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Modulation of promoter occupancy by cooperative DNA binding and activation-domain function is a major determinant of transcriptional regulation by activators *in vivo*

(*Saccharomyces cerevisiae*)

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ABSTRACT **Binding of transcriptional activators to a promoter is a prerequisite process in transcriptional activation. It is well established that the efficiency of activator binding to a promoter is determined by the affinity of direct interactions between the DNA-binding domain of an activator and its specific target sequences. However, I describe here that activator binding to a promoter is augmented *in vivo* by the effects of two other determinants that have not been generally appreciated: (*i*) the number of activator binding sites present in a promoter and (*ii*) the potency of activation domains of activators. Multiple sites within a promoter can cooperatively recruit cognate factors regardless of whether they contain an effective activation domain. This cooperativity can result in the synergistic activation of transcription. The second effect is the enhancement of activator binding to a promoter by the presence of activation domains. In this case, activation domains are not simply tethered to the promoter by the DNA-binding domain but instead assist the DNA-binding domain being tethered onto the promoter. This effect of activation domains on DNA binding is instrumental in determining how potent activators can induce steep transcriptional increases at low concentrations.**

Activation of transcription in eukaryotes is a complex process, involving numerous molecular interactions between components such as transcriptional activator proteins, general transcriptional factors, and promoter DNA (for reviews, see refs. 1–4). Levels of activation can be modulated by variations in these interactions, resulting from changes in diverse parameters such as promoter structure (e.g., the number of activator-binding sites), activator properties (e.g., activation potency), and cellular environment (e.g., activator concentration). Indeed, it is the combined effects of many parameters such as these that ultimately determine the level of transcription of a gene under a given cellular circumstance.

The combined effects of these multiple parameters have been investigated *in vivo* in diverse systems (5–10). While the results of these studies have shown that transcriptional effects caused by changes in these various parameters are often interdependent (5–10), they have recurrently revealed one important aspect in transcriptional regulation—synergistic transcriptional responses to parameter changes. Thus, an increase in the number of activator-binding sites often results in synergistic increases in transcription (5, 8–10), and a moderate increase in activator concentration can induce steep transcriptional responses (6).

In theory, the levels of transcription from a promoter can be modulated by either changes in the levels of activator binding to a promoter or changes in the efficacy of the promoter-bound activators to give rise to distinct transcriptional outcomes (e.g., synergistic transcriptional increases). In the case of the yeast GAL4 activator, it was shown that cooperative activator binding to a promoter is predominantly responsible for synergistic transcriptional activation by multiple activator-binding sites (5). It is not generally known, however, if alterations in transcriptional outcomes are produced through differences in either activator binding or stimulation of transcription by promoter-bound activators. To investigate further the mechanism through which the effects of multiple parameters are integrated on a promoter to produce specific transcriptional outcomes in yeast cells, I have studied the effects of varying three selected parameters—(*i*) the number of activator binding sites present in a promoter, (*ii*) activation potency of activators, and (*iii*) activator concentration—on both transcriptional activator binding to a promoter and transcription from the promoter *in vivo*. This analysis revealed that activator binding to promoter DNA is modulated *in vivo* by both the number of binding sites and activation potency of activators, emphasizing this DNA-binding step as a key regulatory point in transcriptional activation.

MATERIALS AND METHODS

Plasmids and Yeast Strains. Reporter plasmids were derived from the plasmid pJP160 (provided by R. J. Reece, J. Pearlberg, and M. Ptashne, Harvard University, Cambridge, MA), and contained one, two, or six tandem copies of the octamer binding sites (11), the *CYC1* promoter segment (−67 to +49; +1 corresponds to the most upstream transcription initiation site; ref. 12) upstream of the *Escherichia coli lacZ* reporter gene. Yeast reporter strains were produced by integrating the yeast reporter plasmids into the genome of *Saccharomyces cerevisiae* YPH499 (13). The effectors contained the epitope tag and the nuclear localization signal (Met-Ser-Ser-Tyr-Pro-Pro-Tyr-Asp-Val-Pro-Asp-Tyr-Ala-Ser-Leu-Gly-Gly-Pro-Ser-Ser-Pro-Pro-Lys-Lys-Lys-Lys-Lys-Arg-Lys-Val-Ser-Ser; refs. 14 and 15), followed by the Oct-2 POU-domain sequence (195–357 in the Oct-2 amino acid sequence; ref. 16) and various transcriptional activation domains. These activation domains were produced by tandemly reiterating small subsegments within the activation domain of the herpes simplex virus activator VP16 (17–19) (see Figs. 1 and 3 for the amino acid sequences of each segment). The coding sequence was cloned into the plasmid pRS305 (13), together with the flanking *GAL1* promoter and *ADH1* terminator sequences (20), to produce effector plasmids. These effector plasmids were integrated into the genome of the yeast reporter strains described above.

