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# Transcriptional Enhancers: Intelligent Enhanceosomes or Flexible Billboards?

**David N. Arnosti[1,2]* and Meghana M. Kulkarni[3]**

[1]Department of Biochemistry and Molecular Biology, Michigan State University, East Lansing, Michigan 48824-1319
[2]Program in Genetics, Michigan State University, East Lansing, Michigan 48824-1319
[3]Department of Genetics, Harvard Medical School/HHMI, 77 Avenue Louis Pasteur, Boston, Massachusetts 02115-6092

**Abstract** In higher eukaryotes, transcriptional enhancers play critical roles in the integration of cellular signaling information, but apart from a few well-studied model enhancers, we lack a general picture of transcriptional information processing by most enhancers. Here we discuss recent studies that have provided fresh insights on information processing that occurs on enhancers, and propose that in addition to the highly cooperative and coordinate action of ''enhanceosomes'', a less integrative, but more flexible form of information processing is mediated by information display or ''billboard'' enhancers. Application of these models has important ramifications not only for the biochemical analysis of transcription, but also for the wider fields of bioinformatics and evolutionary biology. J. Cell. Biochem. 94: 890–898, 2005. 

**Key words:** enhancer; transcription; enhanceosome; Cis-regulatory element

## ENHANCERS: A DEVELOPING PICTURE

Enhancers are classically defined as *cis*-acting DNA regulatory elements that stimulate transcription, independent of their position and orientation with respect to the transcriptional initiation site [Banerji et al., 1981]. This stimulatory role distinguishes enhancers from basal promoter elements, sequences that bind the basal transcription machinery and determine the site of transcriptional initiation [Smale and Kadonaga, 2003]. The original description of enhancers emphasized activation, however, many cellular enhancers are found to interact with both transcriptional activators and repressors, thus a complete description of these regulatory elements must necessarily take into account both activation and repression [Barolo and Posakony, 2002]. Since their identification in the early 1980s, transcriptional enhancers have been the subjects of numerous studies because of their ubiquitous roles in higher eukaryotic gene regulation. Despite this interest, we lack a detailed picture of the activity and structure of most cellular enhancers.

As discussed here, recent studies have provided fresh insights on the type of information processing that occurs on enhancers, ranging from highly cooperative and coordinate action of "enhanceosomes," which process the information of bound transcription factors and give unitary outputs, to the flexible information display characteristic of "billboard" enhancers. A billboard provides a display of symbols, but that information is processed into a message only by the active perception of an observer. Similarly, a billboard enhancer displays potential transcriptional information that is processed by interaction with the basal transcriptional machinery, and exact positioning of bound transcription factors plays a less important role than with an enhanceosome.

This review is dedicated to Prof. Walter Schaffner on the occasion of his 60th birthday.

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*Correspondence to: David N. Arnosti, 413 Biochemistry, Michigan State University, East Lansing, MI 48824-1319. E-mail: arnosti@msu.edu

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In a sort of molecular shorthand, enhancers are usually defined solely by DNA sequences, but in reality these elements function as nucleoprotein complexes, which modify chromatin structure and interact with components of the basal machinery. Enhancer associated proteins can bind in sequence-specific or sequence-nonspecific manners, as well as indirectly through protein–protein contacts. Typical enhancers span 200–1,000 bp, and bind to dozens of sequence-specific proteins upwards [Carroll et al., 2001; Davidson, 2001]. Enhancers are suggested to affect gene expression in either a binary, "stochastic" manner, or in a continuous, "rheostatic" manner. In the former case, the transcriptional rate of a gene has two possible states, either "off" or "on," and the activity of an enhancer shifts the balance to the active state [Blackwood and Kadonaga, 1998]. Compelling evidence for this sort of model comes from studies by Whitelaw and colleagues. Here, the authors found in a transgenic mouse model that a globin enhancer allowed a greater percentage of erythrocytes to express a *lacZ* reporter gene without changing expression levels of the activated gene [Sutherland et al., 1997; reviewed in Fiering et al., 2000]. The "rheostat" model suggests that enhancers might also quantitatively regulate transcription rates through a continuous spectrum, depending on the amount and nature of bound factors. Direct manipulation of transcription factor concentrations on synthetic enhancers supports this notion [Rossi et al., 2000; Biggar and Crabtree, 2001]. Sequence changes affecting individual factor binding sites within an enhancer can also quantitatively affect the strength of the activation, and such mutations are suggested to underlie many quantitative differences between expression of alleles in a population [Knight, 2004].

