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Threshold Phenomena and Long-Distance Activation of Transcription by RNA Polymerase II

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To explore the underlying mechanisms by which genes are regulated in eukaryotes, long-distance transcriptional activation and threshold effects were reconstituted in vitro. Long-range activation of transcription by GAL4-VP16 protein located 1300 base pairs upstream of the RNA start site was dependent on packaging of the template into histone H1–containing chromatin. A transcriptional threshold effect by GAL4-VP16 was observed with repressed chromatin templates but not naked DNA templates. The experimental data with the chromatin templates were similar to the theoretical activation profile that is predicted if the action of each DNA bound protomer of GAL4-VP16 were independent and additive in terms of free energy.

The proper growth and development of an organism are dependent on a tiered array of processes by which genes are spatially and temporally regulated. Packaging and compaction of DNA render genes refractory to transcription, and the early stages in the pathway leading to gene activation appear to involve alterations in chromatin structure (*1*). Biochemical studies of RNA polymerase II transcription have revealed that basal transcription requires RNA polymerase II and several auxiliary factors (*2*), whereas the activity of the basal machinery is controlled by the combined action of sequence-specific DNA binding factors (*3*) and another class of factors known as coactivators or mediators (*4*). A major weakness of the biochemical experiments, however, has been the inability to recreate in vitro a number of phenomena that are observed in vivo. For instance, it has been difficult to achieve long-range activation of transcription, such as the action of enhancers in vitro (*5*). Moreover, threshold phenomena in which shallow gradients of transcription factors mediate sharp boundaries of gene activation have been well characterized in vivo (*6*) but not demonstrated in vitro for RNA polymerase II transcription.

In general, in vitro transcription experiments have been carried out with naked DNA templates, although reconstituted chromatin templates are probably a better model for the natural state of the DNA in the nucleus. To examine the role of chromatin structure in transcriptional activation, we have reconstituted and characterized chromatin templates prepared from purified components (*7*). Basal transcription is repressed with histone H1–containing chromatin, and the sequence-specific factors Sp1 and GAL4-VP16 both counteract the chromatin-mediated repression (antirepression) and facilitate the inherent transcription reaction (true activation). Thus, with transcriptionally repressed chromatin templates, transcriptional "activation" by a sequence-specific factor is the combination of both antirepression and true activation. Under such conditions of repressed basal transcription, the magnitude of transcriptional activation by Sp1 and GAL4-VP16 is similar to that observed in vivo.

Transcriptional enhancers are often key elements in the spatial and temporal regulation of genes, and the mechanisms by which enhancers activate transcription from relatively long distances (greater than 1 kb from the RNA start site) have been a subject of considerable investigation (*5*). As a first step in the biochemical analysis of enhancer function, we sought to reconstitute long-range activation of transcription. Typically, when the binding sites for sequence-specific factors are located more than 200 bp upstream of the RNA start site, the factors cannot activate transcription in vitro. There is, however, one report of long-range (1300 bp upstream of the start site) activation of transcription in vitro with the GAL4-VP16 hybrid activator protein (*8*). We attempted to reproduce this experiment but were not successful in recreating the long-distance activation (*9*). Although the molecular basis of this discrepancy in the data is not obvious, it is possible that the DNA template may have been assembled into a chromatin template in the previous study (*8*). Reconstitution of DNA into chromatin has been observed with in vitro transcription extracts (*10*), which contain histone H1, a repressor of RNA polymerase II transcription (*11*). We therefore examined the role of chromatin structure in long-range activation of transcription by GAL4-VP16.

