A synergistic increase in potency of a multimerized VP16 transcriptional activation domain

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Communicated by E.De Robertis

Transcriptional synergy in eucaryotes provides a means to control both the level and diversity of gene expression. The mechanism by which multiple activators elicit such effects is unknown. To address this problem we considered whether multimerizing an activator's binding sites on DNA. Synthetic activators bearing one, two or four VP16 'core' activation domains, fused to the GAL4 DNA binding domain, were co-transfected into Cos-1 cells with CAT gene reporter templates containing one, two or five upstream GAL4 binding sites. Our results demonstrate that all of the activators elicit synergistic effects when comparing the amounts of transcription on multiple sites versus a single site. In contrast, the multimerized activation domains did not stimulate transcription significantly on a template bearing a single site; a synergistic increase in potency was, however, apparent on a template bearing two sites. Introducing the flexible λ repressor linker region in between the activation domains increased the ability of activators bearing two or four VP16 domains to stimulate transcription from the single-site template. We discuss the mechanistic implications of this study on gene activation and synergy.

Key words: RNA polymerase II/synergism/VP16

Introduction

Eucaryotic RNA polymerase II promoters bind a complex array of proteins that regulate transcription initiation and elongation (Johnson and McKnight, 1989; Ptashne and Gann, 1990; reviewed in Carey, 1991). A typical regulated promoter contains a TATA box and two or more binding sites for gene activators. Activators turn on transcription of genes during development and differentiation and in response to selected extracellular stimuli. Activators are composed of one domain that recognizes DNA and at least one other that stimulates transcription (reviewed in Ptashne, 1988). These domains are either contained within a single polypeptide or constitute distinct subunits in a multiprotein complex (reviewed in Ptashne and Gann, 1990; Carey, 1991). Activation domains are generally classified according to amino acid composition; the prototype is the yeast GCN4 activation domain characterized by an abundance of acidic amino acids and a net negative charge (Hope and Struhl, 1985). Recent studies suggest that the acidic activation

domains function by directly contacting components of the general transcription machinery to help assemble a transcription complex at the TATA box (Lin and Green, 1991; Wang *et al.*, 1992). Biochemical analysis of the mammalian transcription machinery has shown that in addition to RNA polymerase II, the complex contains the TATA binding factor (TBP), several uncharacterized TBP-associated factors (TAFs), TFIIA, TFIIB, TFIIE, TFIIF, TFIIH and TFIJ (reviewed in Zawel and Reinberg, 1992). The TAFs appear to mediate transcriptional activation but are not required for basal transcription (Pugh and Tjian, 1990).

The mechanism of transcription stimulation by acidic activators has been studied extensively in mammalian cell-free systems. Lin and Green (1991) showed that a GAL4-derived acidic activator does not affect binding of TFIID (a complex of TBP and TAFs) to a reporter template bearing a TATA box. In contrast, the activator strongly stimulated association of TFIIB with DNA-bound TFIID. The remaining general factors and RNA polymerase II then bind to form what is referred to as the closed complex. In the presence of ATP the DNA at the start site becomes melted to permit initiation by RNA polymerase (Wang *et al.*, 1992).

When two or more activators elicit a transcriptional response that is greater than the sum of the effects measured when each of the activators is assayed individually, they are said to act in synergy or to act cooperatively (reviewed in Carey, 1991; Ptashne, 1992). Many cellular and viral RNA polymerase II promoters contain multiple upstream binding sites for activators that mediate synergistic effects on transcription. Synergy allows particular combinations of activators to give rise to unique programs of gene expression, a phenomenon called combinatorial control, and is believed to be one of the predominant mechanisms for differential gene transcription in eucaryotic cells. Synergy also plays a role in regulating the level of transcription by master control switches. The homeodomain protein bicoid, for example, acts synergistically in directing formation of a gene expression gradient during development of the anterior–posterior body axis in *Drosophila* (Driever *et al.*, 1989; Struhl *et al.*, 1989).

The phenomenon of synergy has been recreated in many systems using simple artificial promoters bearing one or more upstream activator binding sites. Synthetic promoters bearing one or more recognition sites for GAL4 derivatives (Carey *et al.*, 1990), ZEBRA (Carey *et al.*, 1992), NF κ B (Pettersson and Schaffner, 1990), Sp-1 (Courey and Tjian, 1988), progesterone receptor (Tsai *et al.*, 1989), bicoid (Driever *et al.*, 1989; Struhl *et al.*, 1989), as well as other activators, have revealed that one binding site cannot effectively mediate gene activation; two or more sites are required to observe a significant, synergistic effect. Different combinations of eucaryotic activators also stimulate transcription synergistically in these model systems

(Davidson *et al.*, 1988; Ondek *et al.*, 1988; Schule *et al.*, 1988; Strahle *et al.*, 1988).