Enhancers can thus generate a step function or a continually variable output, and this activity is clearly a function of the presence of various regulatory proteins in the nucleus. However, it has remained unclear at what level this information about nuclear transcription factors is directly processed by enhancers. To what extent do the presence of and arrangement of binding sites in enhancers facilitate the workings of transcriptional regulatory proteins? In effect, is the arrangement of binding sites a critical element in information processing? As we discuss below, the answer to this question has important ramifications not only for the biochemical analysis of transcription, but also for the wider fields of bioinformatics and evolutionary biology.

At a general level, there are two basic structural features that apply across the spectrum of enhancers found in multicellular organisms: factor binding sites are typically within 100 kbp of a gene, and they are usually clustered. (enhancers are generally linked in *cis* to their targets, although examples of *trans* regulation—generally via pairing of sister chromosomes—are documented [Duncan, 2002; Kennison and Southworth, 2002]). The clustering of binding sites seems to be a common feature of enhancers; single factor binding sites are in general insufficient to drive gene expression, a mechanistic feature that appears to prevent unwanted activation by randomly occurring binding sites. Instead, multiple, clustered sites are a hallmark of enhancers, and presumably reflect the synergy required for important, but weak, protein–protein interactions to occur [Dröge and Müller-Hill, 2001].

## TWO MODELS: ENHANCEOSOMES AND BILLBOARDS

Beyond these general features, is there importance to the exact nature of the transcription factor binding sites situated within transcriptional enhancers? Two general models have been proposed to describe the nature of how binding sites might work together (Fig. 1). The "enhanceosome" model proposes that the DNA sequences of the enhancer operate as a scaffold to form a unified nucleoprotein complex (hence the "-some" designation). The enhancesome features a high degree of cooperativity between enhancer-bound proteins, such that alterations in individual binding sites can have drastic effects on enhancer output. The function of the enhanceosome is thus more than the sum of individual factor contributions, but emerges from a network of interactions [Thanos and Maniatis, 1995; Merika and Thanos, 2001]. In contrast, the billboard model suggests that binding sites are flexibly disposed, because the proteins bound to the enhancer do not operate as a single unit, with a strictly defined overall architecture, but rather as an ensemble of separately acting factors or small groups of factors that independently interact with their targets, for example the basal transcriptional machinery [Arnosti,

