The dangers of 'splicing and dicing': on the use of chimeric transcriptional activators *in vitro*

Chimeric transcription factors composed of heterologous DNA-binding and activation domains are often used to study the regulation of gene expression. The fact that such preparations also contain molecules in which only one of the two domains is functional is often overlooked, but a surprisingly small proportion of inactive domains could cause serious problems in the interpretation of quantitative data.

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Understanding the molecular mechanisms involved in the regulation of eukaryotic gene expression is a central problem in molecular biology. In most cases, regulation occurs at the level of transcription. Not only is the proper regulation of RNA biosynthesis central to development and the appropriate response of cells to metabolic signals, but it is now clear that many human cancers involve aberrant gene regulation. The transcription of almost all genes is controlled, at least in part, by gene-specific activators. This ubiquitous class of proteins can stimulate the transcription of target genes by 1000-fold or more in response to the appropriate signal. No one knows exactly how activators bring about this huge increase in the frequency of transcript synthesis, but some commonalities between the hundreds of activators characterized to date have become clear. Most importantly, activators must both contact specific DNA sequences in the target promoter and bind other transcription factors, including one or more proteins in the general transcription complex. Several years ago it was demonstrated that these activities reside on domains that are separable and functionally independent [1–4]. A particularly striking demonstration of this was the finding that chimeric transcription factors composed of DNA-binding domains from one protein and protein-interaction (activation) domains from another could activate transcription *in vitro* and to some extent *in vivo* [5,6]. Because the functions of the two domains are separable, however, they can also be inactivated separately. This can pose problems when mixtures of fully and partially active chimeric activators are used to study activator function *in vitro*.

Activators bind to DNA sites far from the transcription start site

The binding sites for activators and for the general transcription machinery are usually separated by hundreds or thousands of base pairs in natural promoters. Therefore, interactions between activators and the proteins responsible for transcription require 'looping' of the intervening DNA (Fig. 1) to bring the proteins close enough to bind. Although there is a general consensus in the transcription community that the looping model [7] is basically correct, it is now appreciated that the events involved are far more complex than this simple picture would suggest. For example, the stability of this looped complex may be affected by DNA-bending or wrapping proteins such as histones, providing yet another layer of control [8]. Even more problematic is that proteins known as coactivators are required to generate a response to activators *in vitro* [9–13]. One well characterized coactivator, the SUG1 protein of *Saccharomyces cerevisiae* [14,15], resides in a large (~1000 kD) complex that includes about 19 other proteins and has an ATPase activity that is required for high-level transcription [16] (R. Kornberg and colleagues, personal communication). Despite the complexity of the phenomenon, a central near-term goal in this area is relatively simple: it is to identify the protein–protein contacts between activators and other transcription factors, and the order in which they occur, so as to build up a picture of how a functional complex assembles.

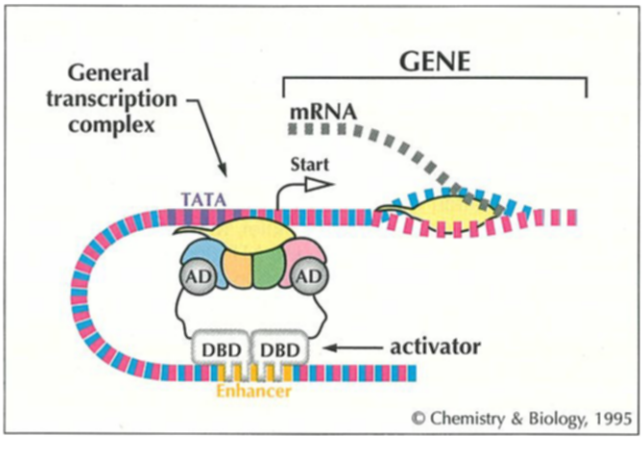

Fig. 1. Activation of transcription requires DNA looping. The activator binds to a specific DNA sequence (the enhancer) upstream of the transcription start site. For activation of transcription to occur, the DNA must form a 'loop' so that the activator is close enough to the transcription start site to form specific protein–protein contacts with the transcription complex bound there. After binding, DNA melting occurs and the transcription complex can initiate synthesis of mRNA.

