A single GAL4 dimer can maximally activate transcription under physiological conditions

(synergy/transcription activation/VP16/cooperative binding/DNA binding)

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ABSTRACT Most eukaryotic promoters contain multiple binding sites for one or more transcriptional activators that interact in a synergistic manner. A common view is that synergism is a manifestation of the need for many contacts between activators and the general transcription machinery that a single activator presumably cannot fulfill. In this model, various combinations of protein–protein interactions control the level of gene expression. However, we show here that under physiological conditions, a single binding site and presumably binding of a single dimer of the prototypical yeast activator GAL4 can activate transcription to the maximum possible level in vivo. Synergistic effects in this natural system are shown to be consistent with cooperative DNA binding. These results point to DNA occupancy as the major element in fine tuning gene expression in the galactose regulon.

Most eukaryotic promoters are complex and contain multiple binding sites for one or more transcriptional activators. These sites generally produce synergistic effects; that is, the level of transcription is greater than the expected sum of the contributions of each individual site (1–12). A fundamental question in transcriptional regulation is how the activators bound to these sites interact with one another and the general transcription complex to exert these synergistic effects.

One model commonly proposed to explain this phenomenon suggests that multiple contacts between regulatory factors and the general transcription apparatus are required to stimulate transcription maximally and that a single activator or activator dimer cannot fulfill all of these interactions (13, 14) (Fig. 1). This has been termed the “multiple contact” model. These multiple protein–protein interactions could be required to recruit several different general transcription factors to the promoter or to catalyze different fundamental steps in the transcription cycle. The results of some experiments designed to probe the mechanistic basis of synergistic effects appear consistent with this idea, while some do not. However, these studies have suffered from using artificial or poorly characterized activators (usually chimeras composed of heterologous DNA binding and activation domains), very high nonphysiological levels of activators, nonnative promoters, or a combination of these factors. Here, we report that in vivo, under normal physiological conditions, a single dimer of the well-characterized yeast activator GAL4 is able to maximally stimulate transcription of a target gene. Quantitative in vivo probes of GAL4–DNA binding are also described. The data rule out the multiple contact model in the case of GAL4-activated transcription and argue strongly that synergistic effects are due solely to cooperative DNA binding.

In Vivo Transcription Assays. The reporter plasmid pJLb contains a CYC1 promoter (lacking an upstream activation sequence (UAS)) linked to the bacterial lacZ gene. An oligonucleotide including the weak GAL4 binding site from the GAL1-10 promoter region (the third of four 17-mers, obtained by annealing two oligonucleotides with the sequences 5'-TGCAGAGGAAGACTCTCCTCCGG-3' and 5'-TGCAGCGGAGATGCTTCTC-3') was inserted into the Xma I site located ~100 bp upstream of the “TATA” box. The plasmid carrying the consensus GAL4 binding site (obtained by annealing the oligonucleotides 5'-TGCAGAGGAAGACTGCTCCTCCGG-3' and 5'-TGCAGCGGAGATGCTTCTC-3') was constructed in a similar fashion. Tandem repeats of two or three weak sites, or two strong sites, were obtained by ligation of the corresponding single site. The resultant plasmids were introduced into wild-type yeast strain 21R (GAL4, GAL80, ura3-52, leu2-3,112, ade1, MEL1) or the same strain with a multicopy plasmid that expresses GAL4 (the GAL4 expression plasmid was obtained by inserting the wild-type BamH1–HindIII GAL4 fragment into the multicopy plasmid YEP351). β-Galactosidase activity was assayed as described (15) and expressed as Miller units/mg of total protein. The values represent the average of at least three experiments. There was <10% variation in the determinations.

Plasmid Titration Experiments. One, two, or three copies of the UAS3 or cUAS sites were cloned into the Sal I site of plasmid YEP352 or YEP351. The plasmid was introduced into

Abbreviations: UAS, upstream activation sequence; VP16, viral protein 16.