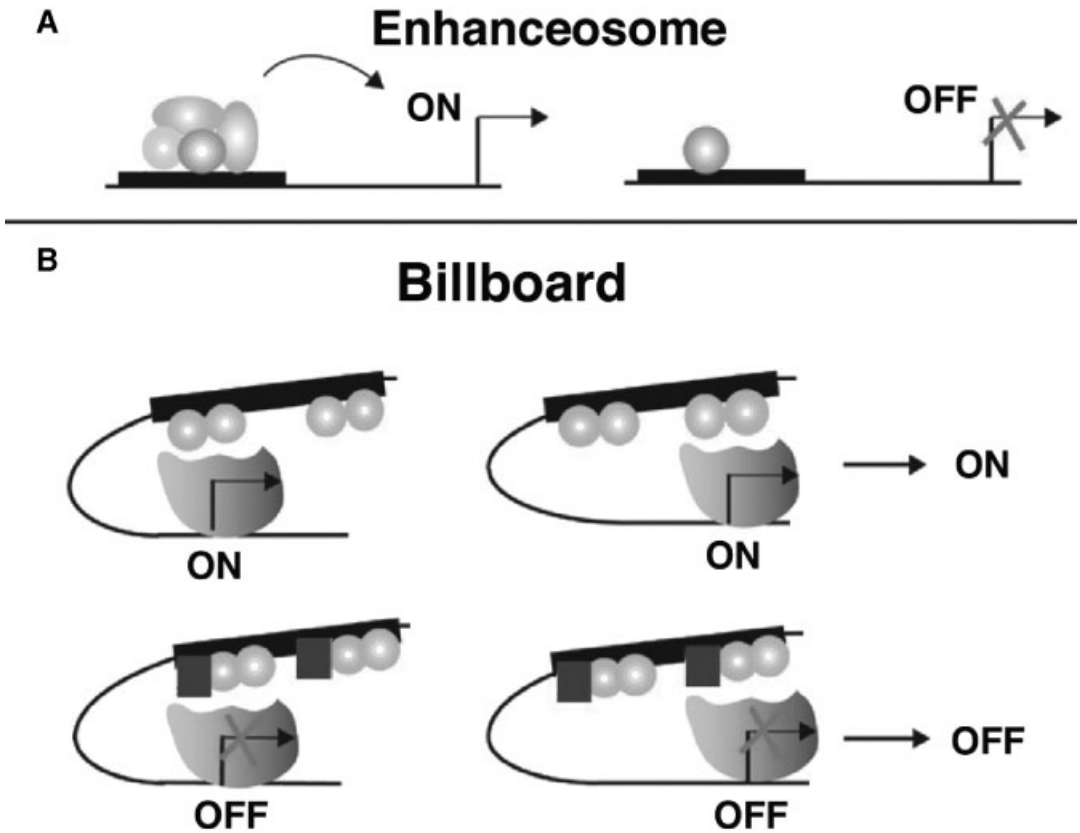


**Fig. 1.** Two models of enhancer action. **A**: In the Enhanceosome model, the binding sites within the enhancer allow for a highly cooperative assembly of transcription factors (colored ovals), leading to gene activation. Disruption or displacment of a single binding site, or the absence of one regulatory protein, causes the element to be inactive. **B**: In the Billboard model, the enhancer contains multiple functional units that are able to independently regulate gene expression. Above, activators (colored ovals) located in separate portions of the enhancer are ''sampled'' by the basal machinery, and the integration of such interactions results in total gene output. Below, regulation by short-range repressors. Individual subelements of the enhancer are repressed by the action of short-range repressors (red squares) located near each cluster of activators.

2003; Kulkarni and Arnosti, 2003]. The term billboard emphasizes that the enhancer is a unit of information display, whereby elements are arranged for interpretation by the basal machinery, which plays an active role in deciphering the message, much as an observer reads a billboard. Just as different arrangements of words can convey similar meanings in advertisements, so can the elements of a billboard enhancer be redistributed without destroying the overall function. A closer examination of these models will reveal how they can guide our analysis of *cis* regulatory information in the future.

## Enhanceosomes

The mammalian interferon beta *cis* regulatory element is arguably the best-studied example of an enhanceosome. This small 65 bp enhancer binds to Rel family NF-κB proteins, the ATF-2/c-jun heterodimer, and proteins of the interferon regulatory factor (IRF) family [Merika and Thanos, 2001]. HMG-I(Y) binding proteins play what is termed an architectural role, facilitating the loading of the other factors by DNA bending [Yie et al., 1999]. Single point mutations that move or remove the binding site of individual proteins disable this enhancer, suggesting that an overall protein/DNA superstructure is critical [Thanos and Maniatis, 1995]. Negative regulatory elements can also exhibit the cooperative assembly properties of enhanceosomes. Control elements of the *zen*, *dpp*, and *tld* genes in *Drosophila* that mediate repression in the ventral regions of the early embryo feature Dorsal protein binding sites with closely linked AT-rich sequences that bind to Cut and Dri proteins [Valentine et al., 1998]. In this context Dorsal mediates repression by recruiting the Groucho corepressor; however,

minor changes in spacing between the Dorsal and flanking sites disrupts the repression activity of this regulatory module, exposing the default activity of Dorsal as an activator [Cai et al., 1996].