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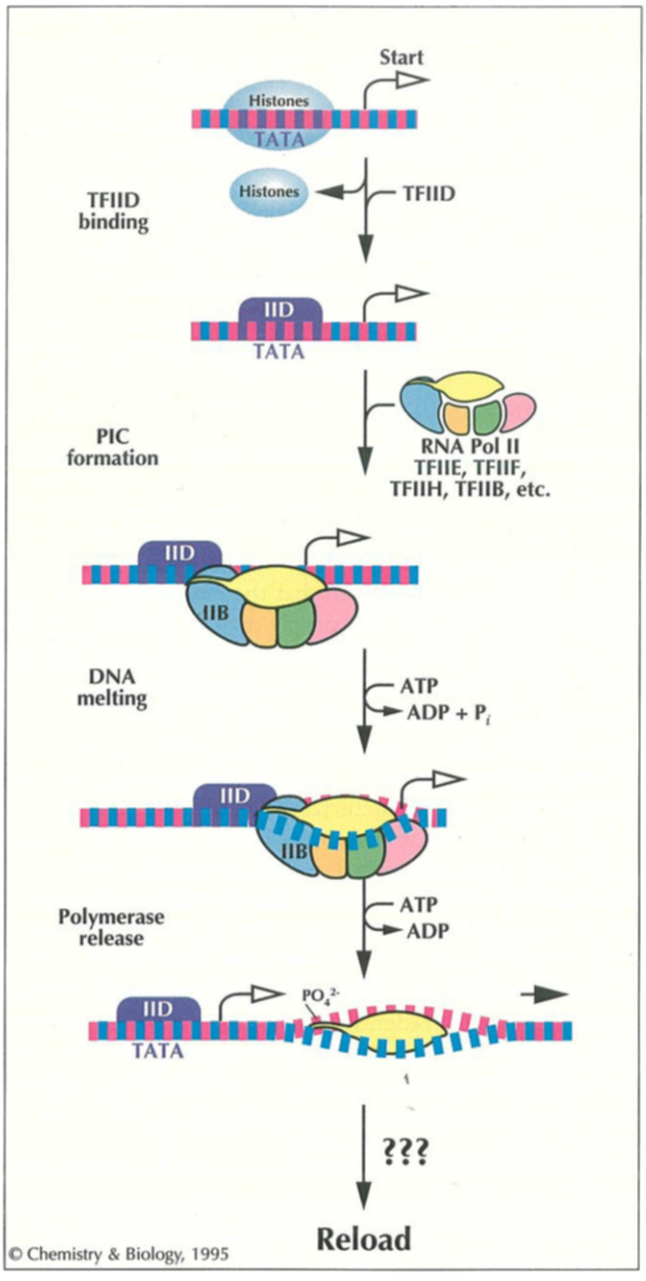

Fig. 2. Several steps in the formation of an active transcription complex might be accelerated by activator binding. The first step in complex formation may be binding of the transcription factor TFIID to the TATA region of the promoter, followed by formation of the pre-initiation complex (PIC) and ATP-dependent melting of the DNA helix, after which transcription can begin. Activators might also enhance release of the polymerase from the promoter, a step which results in phosphorylation of the polymerase.

Studying activator function

It is generally assumed that activators (in concert with the appropriate coactivators) accelerate an otherwise rate-limiting step in the transcription cycle. The rate-limiting step has not been identified, but possible target steps include association of RNA polymerase and various general transcription factors [17] with the promoter to form a pre-initiation complex (PIC), melting of the double helix in the promoter region to allow association of the polymerase with the coding strand, and release of the polymerase from the promoter to allow elongation of the transcript (Fig. 2). The most straightforward way to test these possibilities is to set up an *in vitro* system that will allow the rate or equilibrium constant of any particular step to be measured in the presence and absence of an activator. In practice, this biochemical approach is fraught with technical problems, not the least of which is that native activators tend to be large, poorly behaved proteins that are very difficult to purify in reasonable quantities. This unfortunate property has led to the almost exclusive use of artificial chimeric activators for detailed *in vitro* investigations. Particularly popular are species composed of the dimeric GAL4 DNA-binding domain (consisting of the amino-terminal ~100 residues of this 881 amino acid protein) [18,19] fused to activation domains from other transcription factors, such as the herpes simplex virus VP16 transactivator [20]. These chimeras are generally highly soluble and can be easily purified in milligram quantities.

When used appropriately, chimeric activators are useful tools. But as they are artificial constructs composed of completely unrelated domains, they represent extreme examples of multifunctional proteins. A central characteristic that distinguishes such chimeras from simple monofunctional proteins with a single globular domain is that purified preparations of chimeric activators are likely to contain significant quantities of partially active molecules, proteins that bind DNA but do not activate and *vice versa*. The consequences of this simple fact are potentially quite profound, yet are often ignored.