Transcriptional synergy was first characterized in procaryotes where the phage λ repressor activates transcription from a promoter called P_{RM} by binding cooperatively to two adjacent sites called O_{R1} and O_{R2} (reviewed in Johnson *et al.*, 1981). In this case, the role of repressor bound to a high affinity site O_{R1} is to promote binding of another repressor molecule to a low affinity site O_{R2} ; repressor bound at O_{R2} then contacts RNA polymerase and stimulates transcription (Hochschild and Ptashne, 1986). High concentrations of repressor drive binding to O_{R2} in the absence of O_{R1} and turn on the *ci* gene downstream of P_{RM} . Thus, in procaryotes, excess

activator can overcome the need for additional DNA sites. Several eucaryotic activators, including HSTF and the progesterone receptor, bind DNA cooperatively *in vitro*. It has been proposed that this cooperativity contributes to the transcriptional synergy mediated by these activators *in vivo* (Tsai *et al.*, 1989; Xiao *et al.*, 1991).

In contrast, some eucaryotic activators do not appear to bind cooperatively to DNA yet activate transcription synergistically on templates bearing two or more recognition sites (Carey *et al.*, 1990; Petersson and Schaffner, 1990; Oliviero and Struhl, 1991). GAL4–VP16, for example, is an activator constructed by fusing the acidic activation domain of the HSV VP16 transactivator to the DNA binding domain of GAL4 (Sadowski *et al.*, 1988). GAL4–VP16

Fig. 1. Multimerized VP16 activation domains stimulate transcription synergistically in mammalian cells. (A) Schematic representation of GAL4 derivatives used in our study. The CAT reporter plasmids used in the co-transfection assays are shown. One, two or five GAL4 sites (indicated by open boxes) are positioned 23 bp upstream of an adenovirus E4 TATA box–CAT gene fusion. The effector plasmids contain one, two or four copies of the activation domain of VP16 (413–454) fused to the DNA binding domain of GAL4 (amino acids 1–147) cloned into an expression vector under the control of SV40 enhancer/promoter. (B) CAT assays were performed on Cos-1 cell lysates 72 h after lipofectin-mediated co-transfection of 100 ng of reporter plasmid ($G_1E4TCAT$, $G_2E4TCAT$, $G_5E4TCAT$) and 100 ng of effector plasmid directing the synthesis of either GAL4–VP1, GAL4–VP2 or GAL4–VP4. The amounts of effector plasmid were optimized in a dose response experiment (not shown). The products of the CAT assay were fractionated on a TLC plate. The acetylated product was excised from the plate and the radioactivity was counted in a liquid scintillation spectrophotometer. The c.p.m. of acetylated product were plotted against the number of template sites for each activator. This experiment was repeated seven times in Cos-1 cells, and each time similar results were obtained. Note that in the side-by-side comparison graphed above, the values on the five-site template are an underestimate. In order to detect background and stimulated levels on the single-site template the assay was performed for 3 h bringing the CAT activity levels measured on five sites out of the linear range. However, in a 15 min assay the levels of stimulated transcription by each of the activators were identical (data not shown). (C) All three activators are expressed at the same level in Cos-1 cells. Extracts from [^{35}S]methionine- and [^{35}S]cysteine-labeled Cos-1 cells co-transfected with the $G_2E4TCAT$ reporter plasmid (100 ng) and effector plasmids expressing GAL4(1–147), GAL4–VP1, GAL4–VP2 and GAL4–VP4 (100 ng) were incubated with antibody specific to GAL4(1–147). The immunocomplexes were precipitated with protein A and resolved by SDS–PAGE. An autoradiogram of the gel is shown. The positions of GAL4(1–147) alone, GAL4–VP1, GAL4–VP2 and GAL4–VP4 are indicated by the arrows to the right of the autoradiogram.

stimulates transcription synergistically in mammalian cells, both *in vitro* and *in vivo*, when activation from a template bearing a single site was compared with that from a template comprising two or more sites (Carey *et al.*, 1990). The *in vitro* experiments revealed no significant difference in affinity of GAL4-VP16 for one site compared with multiple sites. Furthermore, unlike the λ repressor, synergy was observed in the presence of excess activator; conditions in which the sites, on the templates being compared, were saturated as measured by DNase I footprinting and transcription assays. These observations strongly suggested that cooperative DNA binding was not the cause of the observed synergy.