Rel family proteins interact with a number of regulatory elements that seem to exhibit the properties of enhanceosomes, perhaps consistent with these proteins' roles in inflammation and infection, where rapidly inducible transcriptional responses are desirable. A recent study of immune regulated genes in *Drosophila* suggests that these genes are activated by the combined activity of the Rel family and GATA factors, and that the conserved positions and orientations of the two binding sites for the two factors comprise a regulatory "grammar" that make it possible to identify such elements in genomic sequences [Senger et al., 2004]. In addition, a common set of features was found in the enhancers of *Drosophila* neuroectoderm-specific genes controlled by the Rel family member Dorsal: a binding site for Dorsal, associated with one or two Twist activator sites within 20 bp, and a binding site related to the Dorsal interacting protein 3 (Dip3) factor 108-153 bp 3′ of the Twist site, with an apparent periodicity of 15 bp. The elements also feature a conserved Su(H) binding site [Erives and Levine, 2004]. Whether these neuroectoderm enhancers exhibit properties of enhanceosomes remains to be seen, but the overall structural conservation indicates that they might. In general, combinations of Rel family proteins such as NFAT or NF-κB appear frequently in a compilation of "composite elements" (CE's), binding site pairs that are found to frequently functionally interact in positive or negative fashions [Kel-Margoulis et al., 2002], suggesting that Rel proteins may be particularly prone to assemble into enhanceosome type complexes. The constrained organization of transcription factor binding sites indicates that these enhancers are likely to form highly ordered nucleoprotein complexes.

## Billboard Enhancers

Enhanceosomes may represent a subclass of genetic switches that are poised for hairtrigger activation, while other enhancers, for example those required for generating complex patterns of expression during development, may function via alternative mechanisms [Merika and Thanos, 2001; Struhl, 2001]. We have recently found that these other enhancers might be operating by a more flexible mechanism, representing an information display or billboard model of enhancer action. The key difference is that with a billboard enhancer, the entire element need not function as a cooperative unit, but rather as an ensemble of separate elements that can independently affect gene expression. The separate subelements would represent either single or smaller groups of binding sites that may function together—indeed, this model does not rule out enhanceosome-like subelements—but such cooperativity is not a necessity. Support for this notion was provided by our recent study of simple, defined genetic switch elements in *Drosophila*. Here, repressor and activator sites were juxtaposed on defined elements, and it was observed that the readout of a 200 bp element could be differentiated into at least two functionally distinct components. In the same nucleus, one group of transcription factors was actively repressed, while an adjacent group activated transcription of the reporter gene. The readout of this element is an integration of these two states, so that in nuclei where contrasting information was present, a weak activation of the gene was achieved, while strong activation was observed where both elements were active. These results indicate that the element, which exhibited the classical properties of an enhancer to act in a distance- and orientation-dependent manner, contains two distinct units with separate information content [Kulkarni and Arnosti, 2003]. Many endogenous enhancers appear to contain redundantly acting independent subelements. When these enhancers are active, independent interactions with targets of the transcriptional machinery would sum up to provide the total, integrated transcriptional output, suggesting that a billboard model, rather than an enhanceosome, might better describe these elements.

Both types of activities ascribed to enhancers, that is, modification of chromatin structure to permit binding of the transcriptional machinery, and direct recruiting of components of the basal machinery to the promoter, are compatible with the billboard model of enhancer action. For instance, factors bound to separate subelements might independently contact a given component of the RNA polymerase holoenzyme in sequential fashion, each contributing to complex stability during a given period of time.

Alternatively, subelements might independently recruit chromatin modifying or remodeling factors to favor the establishment of a favorable chromatin configuration.