***In Vivo* Dimethyl Sulfate (DMS) Methylation Protection Assay.** Yeast cells were treated with 30 mM DMS at 30°C for 5 min, followed by extraction of genomic DNA. The extracted DNA was subjected to piperidine treatment, primer extension,

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Abbreviation: DMS, dimethyl sulfate.

and ligation-mediated PCR amplification, followed by an additional primer-extension reaction with an end-labeled primer that annealed upstream of the octamer site(s) (21, 22). Extents of *in vivo* site occupancy by activators were calculated from ratios of band intensity of protected guanine residues (indicated by filled triangles in Fig. 2*a*) to reference guanine residues, which produced bands immediately below each protected guanine residues (see also Fig. 1*b*). The band intensity was quantitated with Fujix bio-imaging analyzer BAS1000.

Immunoblot and β-Galactosidase Assays. Protein samples were prepared in Laemmli sample buffer (23) and analyzed by SDS/PAGE, followed by immunoblot analysis. The blot was probed with the YS123 monoclonal antibody (provided by W. Herr, Cold Spring Harbor Laboratory, Cold Spring Harbor, NY), which recognizes the Oct-2 POU domain, and the signals were detected by enhanced chemiluminescence (Amersham). Levels of activators were determined by comparing band intensity of experimental samples to quantitation standards, which contained *E. coli*-expressed Oct-2 POU-domain protein serially diluted two-fold in mock yeast extracts. The band intensity was quantitated with IS-1000 digital imaging system (Alpha Innotech, San Leandro, CA). The β-galactosidase activity was assayed according to a published procedure (24).

RESULTS

Experimental Design. Fig. 1 depicts the experimental system. A set of transcriptional activators were produced (Fig. 1*a*) by fusing the mammalian Oct-2 POU DNA-binding domain (16) to tandemly reiterated copies of an 8-aa activation module (referred to here as the VN^8 module) derived from the herpes simplex virus transactivator VP16 (17–19). This reiteration strategy allowed modulation of activation potency by varying the number of VN^8 modules without introducing a qualitative alteration in activation-domain function; activators with increasing number of reiterated VN^8 module activated transcription more efficiently, whereas the POU domain alone, although selectively localized in the nucleus, did not activate transcription efficiently (see below; and data not shown). The VN^8 module was one of the most potent in activating transcription among several tested (e.g., see Fig. 3), yet it had no effect on yeast cell growth under the experimental conditions described in the present work. To modulate activator concentration, expression of these POU-domain-containing activators was regulated with the inducible *GAL1* promoter (Fig. 1*a*). Finally, to examine the effects of the number of activator-binding sites in a promoter, a set of test promoters, which contained one, two, or six tandem copies of the Oct-2 POU-domain-binding site (the octamer sequence), were fused upstream of the *E. coli lacZ* reporter gene (Fig. 1*b*). For each combination of activator potency, concentration, and number of binding sites, the levels of transcriptional activation were determined by quantitating expression of the *E. coli lacZ* reporter gene. In parallel, binding of the activators to their cognate sites was examined *in vivo* by DMS methylation protection assay; a guanine residue within the octamer motif was specifically protected from methylation by binding of POU-domain proteins both *in vivo* and *in vitro* (see below; and data not shown).