To explain this novel form of cooperativity, it was proposed that multiple activators could simultaneously interact with one or more components of the pol II transcription machinery (Ondek *et al.*, 1988; Carey *et al.*, 1990; Lin *et al.*, 1990; Petersson and Schaffner, 1990). These contacts would, in turn, lead to a cooperative increase in assembly of the transcription complex at the TATA box. The model is compelling in that *in vitro* experiments support the idea that the synergistic increase in transcription correlates with a cooperative increase in the number of pre-initiation complexes (Wang *et al.*, 1992). Furthermore, the model can accommodate homo-synergy between identical molecules of activator as well as the promiscuous hetero-synergy exhibited between diverse combinations of mammalian activators (Davidson *et al.*, 1988; Lin *et al.*, 1988, 1990; Kakidani and Ptashne, 1988; Schule *et al.*, 1988; Strahle *et al.*, 1988).

One prediction of the simultaneous contact model is that a multimerized activation domain should act synergistically when compared with a single activation domain. We tested this prediction by fusing multimers of the VP16 activation domain to the GAL4 DNA binding domain; the potency of these activators was measured on GAL4 responsive templates. Our data suggest that multiple domains can act synergistically, but that steric constraints limit the effectiveness of the interactions in mammalian cells; increasing the spacing between domains augments the ability of the domains to function.

Results

Figure 1A is a schematic illustration of the DNA constructs used in our study. The GAL4 DNA binding domain, GAL4(1–147), was fused to either one, two or four copies of the core VP16 activation domain, VP16 (413–454). The core VP16 activation domain stimulates transcription less efficiently than the entire 89 amino acid activation domain (411–490) (Triezenberg *et al.*, 1988). For simplicity we will refer to the proteins containing one, two and four domains as GAL4-VP1, -VP2 and -VP4 respectively. Note, however, that the GAL4 DNA binding domain recognizes its site as a dimer (Carey *et al.*, 1989; Marmorstein *et al.*, 1992) and therefore the dimers contain two, four and eight activation domains, respectively. Also note that GAL4(1–147) contains a cryptic activation domain that stimulates transcription *in vitro* (Lin *et al.*, 1988). Although this domain does not activate transcription *in vivo* it may contribute to the stimulatory potential of activation regions fused near it. The genes encoding the fusion proteins were placed under control of the SV40 enhancer/promoter. These constructs were co-transfected into Cos-1 cells with reporter templates bearing either one, two or five 17 bp GAL4 binding sites (17mers) positioned 23 bp upstream of an adenovirus E4 TATA box—CAT gene fusion; CAT activity was used as a measure of transcription. Dose-response curves were generated to determine the optimal amount of effector and reporter plasmids and these amounts were used in subsequent experiments.

Figure 1B is a plot of the transcription signal against the number of template sites from a representative experiment. The transcription signal is illustrated as the c.p.m. of acetylated chloramphenicol, the product of the CAT enzyme assay. When compared with the GAL4 DNA binding domain alone, the fusion proteins stimulated transcription only marginally above background levels on a template containing only one upstream GAL4 binding site. However, on templates bearing two sites, GAL4-VP2 and GAL4-VP4 elicited strong stimulatory effects compared with GAL4-VP1. The results on this template best illustrate the

Fig. 2. Graphical representation of site- and domain-synergy. (A) All three activators stimulated transcription synergistically on the two- and five-site templates relative to a single-site template. This bar graph represents the site synergy. The black bars represent the signals elicited by the indicated activators on the lower number of sites, the white bars represent the transcriptional signals elicited on the templates bearing the greater number of sites and the cross-hatched bars represent the expected values if the effect of sites was additive. In this bar graph, all of the possible combination of sites are examined. (B) The VP16 activation domains function synergistically on the two-site template. This bar graph represents the activation domain synergy. The black bars represent the signals elicited by the activator bearing the lower number of domains, the white bars represent the transcriptional signals elicited by the higher number of activation domains and the cross-hatched bars represent the expected values if the activation domains were acting additively. The fold-synergy is indicated below each comparison (the ratio of the white bars to the cross-hatched bars). The value of the basal level transcription was subtracted from all the activated levels in our measurements.

increased potency of activators bearing two or four activation domains compared with an activator bearing a single domain. On five sites, all three activators stimulated the same amount of transcription. We observed similar transcriptional results in seven independent experiments. The significant differences in activity observed between the activators on the two-site template were not a consequence of variability in the amounts of activator. Immunoprecipitation reactions showed that all three activators were expressed at the same level (Figure 1C).