Composition of chimeric activator preparations

Proteins can suffer any number of debilitating reactions during the course of purification and storage, including denaturation and/or chemical modification of various amino-acid side chains [21]. Many of these processes can abolish activity without obviously changing the chromatographic properties of the protein or its electrophoretic behavior in denaturing gels. Thus, even preparations that appear as a single band on a gel may be composed of a heterogeneous collection of species, only some of which are active. Before the advent of recombinant-DNA technology, most proteins were purified on the basis of some biochemical activity, so that preparations could reasonably be assumed to have a high ratio of active to inactive proteins. Nowadays, however, many recombinant proteins are expressed at high levels in *Escherichia coli* and their purification is monitored by gel electrophoresis. These preparations may well contain a significant fraction of inactive molecules. This may not be a problem, even in some quantitative studies, so long as the fraction of active protein is known and the inactive protein does not interfere with the active species. This is often the case with monofunctional globular proteins.

Unfortunately, the situation is not so simple for multidomain proteins. Since the heterologous DNA-binding and activation domains do not interact with one another in a functional or structural sense, it is reasonable to

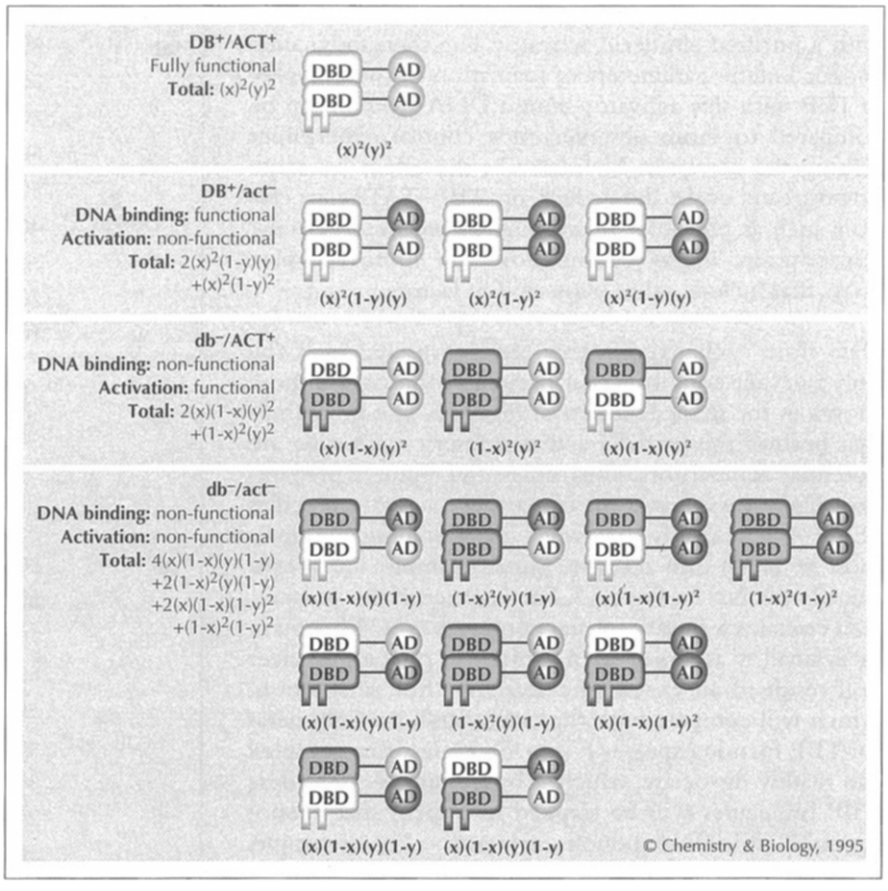

Fig. 3. The sixteen possible dimeric species present in a purified preparation of a dimeric activator of the type DBD–AD, divided into functional classes. The dark shaded domains are those that have been inactivated. We have calculated the relative representation of each species, using x and y to represent the fraction of active DNA-binding and activation domains, respectively. We have assumed that there is no resolution of the various species during purification, that both DNA-binding domains need to be active for the protein to bind to DNA, and that both activation domains are required for high-level transcriptional activation. The fraction of the total population that falls into each functional class, using these assumptions, is given. If only one of either type of domain is required for the function in question, the argument elaborated in the remainder of this article is quantitatively different, but the conclusions are essentially the same.

assume that a purified preparation of chimeric activator could contain, in addition to the completely active and inactive species, molecules that are competent for DNA binding but not activation, and molecules that cannot bind DNA, but contain a functional activation domain.

To understand the consequences of this potential heterogeneity, let us consider the possible composition of chimeric activator preparations. Figure 3 illustrates the sixteen dimeric species that may be contained in a preparation of a chimeric activator such as GAL4–VP16, which contains a DNA-binding domain (DBD) fused to an activation domain (AD). In our model, we have made the simplifying assumption that all of the molecules in the preparation are dimeric. If a third variable for dimerization activity were included it would alter the details of our discussion, but would not affect the fundamental conclusions.