Cooperative Activator Binding to Multiple Sites in a Promoter *in Vivo*. Fig. 2*a* displays the result of the *in vivo* DNA-binding analysis. Each activator was expressed at three different levels, as detected by immunoblot analysis (Fig. 2*b*), and assayed on the 1×, 2×, or 6× site test promoters. In this analysis, different activators were expressed specifically at levels that produced similar extents of site occupancy *in vivo*. A comparison of promoters containing different numbers of sites revealed that for all the effector proteins—regardless of whether they contained an activation domain—*in vivo* site occupancy was greater for the 6× site promoter than for the

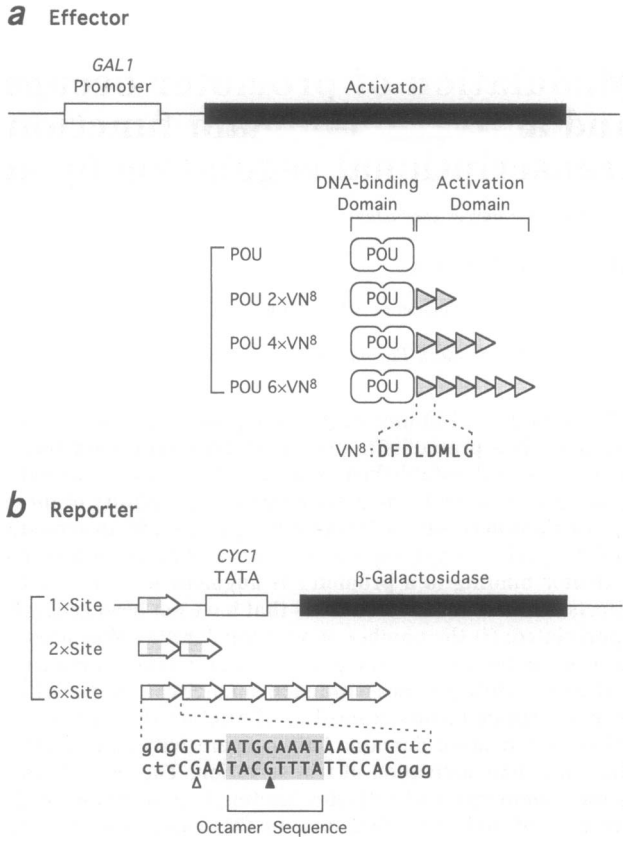

FIG. 1. Effectors and reporters. (*a*) The Oct-2 POU domain was fused at its C-terminus to either a stop codon or to reiterated copies of the VN^8 activation module. The activator-encoding sequences were placed downstream of the regulatable *GAL1* promoter. (*b*) The reporter-gene promoter contained the *CYC1* TATA boxes and one, two, or six tandem copies of a sequence containing a canonical octamer sequence (shaded) derived from the human histone *H2B* promoter (shown by uppercase letters in the DNA sequence) fused upstream of the *E. coli* β-galactosidase reporter gene. The filled triangle below the DNA sequence indicates the guanine residue that was protected by the POU-domain proteins from DMS modification. The open triangle indicates the guanine residue used as a reference for quantitations (see Fig. 2).

2× site promoter (e.g., compare lanes 19–21 to lanes 15–17 in Fig. 2 *a* and *b*). In contrast, all the effectors failed to bind effectively to the 1× site promoter (lanes 11–13, 23–25, and 35–37; see also a quantitation in Fig. 2*c*). This result demonstrates that activator binding is augmented by the number of binding sites present in a promoter, indicating that activators cooperatively bind to their cognate sequences *in vivo*, and, further, that this cooperativity does not require the presence of an effective activation domain.