The data were further quantified in two ways. First, we compared the effects of a fixed number of activation regions on different numbers of sites. We refer to this as site synergy. Secondly, we compared the effects of multiple domains with that of a single domain on the two-site template. We refer to this as domain synergy. The results of the comparisons are shown as bar graphs in Figure 2A and B.

The black bar in Figure 2A represents the signal observed on a template bearing the fewest sites. The white bar is the signal observed on the template bearing the greatest number of sites. The cross-hatched bar represents the signal expected if the activators were acting additively on the template bearing more sites with respect to the template bearing fewest sites. When the observed signal is greater than the predicted signal then the activator is considered to be acting synergistically. The ratio of the two bars, termed the fold-synergy, is a relative measure of the effect. All three activators stimulated transcription synergistically when comparing the signals seen on five and two sites with the signal observed on a single site. The signal observed on a single site was so near to background that it was difficult to calculate the fold-synergy precisely. The site synergy was most evident comparing GAL4–VP2 on five sites with its effect on a single site. The synergy drops off when comparing GAL4–VP2 on five sites with two sites because the transcription on the two-site template is much higher than on a single site reporter. In contrast, the synergy with GAL4–VP1 is most evident when comparing five sites with two sites because the protein is only marginally active on a template bearing two sites, whereas it is as active as GAL4–VP4 on the five-site template. Thus the synergistic

increase from trace to high levels of transcription occurs on fewer sites when the activator bears two or more VP16 activation domains.

Figure 2B shows that when measuring the ability of GAL4–VP2 to stimulate on the two-site template (white bar), the effect was 8-fold greater than the signal elicited by GAL4–VP1 and 4-fold greater than what we would predict if the domains were working additively. We refer to this as 4.5-fold synergy. There was 3-fold synergy when GAL4–VP4 was compared with GAL4–VP1. We conclude that on this particular template, the two- and four-domain fusions function synergistically relative to an activator bearing a single domain. The perplexing result was the observation that GAL4–VP2 and GAL4–VP4, although potent activators on two sites, do not significantly stimulate transcription on a template bearing a single site. Taken together, the data suggest that multimerizing the VP16 activation domain generates a stronger interaction with the target protein(s), but that the energy is still not sufficient to trigger transcription from a single-site template; at least two sites are necessary.

To explore this result further, we considered the possibility that the multiple domains were affecting the activator's affinity for the target, but the interaction was not optimal, perhaps due to steric constraints, i.e. the proximity or relative flexibility of the two domains. Note that the center-to-center distance between neighboring GAL4 sites in the two- and five-site constructs is 19 bp or ~ 67 Å. In contrast, the linker region between the repeated domains in our constructs is only three amino acids long, which corresponds to ~ 4.5 Å in an α -helix. Thus it was conceivable that the distance between the DNA sites imparted enough flexibility to adjacent activators such that they could effectively interact with their target(s), whereas the lack of spacing between adjacent domains in the multimers was limiting their activity. To test this hypothesis we chose to introduce a linker between the VP16 domains. We used the 40 amino acid linker that normally separates the amino- and carboxyl-terminal domains of λ repressor. This portion of the repressor is believed to be relatively flexible (Pabo *et al.*, 1979). If this region were to fold into an α -helix it would be ~ 60 Å in

Fig. 3. The λ repressor linker increases the potency of multimerized domains. (A) A schematic representation of the effector plasmid constructs and the template used in the co-transfection studies in B. There is now an insertion of the 40 amino acid linker of λ repressor (92–132) between the VP16 activation domains (compare with Figure 1A). (B) The λ repressor linker causes a modest increase in stimulation by the activators containing two or four activation domains. Cos-1 cells were co-transfected with 100 ng of the effector plasmids directing the synthesis of GAL4(1–147), GAL4–VP1, –VP2, –VP4, – λ VP1, – λ VP2 and – λ VP4 with a reporter template containing a single GAL4 binding site. 72 h after transfection the cells were harvested and subjected to a CAT activity assay. An autoradiogram of a typical assay is shown. The degree of relative transcription is indicated below each panel.

length; if it were a β -sheet or random coil, it would be over twice as long. A schematic illustration of the resulting construct is shown in Figure 3A.