The equations derived in Figure 3 can be used to calculate the relative amounts of the fully functional, DNA-binding and activation-competent species. In Figure 4, the expected representation of each species is shown for several values of the fraction of active DNA-binding (x) and activation (y) domains.

As can be seen from Figure 4, even modest levels of inactivation of the DNA-binding or activation domains can lead to a preparation in which the completely active species comprises a minority of the population; the curve in Figure 4a is very steep. More importantly, it is clear that for certain values of x and y, the two partially active species, DB^+/act^- and db^-/ACT^+, are present in excess over the fully functional chimera. For example, if x = y = 0.5, the ratio of DB^+/act^- dimers to DB^+/ACT^+ molecules is 3:1. Obviously, this can have important consequences for the number of productive complexes formed, because of competition. This reduction of signal to noise would not be serious if the unproductive complexes were functionally silent. But, as we illustrate below, the presence of such species may well distort our whole picture of the activation process.

The assembly of the general transcription complex

One popular model for the mechanism of action of activators is that they bind stably to the enhancer and accelerate the assembly of, and/or increase the stability of, the PIC. Since many biochemical pathways are regulated at an early step, it is particularly appealing to imagine that the activator facilitates binding of the general transcription factor TFIID to the TATA region found in most promoters, as this sequence-specific DNA-binding event is thought to be the first step in the formation of the PIC. This model is consistent with the observation that the ADs of several activators bind the TATA-binding protein (TBP) component of TFIID *in vitro* [22–24]. Therefore, there has been considerable interest in probing the effect of activators on the promoter-binding properties of TBP [25,26].

In theory, this question can be explored *in vitro* in a straightforward manner. A DNA fragment containing

both an enhancer and a TATA box would be saturated with a purified chimeric activator. The thermodynamic and/or kinetic parameters of formation of the complex of TBP with this activator-bound DNA would then be compared to those observed in a control experiment lacking the activator. Alternatively, the effect on some downstream event dependent on TBP–TATA association, such as promoter unwinding or promoter-specific transcription, might be monitored in more complex assays that include other transcription factors.

Data from such experiments are interpreted as if the only relevant equilibria that occur in solution are those shown in the shaded section of Figure 5. The problem is that both of the partially active chimeric molecules are potential competitors. Thus, unless the chimera preparation has a very low level of partially active molecules (x = y = 1, or nearly so), several other binding equilibria must be taken into account. Some examples are shown outside the box in Figure 5. For instance, if the preparation contains a significant fraction of db^-/ACT^+ protein (x is small, y is large), then saturation of the enhancer will result in an excess of active ADs free in solution, which will compete with the enhancer-bound chimeras for TBP, forming species F (Fig. 5). Unless this complex can readily dissociate, which is by no means clear, these TBP molecules will be trapped in a form that cannot respond to DNA-bound activator. For example, complex F might first encounter the DB+/ACT+ chimera–DNA complex B to form complex G. This complex would be non-productive, and for this promoter to become functional one of two dissociation events would be necessary. Dissociation of TBP from the db^-/ACT^+ chimera might occur, giving complex C; alternatively, TBP could dissociate from the TATA box, returning to complex B. Since many TBP–TATA complexes have very long half-lives [27], this exchange might not occur on the time scale of the experiment.

On the other hand, consider the situation in which a preparation containing a large amount of DB^+/act^- protein is employed (x is large, y is small). In this case, much of the DNA will be bound by an activator that cannot interact with TBP (complex E in Fig. 5). For example, if x = 0.8 and y = 0.3, then only 5.8 % of the DNAs will be occupied with chimeras capable of interacting with TBP. If the investigator assumes that the solution contains a homogeneous population of DNA complexes, all of which are occupied by fully functional activators, even a large effect of the presence of the activator on the properties of the TBP–TATA complex D could be missed. Because of these interfering side reactions it would be almost impossible to accurately measure the effect of the bound activator on the kinetic association and dissociation rates of the TBP–TATA complex or its effect on thermodynamic equilibria.