## COMPUTATION OF TRANSCRIPTIONAL INPUTS

These two models have different predictions with regard to the role of enhancer sequences in actual computational activities, that is, the processing and transformation of information represented by a collection of transcription factors. An example of such a transformation would be the building of a unique protein surface that would not normally form in solution, through the stabilizing action of the DNA scaffold [Dröge and Müller-Hill, 2001]. Regardless of the exact mechanism of signal processing, the "enhancer as computer" analogy is rife in contemporary studies of developmentally regulated enhancers [Ghazi and VijayRaghavan, 2000]. Enhancer sequences have been described as the "hardwiring" that responds to and channels different inputs [Davidson, 2001]. This comparison appears to have merit; the concentrations of distinct transcription factors in the nucleus, which change in response to developmental and environmental signals, represent the "input." The enhancer would then send integrated signal outputs, instructing the basal machinery to transcribe a gene at a specific rate. A particularly well-studied example of this process comes from the regulatory modules of the sea urchin *S. purpuratus endo16* gene. This gene is developmentally regulated by a 2.3 kbp *cis* element that can be subdivided into six distinct elements. The transcriptional activity generated by combinations of these elements has been empirically measured with respect to the quantitative and qualitative outputs during development. The output of the different modules can be quantitatively modeled using Boolean operators to represent that action of particular portions of the upstream regulatory region [Yuh et al., 1998; Davidson et al., 2002].

To what extent do these models describe what is actually occurring on DNA elements? The scaffolding of protein complexes by enhanceosomes does in fact represents a type of information processing, because it allows the building of unique protein surfaces and complexes that are not achievable in solution at physiological concentrations. A beguiling picture thus emerges of the enhancer as a information-processing black box, with the basal promoter and machinery as passive recipients of such information. However, two lines of evidence suggest that interactions with the basal transcriptional machinery also play important roles in the decipherment of *cis* regulatory information. First, basal promoter regions are not always interchangeable, suggesting that the composition or conformation of basal machinery at these elements is informational [Li and Noll, 1994; Butler and Kadonaga, 2002]. Second, "billboard" type enhancers are capable of displaying multiple forms of transcriptional information, in some cases contradictory in nature, with opposing outputs by activators and repressors. An integration of these signals by interactions with the basal machinery is critical for establishing the output of these *cis* regulatory regions. By contrast, in an enhanceosome, a unity of output is achieved by specific contacts between the bound factors. In the case of the *zen* ventral repression element enhanceosome, the potential contradictory activation activity by the Dorsal protein is resolved by the careful positioning of flanking neighboring proteins that induce Dorsal to recruit a corepressor protein [Cai et al., 1996; Valentine et al., 1998]. The proportion of natural enhancers that resemble enhanceosomes or billboards is not known, but evolutionary studies give a clue.

## FLEXIBILITY AND REDUNDANCY

From an evolutionary point of view, the billboard model suggests a high degree of functional redundancy for individual binding sites or groups of sites. The billboard model makes two specific predictions that appear to fit observations of a number of natural enhancers: flexibility in binding site positioning and redundancy in function. Binding site flexibility results from independently acting factors being able to contact targets, whether they be the basal machinery or chromatin-modifying cofactors, from many different configurations. Redundancy, or apparent redundancy, stems from the enhancer being designed to provide correct quantitative outputs from the integrated readout of multiple subelements, each of which may provide the correct temporal and positional information.