Figure 3B displays the results of a typical CAT assay from cells co-transfected with the various constructs and a reporter template containing a single GAL4 site. While the linker has no apparent effect on an activator bearing a single domain, it leads to a modest increase in stimulation by activators containing two or four domains. A similar result was observed in four independent experiments. We have previously tested a GAL4- λ repressor fusion containing the linker and it has no apparent effect on transcription (Lin *et al.*, 1988) in a mammalian system. To demonstrate further that the linker was not contributing an additional activation domain, we compared the activity of GAL4-VP2 with GAL4- λ VP2 on one-, two- and five-site templates. Figure 4 is a compilation of two such experiments. In each case, the activator lacking the λ linker is compared with the protein bearing the linker (represented as 100%). Although we observed a small increase in activity of the linker bearing protein on two and five sites, it was negligible compared with the 2- to 4-fold effect it had when the activators were compared on a single site. We conclude that the effect of the linker is not to contribute an additional activation region, but rather to increase the spacing or flexibility between the domains. Surprisingly, the GAL4- λ VP2 construct was consistently stronger than GAL4-VP4 on a single site, suggesting that the additional two VP16 activation domains in VP4 do not act as effective linkers. This may bear relevance to the proposed flexibility of the λ linker relative to other portions of protein.

Discussion

Our data demonstrate that multimerizing the VP16 activation domain leads to a synergistic increase in its ability to stimulate transcription. Hollenberg and Evans (1988) measured a similar synergistic effect when oligomerizing the acidic activation domain of the human glucocorticoid

Fig. 4. A bar graph representation of the relative activity of GAL4-VP2 and GAL4- λ VP2. The data is an average of two representative experiments. The c.p.m. of acetylated chloramphenicol products were determined and background was subtracted. The transcriptional efficiency of the GAL4- λ VP2 was denoted 100%. The ratio of the products elicited by GAL4-VP2 and GAL4- λ VP2 gives a relative measure of the efficiency of the linker in different promoter contexts.

receptor. However, our studies show that, in the case of the core VP16 domain, the increased potency is only evident on the two-site template and is affected to a moderate degree by the spacing between the domains. Below we consider how these results bear on the mechanism of synergy.

Simultaneous contact model

We have previously proposed a model, called the simultaneous contact model, whereby multiple activators concurrently contact a common target and it is this interaction that leads to transcriptional synergy (Carey *et al.*, 1990; Lin *et al.*, 1990). Although the model broadly postulated the existence of multiple contact sites on the transcriptional apparatus it did not consider the relative positioning or capacity of these sites. Our current results begin to define these parameters as described below. We considered three key observations. First, very little gene activation (< 10-fold) is observed from a template bearing a single site; although an activator may contain multiple activation domains, multiple sites are still required for a significant amount of transcriptional stimulation. Secondly, on two sites, activators bearing multiple domains are much more potent than an activator bearing a single domain. Thirdly, on five sites, all activators elicit the same high levels of transcription. Our interpretation of these results is illustrated in Figure 5.

In our model, the transcription apparatus, comprising RNA polymerase II and its associated factors, is considered one contiguous surface, illustrated by the gray box. This is not an unreasonable assumption as the factors constituting

Fig. 5. A model for transcriptional synergy by GAL4-VP16 fusion proteins. Two or more GAL4-VP2 activators simultaneously interact with separate contact points (1 and 2) within the transcription complex (gray box) leading to synergistic increase in the stability of the complex and hence a synergistic effect on transcription. Some of the components of the complex are indicated. Each molecule of GAL4-VP2 could be replaced by two molecules of GAL4-VP1. The thermodynamic basis for the model has been proposed previously by Carey *et al.* (1990) and Lin *et al.* (1990). Namely, when each activator contacts a target protein it releases a certain amount of energy. Interaction of multiple activators would lead to a summing of the free energies. This is at best an approximation because one cannot simply sum interaction energies as each ΔG contains an entropic component that cannot be added (Jencks, 1981). Nevertheless it provides a simple way to conceptualize the idea based on Gibbs equation, $\Delta G = -RT \ln K$, where the summing of the free energies would lead to an exponential increase in the affinity (K) of the complex for the activators. The energy may also be funneled into a conformational change, but this does not significantly change the interpretation. By our model the free energy needed to accomplish the task could not be generated by filling of one contact point by activators as there would be a limit to the number of contacts and hence the energy of interaction at each point. Each point can accommodate two or maybe more activation domains. Only when both points were full, or nearly so, would the energy released be sufficient to trigger transcription. The model is not drawn to scale.

