19). Wild type GAL4, in contrast, was found to be much more efficient at activating transcription from distantly placed sites. It has recently been shown that insertion of a "protein spacer" between the GALA DNA-binding domain and the VP16 activating domain produces a transcriptional activator that is much more efficient at activation from distant sites in vitro. Perhaps the central region, in addition to its other functions, serves as a "spacer" that contributes to activation at a distance.

Experiments performed in vitro have demonstrated that transcriptional activation by GAL4 derivatives from multiple binding sites is much more than an additive effect of individual sites (21). These experiments were done at concentrations of GAL4 derivative at which all of its binding sites were fully occupied, and therefore suggests that multiple GAL4 activators must contact and stimulate the target(s) synergistically. Synergistic activation has also been demonstrated to occur between different activator proteins in vitro (17, 30), and many experiments have demonstrated synergistic transcriptional activation by multiple cooperatively bound GAL4 dimers.

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