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yeast strain Sc18(MATa, GAL4, ∆gal80, ura3-52, leu2-3,112, his3, MEL1), and the decrease in chromosomal MEL1 expression was determined by measurement of α-galactosidase activity as described (16). The values presented represent the average of at least three experiments with <10% variation in the determinations.

RESULTS AND DISCUSSION

Low-Affinity GAL4 Binding Sites Interact Synergistically. The *Saccharomyces cerevisiae* GAL4 protein strongly activates the transcription of several genes in response to galactose (17). Many GAL4-responsive promoters contain more than one 17-bp site [to which GAL4 binds as a dimer (18)]. As shown in Fig. 2A, insertion of two or three GAL4 binding sites upstream of a *lacZ* reporter gene resulted in a synergistic increase in transcription. The 17-bp sequence employed, UAS3, is the third of four activator binding sites found in the promoter of the GAL1 and GAL10 genes. Thus, the galactose regulon of yeast provides an excellent model system for studying the mechanism of synergy in a physiologically relevant setting.

A Simple Test of the Multiple Contact Model. The multiple contact model makes the strong prediction that even sites that are individually saturated by an activator should provide synergistic responses when two or more copies are present. To test this, it was necessary to identify a GAL4 binding site fully occupied in vivo when present at a single copy. This was assessed by comparing the level of GAL4-dependent *lacZ* expression supported by a particular UAS in wild-type cells in which GAL4 is expressed from its natural promoter and in cells where GAL4 is expressed at a 15- to 20-fold higher level from a multicopy plasmid. With a single UAS3 site in the promoter, there was a 26-fold increase in *lacZ* expression when GAL4 was expressed from a multicopy plasmid relative to wild-type cells (50 vs. 1300, compare Fig. 2A and B); so UAS3 binds GAL4 poorly and is clearly not saturated under normal conditions. We therefore turned to a consensus near-symmetric GAL4 binding site, UASc (18), that binds the GAL4 DNA-binding domain with an 8-fold greater affinity than UAS3 in vitro (15). Reporter gene expression supported by cUAS was only ~35% higher when GAL4 was overexpressed (8700 units vs. 6400 units; Fig. 3), showing that cUAS is almost fully occupied in wild-type cells.

When two copies of the cUAS were placed upstream of the reporter gene, only a 1.3-fold higher level of *lacZ* expression was observed (8300 units vs. 6400 units; Fig. 3). This is not even an additive, let alone synergistic, response. The level of reporter gene expression supported by two cUAS sites was not significantly different than that obtained when a single cUAS was saturated by increasing the level of GAL4 expression. These data are inconsistent with the multiple contact model and argue that the major effect of placing two cUAS sites in the promoter is to increase occupancy by GAL4. Finally, when the number of UAS3 sites was increased in cells with elevated GAL4 levels, a less dramatic synergistic effect was observed than was the case in wild-type cells (compare Fig. 2A and B). This is also difficult to explain by the multiple contact model.

Synergistic Effects Are Due to Cooperative DNA Binding. Since the data do not support the multiple contact model, what

![Fig. 2](image1.png)

**Fig. 2.** Synergistic activation by GAL4 from multiple weak UAS3 binding sites. The GAL4 protein was expressed from its endogenous chromosomal gene (single copy GAL4) (A) or at 15- to 20-fold higher levels from the multicopy plasmid YEP351, which contains the endogenous GAL4 promoter (multicopy GAL4) (B). In each case, the cells also contained a reporter plasmid in which the *lacZ* gene was linked to the *CYC1* core promoter downstream of GAL4 binding sites. The level of reporter gene expression was determined by measuring the β-galactosidase (the *lacZ* gene product) activity produced. The solid bars represent the observed levels of activation.