Enhancement of Activator Binding to Promoters by Transcriptional Activation Domains *in Vivo*. In contrast to the activation domain-independent effects of multiple binding sites, a comparison among activators of different potencies revealed that activator binding to a promoter *in vivo* is augmented by the presence of activation domains (Fig. 2). For example, to achieve relatively similar levels of binding to the 2× site promoter *in vivo* (Fig. 2*a*, lanes 15–17, 27–29, and 39–41), the DNA-binding domain alone required significantly higher levels of expression (lanes 15–17 in Fig. 2*b*) than either the strong 6× VN^8 (lanes 39–41 in Fig. 2*b*) or weak 2× VN^8 (lanes 27–29 in Fig. 2*b*) activators. Indeed, quantitation of these results (Fig. 2*c*; note that scales of horizontal axes in the graphs vary) revealed that, with the 2× site promoter, the activator with the 6× VN^8 activation domain (the right graph) occupied the cognate sites at 30-fold lower concentrations than

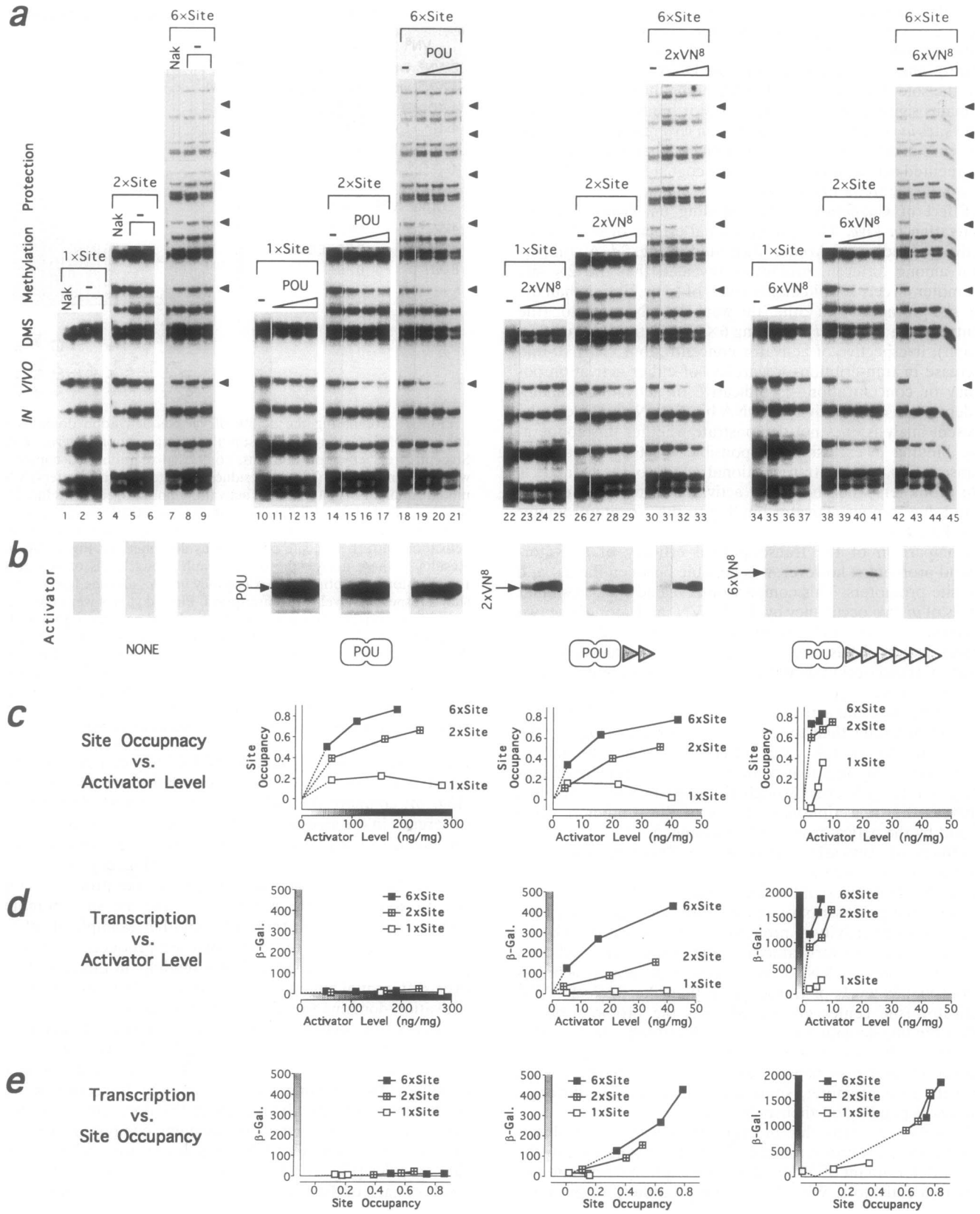

FIG. 2. *In vivo* site occupancy and induction of reporter-gene expression by activator proteins. (*a*) *In vivo* site occupancy by activators. Products of G-specific cleavages of yeast DNA, methylated *in vivo* by DMS, are shown. Positions of protected guanine residues (filled triangles; see also Fig. 1*b*) are indicated at right. The figure consists of four three-panel sets; three panels within each set show the results with the 1×, 2×, and 6× site promoters, as indicated. The first three-panel set (lanes 1–9) shows the results with negative control samples, which contained either yeast DNA methylated by DMS *in vitro* (lanes 1, 4, and 7) or methylated *in vivo* in the absence of effector expression. These *in vivo*-methylated control samples were prepared from cells that contained an empty vector and were grown in a medium supplemented with glucose (lanes 2, 5, and 8) or a galactose/glucose (0.324/0.676) mixture (lanes 3, 6, and 9). The remaining three three-panel sets show experimental results with the POU DNA-binding domain alone (lanes 10–21) and the 2× VN^8 (lanes 22–33) and 6× VN^8 activators (lanes 34–45). Each panel here consists of four lanes; the first lane of each panel is a duplicate of the appropriate negative control sample (i.e., lane 3, 6, or 9), and the following three lanes contain

the DNA-binding domain alone (the left graph), whereas the weak 2× VN^8 activation domain also showed a significant, although moderate, 3-fold effect on DNA binding (the center graph). These results indicate that the DNA-binding domain is not the sole determinant of DNA binding, but that activation domains significantly facilitate activator binding to their cognate sites *in vivo*. In contrast to these *in vivo* results, neither the presence of activation domains nor the number of binding sites augmented activator binding to their cognate sites in a typical *in vitro* assay (data not shown).