the complex are tethered to each other through a vast network of protein–protein interactions and to DNA via TBP. Interaction of an activation region at any point along the structure will lead to stabilization of the whole structure because the various subunits constituting the assembly contact one another. Interactions with multiple points would have a multiplicative effect on the stability. Stability could either refer to stabilizing a conformational change in the complex or simply decreasing the off rate of factors participating in the complex.

Our current data further suggest that the transcription complex contains at least two distinct contact points (shown as 1 and 2) for activators and that these points are located at distinct positions along the surface of the transcription complex. They are separated such that a single molecule of activator, regardless of the number of activation domains, would only be able to approach and interact with one contact point due to steric and/or distance constraints. However, two molecules of activator bound to adjacent sites on DNA would be able to contact different points due to the increased spacing between sites and possibly the rotational freedom of DNA relative to the flexibility, and spacing of activation domains within a protein. This new feature would accommodate our finding that two or more sites are necessary to activate efficiently our reporter genes regardless of the number of activation domains the activator carries.

Furthermore, we hypothesize that each contact point is able to bind at least two, and perhaps more, activation domains. This parameter would be limited by the available surface area and specific amino acid contacts. The critical feature is that filling one contact point with activation domains would still not release enough energy to trigger a significant amount (>10 -fold stimulation, for example) of transcription; only when both contact points were filled would this occur. This feature would accommodate the observation that GAL4–VP2 works efficiently from two sites whereas GAL4–VP4 was only marginally active from a single site. By further postulating that two activators, each bearing a single activation domain, can contact a single point, we could explain our observation that five, but not two, molecules of GAL4–VP1 can turn on the gene to a high level. Only the interaction of a single activator with multiple contact points would be proscribed.

In nature, different acidic activation regions may exhibit different strengths dictated by their surface area and distribution of amino acids able to contact the target. Thus the number of domains necessary to achieve activation may differ among activators (Carey *et al.*, 1990). For example, the intact VP16 activation domain is a more potent activator than the core domain used here (Triezenberg *et al.*, 1988). It is plausible that the intact domain may be equivalent to two or more core domains in this type of assay.

Why does the λ linker increase the ability of the domains to function on a single site? There are two possibilities. The first is that the linker allows an activator to present two or more activation domains to a single contact point more efficiently than an activator bearing two domains but lacking the linker. The second is that the linker allows a single molecule of activator to simultaneously present multiple activation domains to both contact points. In each case the linker is overcoming certain steric restrictions that optimize the presentation of the activation motifs to the transcription complex. In this regard we note that the effect of the linker

is only moderate. For example, the linker is able to allow GAL4– λ VP2 to activate as well on a single site as GAL4–VP1 activates on two sites. However, it cannot fully compensate for the effect of sites so that GAL4– λ VP4 on a single site is still less active than GAL4–VP2 measured on two sites.

The observation that two general transcription factors, TFIIB and TBP, interact with the VP16 activation domain in affinity chromatography experiments (Ingles *et al.*, 1991; Lin *et al.*, 1991) is consistent with our proposal. TBP and TFIIB are known to interact with one another when bound to the promoter as measured in gel mobility shift experiments (reviewed in Zawel and Reinberg, 1992). Thus simultaneous interaction of two activators with these factors is analogous to concurrent interactions with a common target at two distinct contact locations.

Role of the DNA binding domain

Our results using multimerized activation domains lacking a spacer region are similar to those of Oliviero and Struhl (1991). In their experiments, the GCN activation domain was fused to the DNA binding domains of jun and fos. Jun/fos heterodimers containing only one activation domain were compared with jun homodimers containing two activation motifs. The DNA templates were composed of either one or two upstream jun/fos (AP-1) recognition sites. It was shown that in yeast, an activator bearing one activation region was more potent on a template bearing two sites than an activator with two activation regions was when measured on a single site template.