![Fig. 3](image2.png)

**Fig. 3.** Two high-affinity GAL4 binding sites do not activate transcription synergistically. The reporter gene employed was identical to that used in the Fig. 2 experiment except that the weak sites were replaced by the high-affinity cUAS site. The GAL4 protein was expressed either from its chromosomal gene or from a multicopy plasmid as indicated. The hatched bars represent the results expected if the effect of placing two cUAS sites upstream of the reporter gene had doubled the extent of transcription supported by one site.
is the molecular basis of the synergistic effects observed with more than one UAS3 site? One alternative is that the GAL4 protein binds cooperatively to these sites (for previous reports of cooperative binding of GAL4 to DNA, see refs. 19 and 20). In this view, only one activator dimer is necessary for high-level transcription, but it must occupy the UAS efficiently, which is difficult when only a single weak site is present.

To probe this point directly, we employed a "plasmid titration" assay (H.E.X., A. Vonica, T.K., and S.A.J., unpublished data) to examine the occupancy of high- and low-affinity sites. Briefly, this method involves introducing a known number of plasmid-borne GAL4 binding sites, independent of any other promoter sequences, into yeast and measuring the effect on the GAL4-activated expression of the endogenous MEL1 gene (Fig. 4A). Since the amount of GAL4 protein in wild-type cells is the limiting factor in MEL1 expression, the plasmid sites compete directly with the MEL1 UAS for the activator with a resultant decrease in MEL1 expression. A high-affinity plasmid-borne UAS will decrease MEL1 expression more than a weak site. Thus, this assay represents a direct and quantitative measurement of GAL4 binding to sites on the plasmid.

One copy of the weak UAS3 site per plasmid has no detectable effect on MEL1 expression (Fig. 4B). Introduction of plasmids with two or three adjacent UAS3 sites decreased MEL1 expression by 6 and 30%, respectively. These results demonstrate that GAL4 binds cooperatively to low-affinity sites in vivo, providing an explanation for the synergistic effects observed with this site in the previous reporter gene experiments. In contrast, plasmids with one or two copies of the cUAS decreased MEL1 expression by 23 and 50%, respectively. This additive response is exactly the result predicted for individually saturated GAL4 binding sites.

**No More Than Two Viral Protein 16 (VP16) Activation Domains Are Required for Maximal Transcriptional Stimulation.** While the above experiments argue that the synergistic effects in the GAL4 system are due to cooperative DNA binding, this may not be the case for other activators. In particular, several previous studies of synergistic effects, some of which appeared to support the multiple contact model (14, 21, 22), employed artificial chimeric activators composed of the activation domain of the herpes simplex virus VP16 transactivator fused to the GAL4 DNA-binding and dimerization domains (23). To probe whether VP16-mediated activation requires the delivery of many activation domains, we conducted the same experiments as reported for GAL4 (see Fig. 3) but using a chimeric protein in which the 34-residue C-terminal activation domain of GAL4 was replaced by the 78-amino acid VP16 activation domain.

This experiment is more complicated because the protein level of GAL4-(1–841)–VP16 is ~3-fold lower than that of the native activator when expressed from GAL4's native promoter on a single copy plasmid (K. Melcher and S.A.J., unpublished data). This may be due to a reduced half-life in vivo. Thus, it is unlikely that even the cUAS will be saturated under normal physiological conditions. Indeed, two cUAS sites supported a synergistic increase in GAL4-(1–841)–VP16-mediated transcription relative to that observed with one high-affinity site (Fig. 5). Even the cUAS result is consistent with the notion that cooperative binding effects or could indicate that more than one dimer is required for high-level transcription. To distinguish these possibilities, we compared the output of reporter

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**Fig. 4.** Measurement of the affinity of GAL4 for one or more UAS3 or cUAS sites using the "plasmid titration" assay. (A) Schematic depiction of the plasmid titration assay, MEL1 is an endogenous chromosomal GAL4-regulated gene. Expression of MEL1 is limited by GAL4 binding to a single 17-bp sequence (solid box) upstream of the MEL1 gene. Therefore, when plasmid-borne GAL4 binding sites (solid boxes) are introduced into yeast, these sites compete with the MEL1 UAS for limiting GAL4 protein and decrease MEL1 expression. The affinity of the plasmid-borne sites for GAL4 correlates with the magnitude of the decrease in MEL1 expression. MEL1 encodes the enzyme 3-galactosidase, which is easily quantitated. (B) The low-affinity UAS3 sites interact synergistically to decrease MEL1 expression, but the high-affinity cUAS sites have only an additive effect on MEL1 expression. Bars represent the level of observed MEL1 expression.