Effect of Cooperative DNA Binding on Transcriptional Activation *in Vivo*. The transcriptional effects of modulating activator binding *in vivo* are illustrated in Fig. 2*d*. A comparison among different promoters revealed that the 2× site promoter directs 10-fold higher levels of transcription than the 1× site promoter with either the weak 2× VN^8 activator (the center graph in Fig. 2*d*) or strong 6× VN^8 activator (the right graph), irrespective of activator concentrations. This 10-fold increase in transcription—regardless of either activation potency or concentration—is indicative of activation-domain-independent cooperativity in DNA binding. Nevertheless, the present analysis does not demonstrate whether this cooperative binding is exclusively responsible for the increases in transcription, because transcriptional activity of the 1× and 2× site promoters when bound by activators could not be compared due to poor activator binding to the 1× site promoter (see Fig. 2*c*).

Comparison of the transcriptional efficacy of activator-bound promoters, however, was possible between the 2× and 6× site promoters. This comparison revealed that, at similar levels of *in vivo* occupancy by the 2× VN^8 activator, these two promoters direct similar levels of transcription (Fig. 2*e*; the center graph). Thus, the 6× site promoter achieves higher levels of transcription than the 2× site promoter at equivalent activator concentrations (Fig. 2*d*; the center graph) predominantly because the activator is more readily recruited to the 6× site promoter than to the 2× site promoter (Fig. 2*c*; the center graph). In this case, differential transcriptional responses of promoters that contain different numbers of sites are generated primarily through differential activator binding, whereas the transcriptional efficacy of the promoters when bound by the activator is nearly unchanged.

Effects of Activation Domains on Transcriptional Responses. The transcriptional responses displayed in Fig. 2 *d* and *e* also illuminate the significance of activation-domain function in augmenting transcription *in vivo*. First, a comparison of the transcriptional activity of promoter-bound activators (Fig. 2*e*; note that scales of vertical axes in the graphs vary) indicates that the strong 6× VN^8 activator (the right graph) achieves 4-fold higher levels of transcription than the weak 2× VN^8 activator (the center graph), whereas the promoter-bound DNA-binding domain (the left graph) is virtually inactive. This *in vivo* result substantiates a generally assumed function of activation domains—namely, that activation domains, when brought to a promoter, stimulate transcription to different extents depending on their potencies.