Functional and evolutionary studies of enhancers from the *Drosophila even-skipped* (*eve*)

gene support predictions of flexible design. In contrast to the highly cooperative enhanceosome structures, it appears that *eve* enhancers have undergone considerable internal rearrangement over evolutionary time. Experimental manipulation of the *eve* stripe 2 enhancer has shown that it can tolerate a wide variety of changes in activator placement and identity [Arnosti et al., 1996]. Regarding evolutionary changes, comparison of sequences for the 500 bp *eve* stripe 2 enhancer revealed that many, but not all, of the characterized binding sites for activators and repressors were conserved between different species of *Drosophila* [Ludwig et al., 1998]. Furthermore, these enhancers from these diverse species were shown to possess conserved function when tested in *D. melanogaster*. Despite this conservation, however, a chimeric enhancer whose 5′ half was derived from *D. pseudoobscura* and 3′ was from *D. melanogaster* exhibited an aberrant pattern of expression, suggesting that over the ~30 million years that separate these two species, internal modifications had occurred that led to unique arrangements of binding sites on each element [Ludwig et al., 2000]. In light of the billboard model, one can speculate that the overall integrated output of negative and positive inputs from different portions of the enhancer remained constant, while relative contributions of individual subelements had changed. The force of natural selection would act on the net output of the entire enhancer, permitting a wider exploration of sequence space and a faster rate of drift than would be possible with enhanceosomes.

A billboard mechanism also suggests that enhancers might be constructed of independently acting, apparently redundant elements that sum up to provide the correct levels of gene readout. Indeed, redundancy is a commonly observed property of many developmentally regulated enhancers. The 900 bp enhancer that regulates *otd* expression in the *Drosophila* embryo contains a 186 bp Bicoid activated subelement and an 173 bp Bicoid-independent subelement, each of which produce the correct spatiotemporal patterns, but are weaker than the endogenous element. When joined together, they provide a wild-type level of gene expression [Gao and Finkelstein, 1998]. Similar subelements that combine to provide the correct quantitative expression levels have been noted with the SV40 enhancer, wherein the subelements were termed "enhanceons," and in *Drosophila* yolk enhancers [Ondek et al., 1988; Schaffner et al., 1988; Piano et al., 1999], reviewed in Arnosti [2003]. For what purpose might the organism utilize redundant elements to drive transcription? Perhaps it is misleading to term them redundant, because evolutionary pressure is probably at the level of overall output of a given regulatory sequence, and typically we measure responses in the laboratory setting only in a semiquantitative manner. A 10% change in enhancer activity might be missed in most laboratory assays, but may have large effects at the population level.

## PHYLOGENETIC COMPARISONS AND BIOINFORMATICS

The enhanceosome model has been useful in emphasizing the "all or nothing" switch-like operation of the element via highly cooperative protein–protein interactions between factors binding to an enhancer. Designating all enhancers as enhanceosomes, however, blurs a useful distinction between *cis* regulatory elements in general and those that exhibit unitary, cooperative action. The term enhanceosome has been applied indiscriminately to regulatory sequences that may not possess the high degree of cooperativity and single output potential that characterize classical enhanceosomes. In some cases, this may reflect a lack of understanding of the specific biochemical properties that an enhanceosome possesses, or alternatively, an implicit assumption that all enhancers have these properties. In the latter instance, it may come as a surprise how difficult it is to use phylogenetic analysis to characterize a *cis* regulatory "grammar," perhaps because the rules for billboard enhancer construction are less strict than those for an enhanceosome. Lack of phylogenetic conservation should not necessarily be taken to indicate that a noncoding region is not relevant for transcriptional regulation. A recent analysis compared the sequences of 30 well-studied enhancers containing 315 mapped binding sites within ~21 kbp of regulatory sequence from *D. melanogaster* to the corresponding sequences from *D. pseudoobscura* [Emberly et al., 2003]. About 50%–70% of the binding sites were found within conserved sequences, however, this rate of conservation is only slightly greater than that expected by chance. This result suggests that many of these

binding sites are dispensable, possibly because over the course of time, existing binding sites were replaced by newly evolved sites, consistent with the flexibility expected of a billboard type of enhancer. (An alternative explanation, which remains to be tested, is that perhaps many of the previously mapped sites were of little or no importance to start with).