In these experiments, the template DNAs were packaged into chromatin with purified components (with an average of one nucleosome per 200 bp and 0 to 1.5 molecules of histone H1 per nucleosome) (*7*) and then were transcribed in vitro (*12*). To examine long-range activation of transcription, we used a template DNA containing five tandem GAL4 binding sites located 1300 bp upstream of the adenovirus E4 promoter (pG5I1300AE4T) (*8*). In this simplified transcription factor–promoter system, long-distance transcriptional activation (antirepression) by GAL4-VP16 was

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observed when the template was transcribed as histone H1–containing chromatin (Fig. 1A) but not when the template was transcribed as naked DNA, chromatin in the absence of H1 or H1-DNA complexes (histone H1 added to naked DNA) (9). As a control, GAL4-VP16 did not mediate either true activation or antirepression with the template DNA, pG0I1300AE4T, which is identical to pG5I1300AE4T, except that it does not contain the GAL4 binding sites (Fig. 1B) (*13*). These results indicate that long-range activation of transcription can be achieved in vitro and is dependent on both packaging of the template into chromatin and repression of basal transcription by histone H1.

These data suggest a mechanism for long-range activation of transcription by enhancer elements. Packaging of naked DNA into the 10-nm chromatin fiber results in a sevenfold compaction in the length of the DNA. In the long-range activation experiment, GAL4-VP16 functioned to counteract H1-mediated repression in a chromatin template (Fig. 1A). We thus suggest that the long-distance activation by the GAL4-VP16 activator was due to transcriptional antirepression by GAL4-VP16 that was brought into the proximity of the RNA start site by packaging into chromatin. This proposed model for enhancer function may be one of a variety of means for achieving long-range activation of genes in vivo.

Spatial and temporal boundaries in biological systems are sometimes formed by the conversion of a shallow gradient of an activator into a sharp increase in a species whose production is affected by the presence of the activator. A well-characterized example of such a threshold phenomenon is the activation of the *Drosophila hunchback* gene by the protein encoded by the *bicoid* gene, which is present in an anterior-posterior gradient in the early *Drosophila* embryo (6). Despite their fundamental importance, the underlying biochemical mechanisms of transcriptional threshold effects, such as the role of chromatin structure, have yet to be elucidated. We have investigated threshold phenomena in the activation of transcription with either naked DNA or chromatin templates in the simple model system with the GAL4-VP16 protein.

In these studies, we examined the effect of variation of the concentration of GAL4-VP16 on activation of transcription from the adenovirus E4 promoter containing one, two, or five GAL4 binding sites immediately upstream of the TATA box of the promoter (*14*). The templates were transcribed as either naked DNA or chromatin containing an average of one nucleosome per 200 bp and one molecule of histone H1 per nucleosome. As a control,