However, whereas this function of activation domains is required to exert transcriptional effects, it is not necessarily the major determinant of differential transcriptional activation by different activators. With the 2× site promoter, for example, the 6× VN^8 activator achieves 40-fold higher levels of transcription than the 2× VN^8 activator relative to activator levels (Fig. 2*d*; compare the right and center graphs). This 40-fold difference in the steepness of transcriptional responses can be attributed to both (*i*) the 4-fold difference in transcriptional activation by promoter-bound activators (Fig. 2*e*), and (*ii*) the 10-fold difference in activator binding to the promoter (Fig. 2*c*). In addition to the previous 2× and 6× site promoter comparison, this result represents another example of differential transcriptional responses that are produced, in a large part, through differential activator binding; in this case, however, differential binding is caused by the differential ability of activation domains to enhance DNA binding.

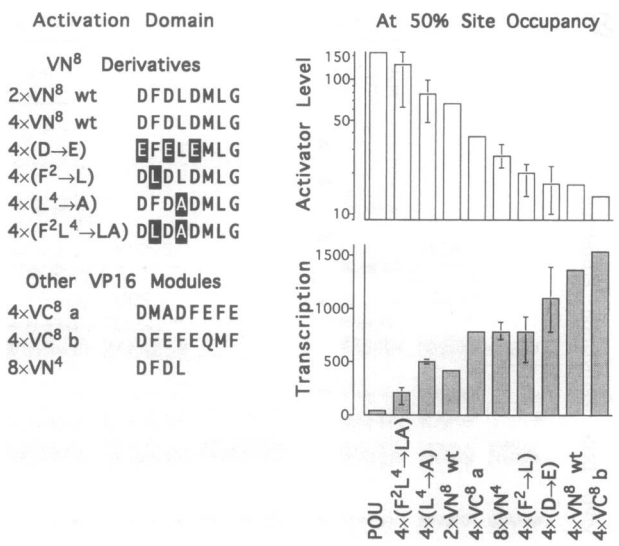

FIG. 3. Comparing the ability of various activation domains to enhance DNA binding and to stimulate transcription *in vivo*. (*Left*) Structures of activation domains, composed of reiterated copies of wild-type and mutant (mutant residues indicated by white letters) VN^8 module and other VP16-derived activation modules; each was fused to the Oct-2 POU DNA-binding domain. These activators were assayed for their levels of expression, *in vivo* site occupancy, and transcriptional activation with the 2× site promoter as described for Fig. 2. (*Right*) Results of these assays; the upper graph shows levels of activators required for half-optimal site occupancy *in vivo*, and the lower graph shows respective levels of transcription. Plotted values were obtained either from a single data point, which displayed near half-optimal site occupancy (46–53% protection), or from the interpolation of a pair of data points, one of which displayed site occupancy lower than half-optimal (29–40% protection) and the other displayed site occupancy higher than half-optimal (58–65% protection). In the latter cases, actual levels of activators and transcription from these pairs of data points are shown as upper and lower limits of error bars.

Correlation Between Abilities of Activation Domains to Enhance DNA Binding and to Stimulate Transcription. The correlation between the abilities of activation domains to enhance DNA binding and to stimulate transcription was further investigated with an array of different activators; these activators contained activation domains composed of reiterated wild-type or mutant VN^8 modules or other VP16 activation modules (Fig. 3). The results displayed in Fig. 3 show the levels of each of these activators required for half-optimal site