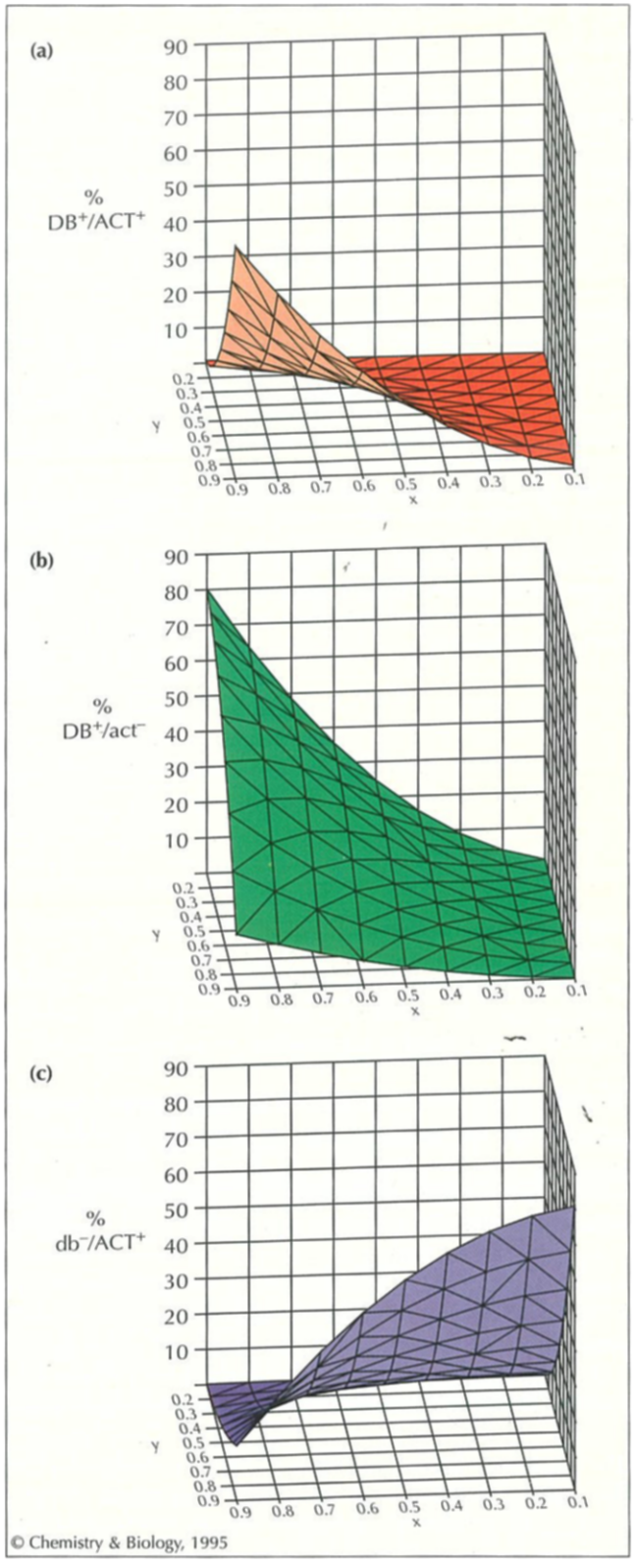

Fig. 4. Percentages of fully and partially active dimeric molecules in a dimeric activator population. **(a)** Fully active; **(b)** DB^+/act^-; **(c)** db^-/ACT^+. We have assumed for the purposes of these graphs that x and y are less than 0.9.

Chimeric activators in transcription reactions *in vitro*

When probing for effects on TBP–TATA interactions, one is asking that the chimeric activator have two specific activities, DNA binding and TBP binding. Thus the fraction of partially active molecules can in theory be determined experimentally, and by using the appropriate