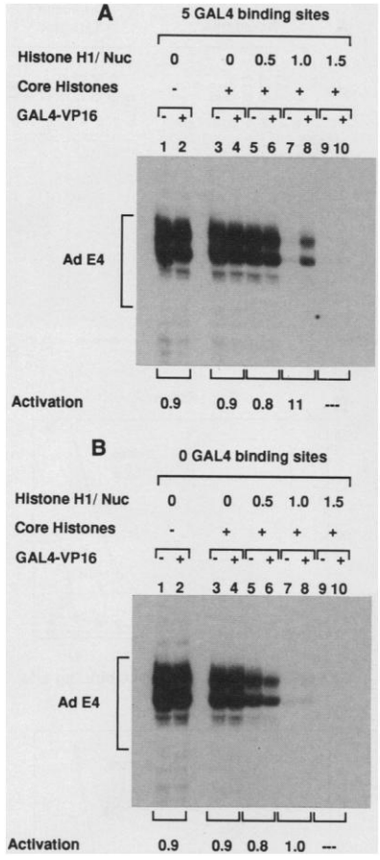

Fig. 1. Long-range activation of transcription in vitro by GAL4-VP16 with chromatin but not naked DNA templates. A mixture of a template plasmid and a pUC derivative (50 ng of DNA of each, as chromatin-containing core histone octamers only) was subjected to salt gradient dialysis with the indicated amounts of histone H1 in the presence or absence of GAL4-VP16 [the rationale for the use of pUC derivatives is described in (*13*)]. The resulting chromatin was transcribed in vitro with the soluble nuclear fraction (*12*). Lanes 1 and 2, naked DNA template (50 ng of template + 50 ng of pUC derivative); lanes 3 and 4, chromatin in the absence of H1; and lanes 5 to 10, chromatin with the specified amounts of H1 (given in molecules of H1 per nucleosome = 200 bp of DNA). The amounts of transcriptional activation mediated by GAL4-VP16 are shown at the bottom. The reverse transcription products of adenovirus E4 RNA are shown. (**A**) Transcription with a template DNA containing five GAL4 binding sites located 1300 bp upstream of the TATA box. A mixture of pG5I1300AE4T (which contains five tandem GAL4 sites upstream of the adenovirus E4 promoter) (*8*) and pUC119 were used. In this experiment, the GAL4 binding sites were located cis to the promoter (*13*). (**B**) Transcription with a control template that does not contain GAL4 binding sites. A mixture of pG0I1300AE4T and pUC119-G5 were transcribed. The pG0I1300AE4T is identical to pG5I1300AE4T, except that it does not contain GAL4 sites. The pUC119-G5 is identical to pUC119, except that it contains five tandem GAL4 binding sites in the polylinker region. In this experiment, the GAL4 binding sites were located trans to the promoter (*13*).

the corresponding template DNA that did not contain GAL4 recognition sequences was not responsive to GAL4-VP16 as either naked DNA or chromatin (*15*) (Fig. 1B). With the template containing five GAL4 binding sites, there was a sharp difference in the transcriptional properties of the naked DNA and chromatin templates (Fig. 2A). As the GAL4-VP16 concentration was gradually increased from 0 to 1.5 protomers per binding site with the naked DNA template, there was a progressive increase in transcription (Fig. 2A). In experiments performed with chromatin templates, however, there was a sharp nonlinear increase in transcription (Fig. 2A). As the amount of GAL4-VP16 in a series of reactions was gradually increased, there was an abrupt increase in RNA synthesis from the repressed chromatin templates but not the naked DNA templates, which were not subject to repression in the absence of activator. This effect was also dependent on the number of GAL4 binding sites in the promoter; it occurred with the chromatin template containing five GAL4 binding sites but not with variants containing one or two GAL4 recognition sites (Fig. 2B). Hence, we were able to reconstitute a transcriptional threshold effect in vitro, but the sharp increase in transcription was dependent on both multiple activator binding sites (more than two) in the promoter and packaging of the template into H1-containing chromatin.

Because the threshold activation occurred with the transcriptionally repressed chromatin templates but not the naked DNA templates, it appears that this effect involves antirepression by GAL4-VP16 rather than by true activation. As the GAL4-VP16 concentration was gradually increased with the chromatin templates, the promoter remained in a transcriptionally repressed state until there was approximately 0.5 protomer of activator per binding site. The mechanism by which the template was rendered transcriptionally competent is not yet known but may be clarified by further analysis of the fundamental process of antirepression. The threshold effect we observed in this work is different, however, from the "synergy" described by Carey *et al.* (*16*). In that study, transcription reactions were carried out in the presence of saturating amounts of GAL4-VP16 with a series of templates (as naked DNA) containing varying numbers of GAL4 binding sites, and the magnitude of GAL4-VP16–mediated activation was found to be more than linearly proportional to the number of GAL4 binding sites in the template to give the effect referred to as "synergy." When they varied the concentration of GAL4-VP16, a gradual increase in transcription was observed rather than a