**Fig. 5.** Activation by GAL4–VP16 from weak and strong binding sites. The GAL4–VP16 protein was expressed from the GAL4 promoter carried on either a single or multiple copy YEp351 plasmid as indicated (33). The reporter genes were identical to those described in Figs. 2 and 3. In this figure all values shown are observed results. The solid, open, and hatched boxes represent values obtained with one, two, and three GAL4 binding sites, respectively.
plasmids containing one or two cUAS sites in cells with elevated levels of the chimeric activator (expressed at ~15-fold higher levels from a multicopy plasmid). Under these more saturating conditions, essentially identical levels of reporter gene expression were supported by one or two GAL4 binding sites (Fig. 5). These results argue that synergistic effects observed with the VP16 activation domain also cannot be explained by the multiple contact model. Given that the same results were obtained with the activation domains of GAL4 and VP16, it seems likely that our conclusions are broadly applicable to many acidic activators.

The fact that a single GAL4 or GAL4–VP16 dimer can activate transcription maximally does not necessarily mean that the activator contacts only one or two general factors in a functionally relevant fashion. It simply demands that however many contacts are made, a single GAL4 or GAL4–VP16 dimer can satisfy them all. Furthermore, our data argue that the protein–protein contact(s) between GAL4 or GAL4–VP16 and their target(s) is saturated when a single UAS is fully occupied. If this were not the case, then an additive increase in transcription would have been observed when two cUAS sites were present, due to an increase in the local concentration of the activation domain.

The Importance of Studying Natural Systems. Previous reports have reached different conclusions regarding the molecular basis of synergistic effects. Some of the data seem to conflict with the multiple contact model (24–28), while some results appear to support it (13, 14, 21, 22, 28, 29). While we do not completely understand the basis of these apparent discrepancies, a number of points are worth mentioning. (i) In many of these studies, it was not clear that the activator binding site employed was saturated when only a single copy was present. As emphasized here, this is a crucial aspect of any experiment designed to probe the mechanism of synergy. (ii) As mentioned earlier, most previous studies employed non-physiological states of the activator proteins (chimeras or high levels). Thus, the results obtained in such experiments (for example, in studies using transient transfection of mammalian cells) may not reflect the properties of a natural system, such as the yeast galactose regulon employed here. In vitro experiments, in particular, may measure a different phenomenon than occurs in vivo. In most in vitro transcription reactions only a small percentage of the templates are active and those that do not reinitiate efficiently, if at all. In these cases, a major role of activators may be to increase the fraction of templates that produce a defined RNA product (30). This is contrasted with the in vivo situation in which all of the templates are presumably functional and activation involves an increase in the frequency with which a gene is transcribed. The level of transcription in vivo is largely a function of the rate of reinitiation. Though it is possible that these two different assays are correlated, it is more likely that they have quite different characteristics and these differences could explain, for example, why Choy and Green (21) observed a synergistic effect in vitro with multiple GAL4–VP16 binding sites under conditions in which a single site was clearly saturated. Thus, while studies using artificial systems or conditions are important, caution should be used in applying these findings to natural regulatory systems. For instance, the synergistic response to multiple GAL4–VP16 activators in vitro was recently found to be enhanced greatly when a chromatin template was employed (31). Furthermore, the results obtained with this more biologically relevant substrate were not consistent with the multiple contact model but were indicative of cooperative DNA binding. Kingston and coworkers (32) have found that chimeras including the GAL4 DNA-binding domain bind cooperatively to histone-complexed, but not naked, DNA. In any case, the results shown here demonstrate that under physiological conditions in vivo synergistic effects in GAL4 and GAL4–VP16-activated transcription are probably due to cooperative DNA binding. It will be interesting to determine whether this is also the case for combinations of different activators.

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