samples that were prepared from cells expressing three different levels of effectors (see *b*). These cells were grown in a medium supplemented with a galactose/glucose mixture of various galactose/glucose ratios (0.012–0.324). (*b*) Expression of effectors. Protein extracts were prepared from portions of yeast-cell samples described in *a*, and effector expression was detected from 10 μg of yeast protein by immunoblot analysis. Quantitations of the results are illustrated in three graphs: *c*, extents of site occupancy *in vivo* versus levels of activator expression; *d*, levels of *lacZ* reporter-gene (β-Gal) expression versus levels of activator expression; and *e*, levels of *lacZ* reporter-gene expression versus extents of site occupancy *in vivo*. The left, center, and right graphs show the results with the DNA-binding domain alone (POU), and the 2× VN^8 and 6× VN^8 activators, respectively; in each graph, the results with the 1×, 2×, and 6× site promoters are indicated by open, crossed, and filled symbols, respectively. Note that scales of some axes were varied, as indicated by different shadings.

occupancy *in vivo* on the 2× site promoter (the upper graph) and the resulting transcriptional activation (the lower graph). This analysis revealed that activators that are more efficient in DNA binding (i.e., those shown toward the right in the graphs in Fig. 3) are generally more efficient in transcription when bound to the promoter. This result indicates that the ability to enhance DNA binding and to stimulate transcription coincides within these various different small activation modules, and, furthermore, it shows that the strength of the two activities correlate with one another.

DISCUSSION

A pioneering study by Giniger and Ptashne (5) previously showed that the potent yeast activator GAL4 binds cooperatively to two adjacent sites *in vivo*. I have described here that activator binding to a promoter is augmented by both activation-domain-independent cooperativity and an activation-domain-mediated effect. It has been also reported that, in the case of the yeast Pho4 activator, the presence of an activation domain is apparently required for it to bind to the *PHO5* promoter and to disrupt nucleosomes (25). In a typical *in vitro* assay, in contrast, neither the number of binding sites nor the potency of activation domains effected on DNA binding of yeast-expressed POU-domain activators (data not shown). Therefore, binding of these POU-domain activators to promoters must be governed *in vivo* not only by the direct interaction between the DNA-binding domain of activators and specific target sequences but also by other interactions that specifically facilitate binding of potent activators to multiple sites. Because cooperative activator binding does not require the presence of effective activation domains, these other interactions constitute two different pathways in modulating activator binding to their cognate sites *in vivo*: a pathway responsible for activation-domain-independent cooperative binding, which perhaps takes effect preceding stimulation of transcription, and a second pathway responsible for activation-domain-mediated enhancement of activator binding.

The comparison of various different activation domains revealed correlations between their effects on DNA binding and transcription (Fig. 3). Therefore, in producing these two effects, activation domains may rely on interactions either with an identical target(s) or, alternatively, with different targets that similarly distinguish different activation domains. In contrast, I have found that the transcriptional effect, but not the DNA-binding effect, of activation domains was diminished by a mutation in the TATA box of a test promoter (unpublished data), suggesting discrete pathways to exert the two effects. Moreover, some *in vitro* studies that use nucleosomal DNA have revealed that histones and regulatory factors such as SWI/SNF proteins (for review, see ref. 26), in the absence of general transcription factors, can modulate activator binding to DNA in a manner that is responsive not only to the number of binding sites (27–29) but also to the presence of activation domains (30). Therefore, although the significance of these *in vitro* effects has yet to be verified *in vivo*, it is possible that activation domains augment activator binding to DNA and transcription through diverse pathways yet via a common or similar target(s).

Transcriptional efficacy of a promoter is determined through integration of numerous parameters via multitudes of protein–protein and protein–DNA interactions. In this entire process, modulation of interactions between promoter-bound activators and general transcription factors have been broadly accepted to produce specific transcriptional outcomes from a promoter. Indeed, by modulating these interactions, potency of activation domains and the number of binding sites can impact on transcription *in vitro* (31, 32). However, the present work indicates that these parameters significantly affect one other step of the activation process *in vivo*—the step of activator binding to a promoter. In the examples described here, as well as in another case with GAL4 (5), these effects on DNA binding are predominantly responsible for different transcriptional responses. These findings suggest that the step of activator binding to a promoter plays a more substantial role than generally realized in sensing the promoter structure and activator quality/quantity to give rise to a specific transcriptional output from a promoter under a particular cellular environment.

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