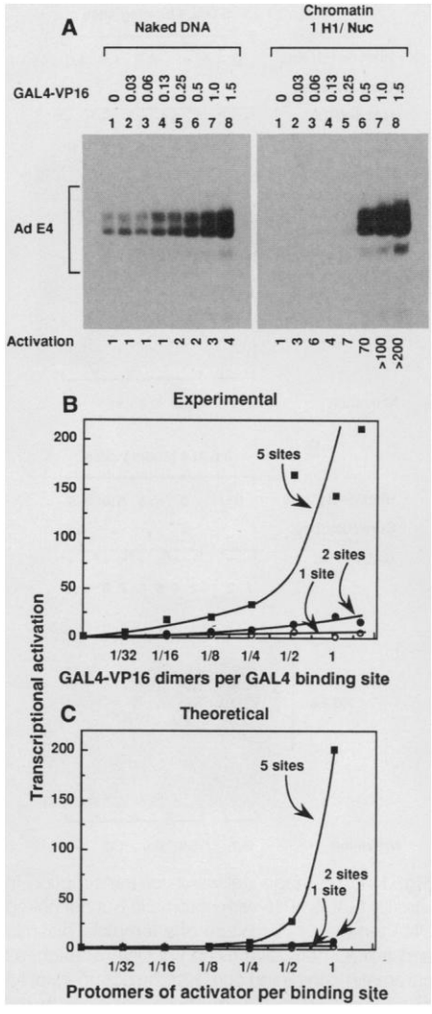

Fig. 2. Reconstitution of threshold phenomena with chromatin but not naked DNA templates. (**A**) Threshold activation of GAL4-VP16 with a chromatin template containing five GAL4 binding sites. Chromatin was reconstituted on pG_5E4T (which contains five GAL4 binding sites upstream of the adenovirus E4 minimal promoter) (*14*) with 1 molecule of H1 per nucleosome and variable amounts of GAL4-VP16, as indicated (as dimers of GAL4-VP16 per binding site). The chromatin templates (50 ng of DNA) were transcribed with the soluble nuclear fraction (*12*) in parallel with a series of naked DNA templates (50 ng) containing identical concentrations of GAL4-VP16. The amounts of transcriptional activation mediated by GAL4-VP16 are shown at the bottom. The reverse transcription products of adenovirus E4 RNA are indicated. When the concentration of GAL4-VP16 was increased to 2.0, 4.0, or 8.0 protomers per binding site, the amount of transcription was similar to that obtained with 1.5 protomers per binding site with both naked DNA and chromatin templates (*15*). Thus, the templates appeared to be saturated with GAL4-VP16 at 1.5 protomers per binding site. (**B**) Summary of transcriptional activation by GAL4-VP16 with chromatin templates containing one, two, or five GAL4 binding sites. The amount of transcriptional activation by GAL4-VP16 versus the number of GAL4-VP16 dimers per binding site is depicted. The data are the average of two to four independent experiments performed with chromatin templates containing one, two, or five GAL4 binding sites. (**C**) Theoretical plot of transcriptional activation versus protomers of activator per binding site. As described in the text and (*17*), transcriptional activation = $(1 - c + cf)^N$ (*17*). In the figure, this equation is shown for $N = 1$, 2, or 5 and for $f = 2.89$ (where, $f^5 = 201.6$, which corresponds to the average of the experimentally observed value for transcriptional activation by GAL4-VP16 with five GAL4 binding sites).

sharp threshold, which is consistent with our results with the naked DNA templates (Fig. 2A). Thus, the threshold effect and synergy are two distinct phenomena, and a transcriptionally repressed chromatin template is required to observe the threshold effect but not the synergy.

We also considered whether the observed threshold effect was due to cooperativity in the activation of transcription by GAL4-VP16. To address this issue, we compared the experimental data with the predicted activation profile that would be obtained if the action of each DNA-bound protomer of GAL4-VP16 were independent and additive in terms of free energy (that is, not cooperative). Under such conditions, the magnitude of transcriptional activation by an activator is described by the equation, $(1 - c + cf)^N$, where N is the number of factor binding sites on the template DNA, c is the number of protomers of activator per binding site ($0 \leq c \leq 1$; for simplicity, it was assumed that all activators were bound to the template), and f is the amount of activation mediated by a single factor (*17*). The theoretical activation curves for $N = 1$, 2, or 5 are presented in Fig. 2C.