One interpretation of the result was that the number of DNA binding domains must be important, perhaps due to requisite interactions with RNA polymerase II or with chromatin. The multiplicative effects of this interaction and the interaction of the activation region with its target would generate the synergistic transcriptional response. This model does not consider the steric limitations that the transcription complex may present. Thus, an alternative explanation of the data is that the spacing between the activation domains is critical and that the effects of a heterodimer bearing two activation regions were not apparent on a single site due to the proximity of the domains, only one of which could be efficiently employed for interaction with its target(s). Our observation that the spacing between the VP16 activation domains can affect their activity provide support for this interpretation.

Cooperative binding to DNA

In addition to the mechanisms described above, alternative modes of synergy exist that may be implemented by the cell to create additional levels of gene regulation. Cooperative binding of two or more activators to DNA sites upstream of a gene, as illustrated by λ repressor, can in principle, generate a synergistic effect on transcription. In this case, direct contact between two or more activators increases their affinity for DNA. This allows a template bearing multiple sites to become saturated at lower concentrations of activator than a template bearing a single site. Cooperative DNA binding is a property of some eucaryotic activators, including the progesterone receptor, HSTF, the BPV E2 protein and Oct-1 (Tsai *et al.*, 1989; Bruggemeier *et al.*, 1991; Li *et al.*, 1991; Xiao *et al.*, 1991) and may play a role in transcriptional synergy. Our previous observations suggest

that GAL4(1–147) derivatives do not bind naked DNA templates cooperatively, regardless of whether they carry an activation region (Carey *et al.*, 1990). However, GAL4 derivatives do appear to bind chromatin templates cooperatively *in vitro* (Taylor *et al.*, 1991), although this does not require an activation region. This form of cooperativity superimposed upon the type described above may create an exquisitely sensitive switch between the on and off states of a gene.

Controlling the strength of an activator

Many eucaryotic activators contain more than one activation region (Courey and Tjian, 1988; Pascal and Tjian, 1991). We argue that to prevent activators from working from a single template site, the normal disposition of repetitive domains within an activator is probably not optimal. Certain gene expression programs require the concerted action of multiple transcription factors. If activators evolved the capability to activate a gene efficiently from a single site, then the cell would not be able to employ synergy as a method of differential gene regulation. To avoid this problem, the cell very probably imposes spacing and steric constraints that can only be overcome by binding of multiple activators to DNA. Our attempts to circumvent these constraints using a flexible protein linker were only moderately successful, suggesting that the reaction requires further optimization.

Materials and methods

Constructs

The reporter template G₅E4TCAT has been described previously by Liu and Green (1990). G₁ and G₂E4TCAT were constructed by replacing the *Hind*III–*Bam*HI fragment of G₅E4T with those of G₁ and G₂E4T (Carey *et al.*, 1990). VP1, VP2 and VP4 were constructed by using PCR to amplify the region between amino acids 413 and 454 of VP16. The PCR primers were engineered to introduce *Bam*HI and *Bgl*II restriction sites at the ends. The fragments were oligomerized by cleaving the PCR products with *Bam*HI and *Bgl*II, purifying the products and then oligomerizing them using DNA ligase and ATP. *Bam*HI and *Bgl*II were included in the ligation mixture to cleave head-to-head and tail-to-tail products thereby driving the equilibrium in favor of the head-to-tail orientation. The products were then isolated and cloned into the *Bam*HI site of pBXG1 (a gift of M. Ptashne and P. Broad) immediately downstream of and in frame to a gene encoding GAL4(1–147) under control of the SV40 enhancer/promoter (ori).

The λ repressor linker between amino acids 92–132 was amplified by PCR. Again the primers contained *Bam*HI and *Bgl*II sites on the ends. The *Bgl*II site on the λ linker fragment and the *Bam*HI site on the VP1 fragment were cleaved and the two fragments were ligated together. To remove homodimers of the two fragments, the ligation reaction was terminated by heat and the products were cleaved with *Bam*HI and *Bgl*II. Only products containing the λ linker and VP1 in a head-to-tail orientation were isolated. This was cloned into *Bam*HI and *Bgl*II cleaved pSP72. The fragment was released by digestion of the plasmid with *Bam*HI and *Bgl*II and oligomerized as described above.