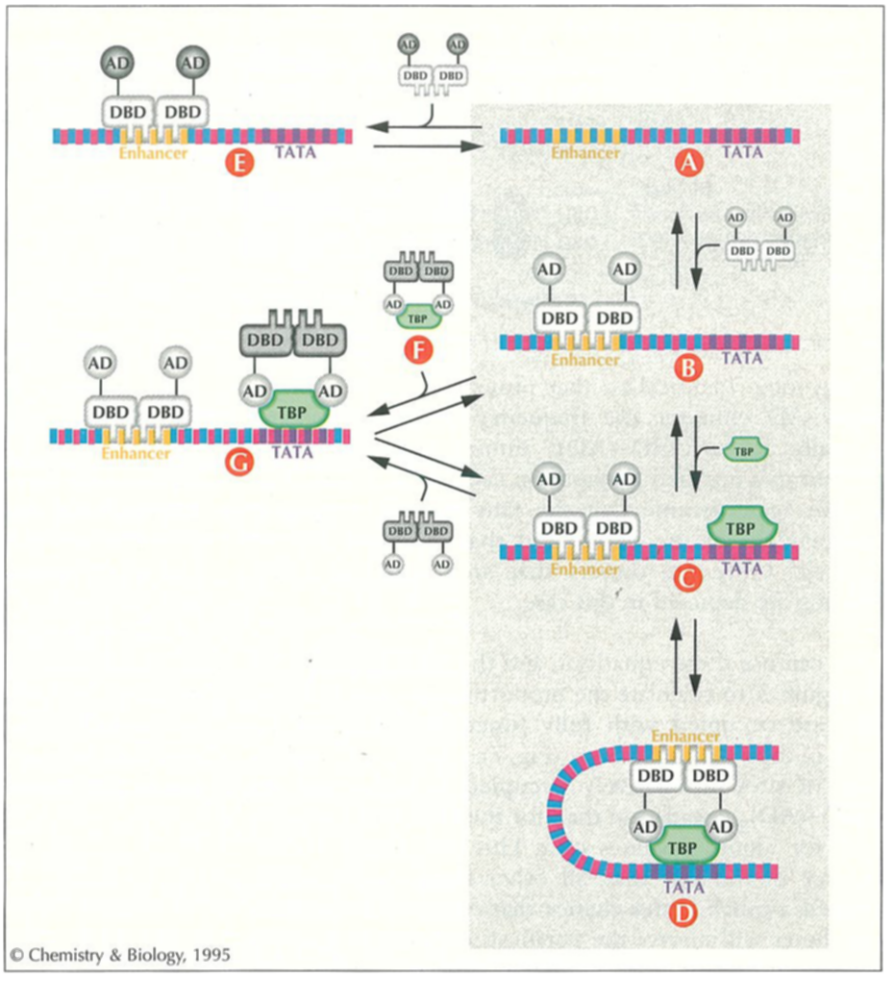

Fig. 5. Some of the equilibria that will occur when TATA-binding protein (TBP) is mixed with an excess of a chimeric activator and DNA containing an enhancer and a TATA region. The shaded box contains the binding events that are ideally the subject of the experiment. However, the events outside the box, as well as other equilibria not shown, must be taken into account if using an activator preparation that contains partially active species. DB^+/act^- proteins would compete for the DNA, forming complex E. Proteins that are db^-/ACT^+ would compete for TBP, forming complex F, which could then compete for complex B to form complex G. Dark shaded domains are inactive.

affinity-purification protocols it may be possible to obtain a very high fraction of completely active molecules (see below). A far more difficult situation arises when chimeric activators are employed in transcription reactions *in vitro*. Since it is unknown how an AD activates transcription, it is impossible to rigorously assay the fraction of ACT^+ species in a purified chimera preparation. Even if a preparation is 90 % active for TBP binding (or binding to any particular transcription factor), it is not clear that this means that 90 % of the molecules are ACT^+. The inability to know the fraction of ACT^+ molecules in a preparation has significant practical consequences in the interpretation of *in vitro* transcription experiments.

The *in vitro* experiments used to probe the molecular basis of the phenomenon of 'synergistic activation' are a case in point. The observation is that genes containing, for example, two activator binding sites in the promoter are often expressed at a level much more than two-fold higher than a gene with a single enhancer [28–33]. The molecular basis of this phenomenon is a very important issue in eukaryotic gene regulation, since most of the promoters contain binding sites for more than one activator. One model that is frequently invoked (the 'multiple-contact' model) is that many specific interactions between the activator and the general transcription apparatus must be fulfilled in order to achieve high-level transcription, and that these cannot be saturated by a single activator dimer [29,34]. An alternative explanation would be that the activator does not saturate a single upstream site, but when more than one site is present cooperative DNA binding results in efficient occupation of the enhancer. In this view, only a single activator dimer would be required for high-level transcription [35], but binding is enhanced by the presence of other dimers.

One imaginative *in vitro* experiment designed to distinguish between these possibilities employed chimeric GAL4–VP16 activators that contained either one, two or four ADs fused to a single DBD [36]. The number of ADs delivered to the target promoter could thus be varied without changing the number of activator binding sites. In this system, if synergistic effects were observed they could not be ascribed to cooperative DNA binding. It was found that a GAL4–VP16 dimer containing a total of four ADs activated transcription about five times as well as the standard dimeric GAL4–VP16 chimera (two ADs) when an excess of the activator was added to an *in vitro* transcription system — a synergistic effect, apparently giving strong support to the multiple-contact model. But consideration of the potential effects of partially active chimeras in such an experiment suggests that another interpretation of these data is possible.