Comparison of the experimental results (Fig. 2B) with the theoretical data (Fig. 2C) revealed that the experimentally observed amounts of activation were similar to those that were expected if each GAL4-VP16 protomer acted independently. With $N = 5$, a sharp increase in transcription at $c = 0.5$ was observed as predicted, whereas with $N = 1$ or 2, a threshold effect was neither observed nor predicted. Hence, in theory as well as in practice, the independent action of multiple activators can convert a shallow gradient of the activator into a sharp increase in transcription. Moreover, the similarity between the experimental and calculated data indicate that the transcriptional threshold effect shown does not necessarily result from cooperative activation of transcription by GAL4-VP16. These findings do not, however, disprove the possible existence of cooperativity in transcriptional activation by GAL4-VP16, but rather they demonstrate that transcriptional threshold phenomena can occur when multiple factors function independently to activate transcription. Furthermore, because transcriptional control regions, such as the promoter of the *Drosophila bicoid* gene (6), typically contain an array of binding sites for transcriptional activators (*3*), it is possible that the independent and additive action of the different activators may be responsible at least in part for threshold phenomena observed in vivo.

The long-term aim of these studies is to reconstitute and to analyze transcriptional regulation in vitro. A key aspect of the strategy that we have used is the attempt to recreate the natural setting of genes in the cell. As a first step in this process, we have been studying the transcription reaction with chromatin rather than naked DNA templates. To simplify the in vitro transcription analyses, we have performed these initial studies with a model system that is regulated by the GAL4-VP16 protein. Thus far, we have been successful in recreating long-distance activation of transcription and threshold effects. Study of naturally occurring transcriptional control regions and reconstitution of the higher order organization of genes should elucidate the pathway leading to activation of genes.

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12. Reconstitution of chromatin and in vitro transcription analysis were performed as described (*7*), and specific conditions were as follows. We de-

posited core histone octamers onto circular template DNA with polyglutamic acid at a histone to DNA mass ratio of 0.8, and we purified the resulting chromatin by sucrose gradient centrifugation. We estimated the concentration of the reconstituted chromatin by extraction of the samples with phenol-chloroform, and then by agarose gel electrophoresis and ethidium bromide staining with DNA standards. The purified chromatin was then subjected to salt gradient dialysis from 0.6 M to 0.05 M KCl in the absence or presence of GAL4-VP16 (*18*) with variable amounts of purified histone H1 from *Drosophila* embryos. Except where indicated, GAL4-VP16 was used at a concentration of 1.5 dimers per binding site. The samples were subjected to in vitro transcription analysis with the soluble nuclear fraction [prepared by extraction of nuclei with 0.1 M KCl instead of the previously recommended 0.4 M potassium glutamate (*19*)]. We assayed synthesis of RNA by primer extension analysis, and we quantitated synthesis by liquid scintillation counting of the appropriate gel slices. With different chromatin preparations, we observed some variation in the relative amounts of transcription with the naked DNA templates compared with the nucleosomal templates. This variation was due to inaccuracy in the determination of the concentration of the reconstituted chromatin after sucrose gradient purification and does not affect the conclusions of experiments based on the magnitude of activation by GAL4-VP16.