Immunoprecipitation of GAL4–VP16 derivatives

Immunoprecipitation experiments were performed 48 h after transfection of Cos-1 cells, grown to 80% confluency. Prior to labeling, the cells were incubated for 1 h at 37°C in DMEM medium, lacking methionine and cysteine (ICN). The medium was then removed and the cells were washed with phosphate-buffered saline (PBS) twice. 3 ml of methionine- and cysteine-free DMEM supplemented with 13 μ Ci of [³⁵S]methionine and [³⁵S]cysteine (Du Pont) were added to the cell monolayers. After 3 h at 37°C, the cells were washed twice with PBS at 4°C and lysed by adding 1 ml of a buffer containing 50 mM Tris, pH 7.5, 150 mM NaCl, 5 mM EDTA, 0.5% NP-40, 0.1% SDS, 10 mM 2-mercaptoethanol, 1 mM PMSF and 10 μ g/ml leupeptin and pepstatin. The lysate was collected by scraping the cells off the plates and debris was removed by centrifugation for 1 min at 4°C in a microcentrifuge. The extracts were incubated overnight at 4°C

with 1.5 μ l of a polyclonal antibody to GAL4. The immune complexes were precipitated with 50 μ l of fixed *Staphylococcus aureus* protein A. After 1 h at 4°C, the complexes were isolated by centrifugation. The pellets were washed three times with 1 ml of the lysis buffer. ³⁵S-labeled immunoprecipitated proteins were fractionated on a 12% mini, SDS–polyacrylamide gel and visualized by exposure of the dried gel to a pre-flashed XAR-5 film.

Transfection and CAT assays

All transfection experiments on Cos-1 cells employed the positively charged liposome lipofectin (Gibco/BRL) and were performed according to the instructions provided by the manufacturer with the following modifications. Prior to transfection, cells grown to 70–80% confluency on 60 mm plates were washed with Opti-MEM reduced serum-free medium (Gibco/BRL). 100 ng of effector and reporter plasmids (the optimum amount obtained from a titration experiment) were mixed in polystyrene tubes with 10–25 μ g of lipofectin reagent in a total volume of 100 μ l. After 15 min at room temperature, the complex was diluted with 3 ml of Opti-MEM reduced serum-free medium, mixed gently and added to pre-washed cells on 60 mm plates. After 24 h of incubation, 3 ml of DMEM supplemented with 10% calf serum were added to the cells. The cells were incubated for 48 h more at 37°C and then harvested.

CAT assays were performed according to the protocol provided by Promega. The culture medium was removed from the plates and the cells were washed with phosphate-buffered saline (PBS) and harvested by scraping in 1 ml of TEN (40 mM Tris/HCl, pH 7.5, 1 mM EDTA and 150 mM NaCl) buffer. The harvested cells were pelleted by centrifugation at 14 000 r.p.m. for 15 s. The cell pellets were then resuspended in 150 μ l of 250 mM Tris–HCl, pH 8.0 and lysed by three consecutive freeze–thaw cycles (3 min each). The lysates were heated to 60°C for 10 min and the resultant insoluble debris was removed by centrifugation at 14 000 r.p.m. for 3 min in a microcentrifuge. The protein concentration of the extracts was measured using the Bradford reagent (Bio-Rad). A typical CAT assay contained 25–50 μ g of cell extract, 0.25 μ Ci [¹⁴C]chloramphenicol, 25 μ g of acetyl co-enzyme A and 65 μ l of 250 mM Tris–HCl, pH 8.0. The reaction mixtures were incubated at 37°C for 3 h. The reaction was terminated by extraction of the acetylated chloramphenicol from the mixtures using ethyl acetate. The ethyl acetate extracts were dried for 30 min in a Speedvac evaporator. The pellets obtained were resuspended in 15 μ l of ethyl acetate and were dotted onto TLC plates. The chromatograph was developed in 150 ml of a 29:1 mixture of chloroform–methanol. The TLC plate was then air dried and autoradiographed by exposure to a Kodak XAR-5 film. To quantify the data, the radioactivity of the acetylated chloramphenicol was scraped off the TLC plates and measured in a liquid scintillation spectrophotometer. We repeated the experiments four to seven times. We also monitored the transfection efficiency by independently transfecting a β -galactosidase reporter template. We observed at best only a 2-fold difference in transfection efficiency (T. Chi, K. Emami and M. Carey, unpublished results) using lipofectin as a carrier.

Acknowledgements

We thank Jeannie Kim for illustrations and Tian Chi, Lin Pei and Yasu Ohashi for comments on the manuscript. This work was supported by a grant from the NIH (GM46424).

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Received on June 3, 1992; revised on August 11, 1992