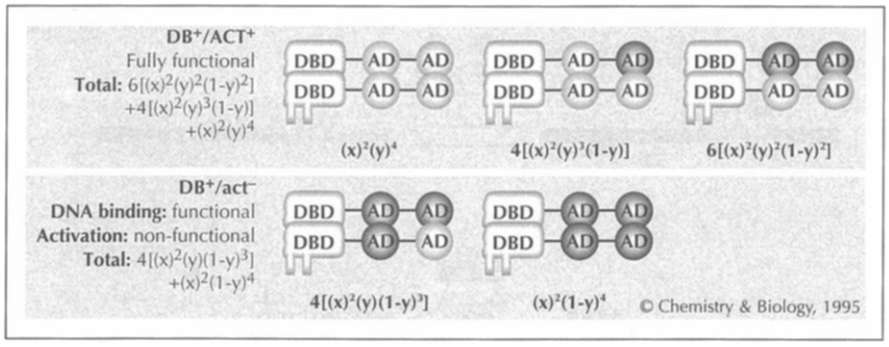

Fig. 6. The five possible species competent for DNA-binding present in a purified preparation of a dimeric DBD–$(AD)_2$-type activator, divided into functional classes. The dark shaded domains are those that have been inactivated. The relative representation of each species is shown below the chimera, using x and y to represent the fraction of active DNA-binding and activation domains, respectively. We have assumed that there is no resolution of the various species during purification.

Using logic identical to that presented for the simple DBD–AD chimera, the frequency of the active and partially active DBD–$(AD)_2$ dimers expected to be present in a purified preparation can be calculated (Fig. 6). We again assume that the fate of each domain is independent of the others and that all molecules are dimeric. Only the dimers that are active for DNA binding are depicted in this case.

One can use these equations, and the equations derived in Figure 3, to calculate the proportion of enhancer sites that are occupied with fully functional chimeras for each of the two populations (Fig. 7). Clearly, the proportion of sites productively occupied is higher for the DBD–$(AD)_2$ population than for the DBD–AD population for identical values of y. This is a simple consequence of the fact that, all other things being equal, there is a much better chance that two out of four ADs per dimer will survive the purification protocol in active form than there is that two of two ADs will retain activity. The two species have the same number of DNA-binding domains, however, so the chances that the proteins will suffer loss of enhancer-binding activity are identical. Thus, the DBD–AD population should have a greater percentage of DB^+/act^- molecules than the DBD–$(AD)_2$ population. This is of central importance if an excess of activator over DNA is employed, which is almost always the case.

To compare the numbers more directly, we have plotted the ratio of DBD–$(AD)_2$ DB^+/ACT^+ molecules to DBD–AD DB^+/ACT^+ molecules for various values of y (Fig. 8). It is clear that DBD–$(AD)_2$ chimeras can produce a much higher content of productively occupied templates for some values of y. For example, if y = 0.2, there is a 4.5-fold difference between DBD–$(AD)_2$ and DBD–AD, close to the experimental result of 5-fold increased activation.

It is, indeed, quite possible that the conclusions originally drawn from this experiment are correct. If the DB^+/act^- species comprises a small fraction of both the DBD–AD and DBD–$(AD)_2$ preparations (the value of y is high in each case), then the published interpretation is correct. The important point, however, is that the value of y is unknowable, since there is no assay to distinguish active from inactive ADs. Therefore, it is impossible to completely rule out 'trivial' interpretations of assays using novel chimeric activators. This experiment alone should not convince us that the multiple-contact model is valid.

Solutions for the real world

Although we cannot tell, at present, whether the use of chimeric activators has led to significant misunderstandings of the molecular interactions involved in transcription, the problem is well worth worrying about. Only two specific examples have been discussed here, but in general all quantitative experiments will suffer from the sort of problems described above. The composition of purified preparations of chimeric activators is almost never reported, leading one to assume that most investigators are unaware of the potential severity of the problem.