3. These experiments were designed to provide the GAL4 binding sites either cis or trans to the adenovirus E4 promoter to maintain a constant concentration of GAL4 recognition sites in each reaction. We accomplished this by mixing equimolar amounts of two plasmids in each reaction as follows: the pG5I1300AE4T (with the GAL4 binding sites in the cis configuration) was transcribed in the presence of pUC119, whereas pG0I1300AE4T (which does not contain GAL4 binding sites) was transcribed in the presence of pUC119-G5, which is identical to pUC119, except that it contains five GAL4 binding sites in the polylinker (in the trans configuration to the promoter). When the pG0I1300AE4T template was transcribed as naked DNA in the absence of the pUC119-G5 plasmid, we observed severalfold activation of transcription by GAL4-VP16, presumably because the excess GAL4-VP16 interacted with cryptic, low-affinity sites in the vicinity of the RNA start sites. In contrast, when the pG0I1300AE4T template was transcribed as naked DNA in the presence of the pUC119-G5 plasmid, GAL4-VP16 did not activate transcription (Fig. 1B). With the pG5I1300AE4T template as naked DNA, we did not observe transcriptional activation either with the template alone or with pUC119 (Fig. 1A), probably because the GAL4-VP16 was bound to the GAL4 recognition sequences located 1300 bp upstream of the RNA start site rather than the cryptic GAL4 binding sites. We have also performed transcription experiments with the pG5I1300AE4T template as chromatin in the presence of an equimolar amount of pUC119-G5 and have observed long-range activation of transcription by GAL4-VP16 (*20*).
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17. The equation, transcriptional activation = $(1 - c + cf)^N$, was derived as follows. First, to exclude either positive or negative cooperativity of binding, we assumed that the protomers of the activator bind randomly to the template according to a binomial distribution. Then, to enable each factor to activate transcription to its full potential, we assumed the action of each of the activators bound to a single template to be independent and additive in terms of free energy (that is, not cooperative). In this manner, the magnitude of transcription at a template with N factors bound relative to that without any factors bound would be f^N, where f is the amount of transcriptional activation by a single factor bound to a template. Next, we define c as protomers of activator per binding site, where $0 \leq c \leq 1$ (for simplicity, it is assumed that all protomers of activator are bound to the template). It follows that c is the probability that a factor is bound to a given site, whereas $(1 - c)$ is the probability that a factor is not bound to a particular site. Combining these terms, we can describe transcriptional activation as a function of c for $N = 3$, for example, by the following equation: transcriptional activation = $(1 - c)^3 + 3c(1 - c)^2 f + 3c^2(1 - c)f^2 + c^3 f^3$, which can be simplified to $[(1 - c) + cf]^3$. Generalization of this formula for N binding sites gives the equation presented above.
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Malignant Transformation by a Mutant of the IFN-Inducible dsRNA-Dependent Protein Kinase

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The double-stranded RNA-dependent protein kinase (dsRNA-PK) is thought to be a key mediator of the antiviral and antiproliferative effects of interferons (IFNs). Studies examining the physiological function of the kinase suggest that it participates in cell growth and differentiation by regulating protein synthesis. Autophosphorylation and consequent activation of dsRNA-PK in vitro and in vivo result in phosphorylation of the α subunit of eukaryotic initiation factor–2 (eIF-2) and inhibition of protein synthesis. Expression of a functionally defective mutant of human dsRNA-PK in NIH 3T3 cells resulted in malignant transformation, suggesting that dsRNA-PK may function as a suppressor of cell proliferation and tumorigenesis.

Interferons induce many proteins (*1*), the best characterized of which are the dsRNA-PK (*2*), the 2′-5′ oligoadenylate synthetase, and the Mx protein (*3*). The first two proteins are thought to participate in mediation of the antiviral and antiproliferative effects of IFNs and require dsRNA for their activation (*3*). The dsRNA-PK is a serine-threonine–specific protein kinase and displays two distinct kinase activities: (i) autophosphorylation and (ii) phosphorylation of the α subunit of the eukaryotic translation initiation factor eIF-2 (*4*), a modification that causes inhibition of protein synthesis (*5*).

Complementary DNAs for human (p68) and murine (p65) dsRNA-PKs have been cloned (*6*, *7*), and the corresponding proteins show 61% sequence identity (*7*). The yeast eIF-2 kinase GCN2 (*8*), which is thought to be the homolog of the mammalian dsRNA-PK, displays 38% identity with its human counterpart in the catalytic domains (*9*). Another mammalian eIF-2 kinase, the heme control repressor (HCR), also exhibits sequence identity (42%) with p68 (*10*). All four kinases contain the 11 kinase catalytic domains that are conserved among all protein kinases (*11*). However, p68, p65, and GCN2 exhibit further homology in the region between catalytic domains V and VI (*7*, *8*). This region contains an invariable sequence of six amino acids (Leu-Phe-Ile-Gln-Met-Glu; residues 361 to 366 in p68); in HCR, the sequence Leu-His-Ile-Gln-Met-Gln diverges at two positions. Although its significance is not clear, this common sequence has been suggested to modulate the kinase activity toward eIF-2α and the interaction with effector proteins (*9*).

To investigate the importance of dsRNA-PK in control of cell growth, we attempted to express wild-type (wt) kinase and a mutant kinase, lacking the six conserved amino acids between catalytic domains V and VI (*12*), in NIH 3T3 cells. We reasoned that the conserved sequence

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