The recently completed rat genome has been analyzed for putative conserved *cis* regulatory elements among rat, mouse, and human [Gibbs et al., 2004]. As authors of this study point out, there are 186 million predicted binding sites in the human genome for 109 transcription factors, but if one focuses on predicted binding sites conserved in all three species, the number is reduced to only 4 million, "a 44-fold increase in specificity." However, if the fluidity of regulatory sequences noted in the *Drosophila* comparison above also pertains to vertebrate sequences, this step does not necessarily represent an increase in specificity, but merely the discarding of most of the data.

For the purpose of population studies, the identification and comparison of *cis* regulatory sequences is of paramount importance, because many of the changes that relate to quantitative differences within populations and that lead to speciation are thought to involve alterations in regulatory, not protein-coding, regions [Tautz, 2000]. A deeper analysis of enhancer design is required to understand the *cis* regulatory grammar underlying most enhancers. At its most extreme, one might suggest that the only constraints on the placement of binding sites within a billboard enhancer are the two general ones noted above, namely, proximity to the gene to be regulated and a loose clustering. Yet functional studies of naturally occurring enhancer sequences that lack tight conservation of binding sites suggest that there is still some amount of information in the positioning of these elements. A key feature of enhancers of the *eve* gene is regulation by short-range repressors. The range of action of these proteins is very limited, so the exact positioning of corresponding binding sites can dictate the extent of repression. Tuning the sensitivity of a gene to these repressors can be achieved by changes that move binding sites ~30 bp [Hewitt et al., 1999]. We have recently identified a number of general principles that dictate short-range repressor effectiveness on defined regulatory elements, rules that constitute a grammar for these proteins. In addition to the exact placement of repressors with respect to activators, the number, affinity, and arrangement of binding sites also dictates repression effectiveness (Kulkarni and Arnosti, in press). While short-range repressors such as Giant and Knirps do not show the same tight constraints in binding site deployment as the proteins found on enhanceosomes, we found that overall rules concerning activator/repressor binding site stoichiometry were critical for dictating enhancer output. Importantly, we find that identical gene outputs are produced by more than one configuration of binding sites, but there are limits to how much the spacing or arrangement of sites can vary before regulatory output changes. By circumscribing the parameters affecting this group of regulatory proteins, we can more effectively search for conserved patterns of binding sites. This process may hold the key to identification and functional analysis of novel genomic sequences. Ultimately, we envision that population and speciation studies will be able to rely on bioinformatic tools that describe the information content of *cis* regulatory elements, and provide indications of evolutionary changes.

## UTILITY OF MODELS

The enhanceosome and billboard models represent two extremes of a continuum that probably describes most cellular enhancers; thus, subelements of a particular regulatory region might exhibit critical cooperative interactions between some of the factors, while other portions of the regulatory region are loosely structured. At what point would we designate a regulatory element, or portion thereof, an enhanceosome? Is there a critical threshold of cooperativity measured in effects on $K_d$, or inflexibility of binding site placement? There is probably no sharp boundary at which one can say an enhancer does or does not exhibit high degrees of cooperativity and unitary action, however for the purposes of designing bioinformatics search tools, we should seek to specify under which circumstances proteins or groups of proteins show tight enough constraints on binding site placement that we can detect these patterns above background, as has been demonstrated in a few cases [Erives and Levine, 2004; Markstein et al., 2004]. In other cases, general clustering of binding sites may be the only signature cur-

rently available [Berman et al., 2002; Rajewsky et al., 2002; Ringrose et al., 2003], but we should be aware that there may be more general elements of a *cis* regulatory grammar applying to billboard enhancers, such as ratios of repressors to activators, waiting to be discovered (Kulkarni and Arnosti, submitted).

When our understanding of protein–DNA and protein–protein interactions at a promoter has progressed to a description at the level of Ångstrom and kcal, the classifications of enhanceosome and billboard will have become obsolete. In the meantime, because of the size and complexity of these nucleoprotein complexes, the differentiation serves a useful purpose in focusing attention on properties of enhancers that can facilitate phylogenetic and population studies.

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