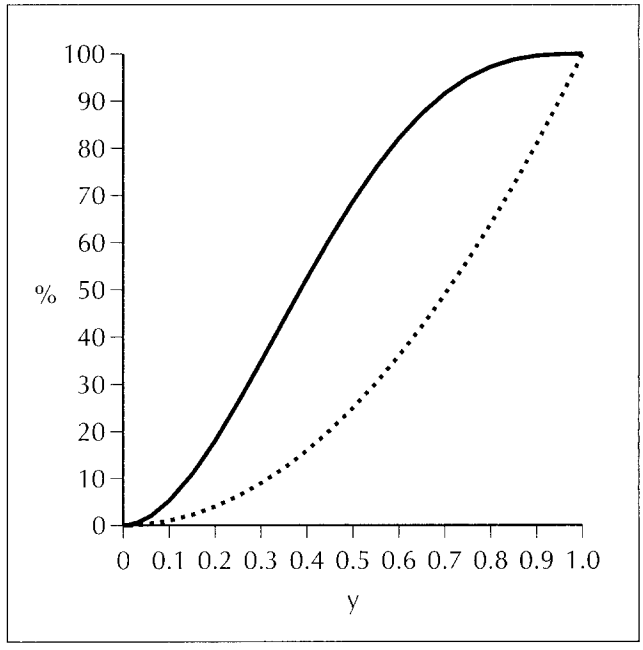

Fig. 7. Percentage of enhancer sites occupied by DB^+/ACT^+ species for DBD–$(AD)_2$ populations (solid line) compared to DBD–AD populations (dotted line). The graphs are calculated by dividing the term for DB^+/ACT^+ by the total number of DNA-binding species (DB^+/ACT^+ + DB^+/act^-). The value of x (the proportion of molecules that can bind DNA) does not affect the graph, since molecules that do not bind DNA are irrelevant to this calculation. If we make the reasonable assumption that the activation-deficient and activation-competent chimeras bind the enhancer with the same affinity, the ratio of DNA substrates occupied with DB^+/ACT^+ molecules to those with DB^+/act^- chimeras will directly reflect their representation in the protein population.

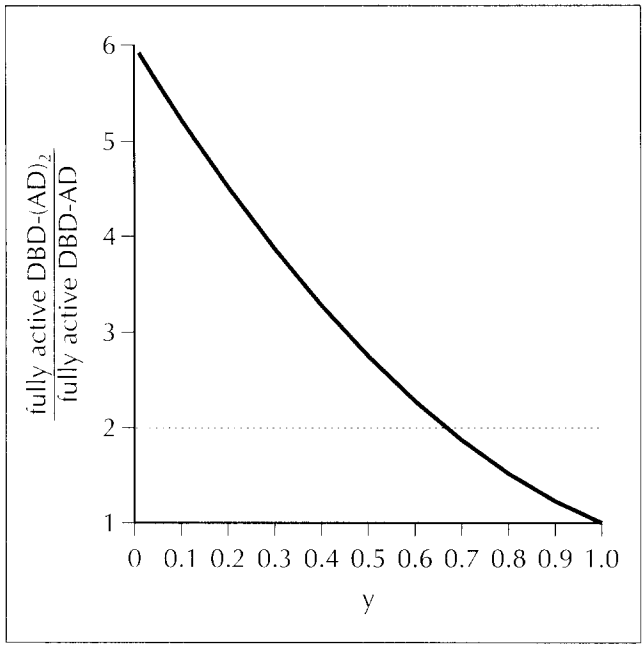

Fig. 8. Ratio of the fully active fraction of the DBD-$(AD)_2$ population to the fully active fraction of the DBD-AD population over a range of values for y (0.01 to 1.0). The dotted line shows the level above which activation is considered 'synergistic'.

How likely is it that these problems are real? It is, after all, conceivable that preparations of chimeric activators have exceedingly low levels of partially active species. We consider this unlikely. In our hands only 3–20 % of the molecules in a preparation of the commonly employed GAL4–VP16 chimera are active for both DNA and TBP binding, depending on the conditions employed and the age of the preparation (D. Fancy and T.K., unpublished observations). These values may or may not prove to be typical for other chimeric activators. It is generally straightforward to measure the fraction of DBD^+ molecules by titrating the appropriate labeled DNA with a limiting amount of chimera at a concentration well above the K_D of the complex. Determining the fraction of active ADs is harder, and sometimes impossible. If the assay in which the chimera is to be employed requires a discrete, measurable activity for the AD, such as TBP binding, then the fraction of active molecules can be determined by some assay that accurately reflects the protein–protein interaction, for example a supershift of the chimera–DNA complex upon addition of TBP in the presence of a large excess of competitor proteins. But it is not clear to us how one would measure the fraction of ADs truly competent to activate transcription.

If it is found that partially active molecules constitute a large enough fraction of the chimera population to complicate the planned experiment, then the completely active species must be purified. The most obvious way to do this would be to incorporate the appropriate affinity chromatography steps in the purification protocol, such as sequence specific DNA affinity chromatography. The problem with this approach is that it would limit the amount of chimeric activator that could be purified conveniently. Another possibility is to employ *in vitro* transcription/translation to produce small amounts of protein directly. We have found that certain GAL4 derivatives produced in this manner have much higher activities than the corresponding species purified from *E. coli* (K. Melchers and S.A.J., unpublished observations). The disadvantage of this method is that the activator is contaminated with many other proteins and, again, only modest quantities can be obtained. It is our hope that, once the significance of this problem is appreciated, more imaginative and practical solutions will be invented.

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