Information display by transcriptional enhancers

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Summary

Transcriptional enhancers integrate positional and temporal information to regulate the complex expression of developmentally controlled genes. Current models suggest that enhancers act as computational devices, receiving multiple inputs from activators and repressors and resolving them into a single positive or a negative signal that is transmitted to the basal transcriptional machinery. We show that a simple, compact enhancer is capable of representing both repressed and activated states at the same time and in the same nucleus. This finding suggests that closely apposed factor binding sites, situated within compact cis-elements, can be independently interpreted by the transcriptional machinery, possibly through successive enhancer-promoter interactions. These results provide clear evidence that the computational functions usually ascribed to the enhancer itself are actually shared with the basal machinery. In contrast to the autonomous computer model of enhancer function, an information-display or 'billboard' model of enhancer activity may better describe many developmentally regulated transcriptional enhancers.

Key words: Enhancer, Cis-regulatory element, Enhanceosome

Introduction

Developmental programs of gene expression are controlled by 'hard-wired' transcriptional circuits composed of modular enhancers that communicate with basal promoter regions (Davidson, 2001). Prior studies in many systems have supported the general notion that an enhancer acts as an information-processing device, or computer, receiving multiple inputs in the form of distinct transcription factors, both activators and repressors, that bind to it (Davidson, 2001; Ghazi and VijayRaghavan, 2000). The analogy of an enhancer as a computer is usually simply that of an element that sorts out inputs (processing) and resolves them into a single output that is instructive to the basal machinery, either turning the gene on or off. An important point is that computational functions – the decision to fire a promoter and at what level – have been ascribed to the enhancer. This is not to suggest that a given enhancer has only a single possible output; depending on signals or cell type, the same enhancer element might activate or repress, and the magnitude of activation signals can be variable, in the manner of a rheostat (Barolo and Posakony, 2002; Biggar and Crabtree, 2001; Rossi et al., 2000). However, it has been thought that enhancers do perform an integrative function and that in a particular nucleus, an enhancer represents a single information state at any given moment.

Such integrative functions have been ascribed to the human interferon-β (IFN-β) enhancer, which drives transcription of the IFN-β gene in response to viral infection (Struhl, 2001). The presence of each transcription factor binding site and its precise arrangement within the regulatory element are critical for the various regulatory proteins (sequence-specific activators and architectural proteins) to assemble through cooperative interactions into a well-defined nucleoprotein complex called the 'enhanceosome'. Assembly of the enhanceosome is essential for the transcription of the IFN-β gene in response to viral infection in cells. In this structured element, the exact arrangement of factor binding sites is critical to dictating the output of the element, so the enhanceosome acts as a molecular computer, leading to a single output directed to the general machinery (Thanos and Maniatis, 1995; Kim and Maniatis, 1997; Munshi et al., 2001). Such a complex might provide a stereospecific interface for interaction with the basal transcriptional machinery, possibly engaging several components of the basal machinery simultaneously to effect synergistic activation (Carey et al., 1990; Chi et al., 1995). With such an enhancer, the target gene would be activated only upon the assembly of a 'complete' complex, providing a precise on/off binary transcriptional switch in response to the appropriate stimulus.

Studies of developmentally regulated genes have also provided examples of enhancers as molecular computers. The developmentally regulated *Drosophila even-skipped* (*eve*) gene is regulated by developmental enhancers that are thought to act in a computational fashion. The reiterated stripe pattern of *eve* expression in the blastoderm embryo is generated by modular enhancers bound by broadly expressed transcriptional activators and regionally distributed repressors (Fujioka et al., 1999; Small et al., 1992; Small et al., 1996). These enhancers interpret gradients of regulatory factors and are active or inactive, depending on the particular set of regulatory proteins present in a given nucleus. The *eve* stripe 2 enhancer is active only in a narrow band of cells where activators Bicoid and Hunchback are present, but repressors Krüppel, Giant and Sloppy-paired are scarce or absent (Andrioli et al., 2002; Small et al., 1992). Key to the functional autonomy of the modular

eve enhancers is the short-range of the repressors that regulate individual enhancers; for example, the short-range transcriptional repressor Krüppel bound to the stripe 2 enhancer in central regions of the embryo does not interfere with the activity of the adjacent *eve* stripe 3 enhancer (Small et al., 1993). An assumption is that each enhancer works as a single computational unit, not a redundant set of independently acting elements. Consistent with this view is the finding that enhancer function is disrupted upon loss of a single activator or repressor site (Arnosti et al., 1996a; Small et al., 1992). However, these experiments have relied on minimal elements that may already represent a subset of the actual regulatory region (see Discussion).

A more detailed picture emerges from the functional dissection of the *endo 16* cis-regulatory region of *Strongylocentrotus purpuratus*. The *endo 16* gene is regulated during development by a 2.3 kb region containing binding sites for factors that contribute to distinct functions such as early widespread activation, late activation, repression of the early element, and potentiation of the repressor sites. Separate portions of the regulatory region can be combined to recreate some or all of the expression pattern, and models based on Boolean logical operators successfully simulate the output of these regulatory regions (Yuh et al., 1998). These studies emphasize the integrative, computer-like processing suggested to be a characteristic of developmental enhancers, and suggest that basal elements respond to signals generated by these molecular logic circuits.

In contrast to this view of the enhancer as an information-processing unit, we find that a single, compact enhancer can serve as an information display, representing on and off states, at the same time and in the same nucleus. This finding suggests that rather than acting as a computer that integrates various inputs, enhancers can simultaneously display both the active and repressed states, which may be interpreted by successive or multiple, simultaneous interactions with the basal transcriptional machinery. In this case, the enhancer does not act in a concerted, computational fashion, and the basal transcriptional machinery plays an active, rather than a passive, role in interpreting signals from the enhancer.

Materials and methods

Plasmid construction

Gal4 (aa1-93) – Gal4 AD (aa753-881)

A *Kpn*I-*Xba*I fragment from pSCTEV Gal4 (1-93)-Gal4 (Seipel et al., 1992) containing the reading frame for the yeast Gal4 activation domain (Gal4 AD) from amino acid residues 753-881, was cloned into *Kpn*I-*Xba*I-cut pTwiggy (Arnosti et al., 1996b) vector, which contains the *twist* enhancer (2×PEe-Et) element, *twist* basal promoter and the Gal4 DNA-binding domain from residues 1-93.

Reporter genes

The plasmid *UAS-lacZ* (Brand and Perrimon, 1993) was modified to contain two **Giant** sites (5′ GGC CGC **TAT GAC GCA AGA** AGA CCC AGA TCT TTT **TAT GAC GCA AGA** GA 3′) or two **Knirps** sites (5′GGC CGC AT**C TGA TCT AGT TT**G TAC TAG ACA T**CT GAT CTA GTT T**CA 3′) 20 nucleotides upstream of the five Gal4 binding sites. The resulting vectors named **M2g5u-lacZ** or **M2k5u-lacZ** (Fig. 1C,D) respectively, consist of two Giant or Knirps binding sites, five tandemly arrayed Gal4 binding sites, followed by the *Hsp70* TATA box and transcriptional start driving *lacZ* expression. These reporters were further modified by introducing oligos containing two Twist and two Dorsal binding sites (Szymanski and Levine, 1995) at the *Not*I site upstream of the Giant or Knirps sites resulting in the **2twi.dl-M2g5u-lacZ** and **2twi.dl-M2k5u-lacZ** reporters (Fig. 1A,B,E,F).

The regulatory element from 2twi.dl-M2g5u-lacZ, containing two Twist sites, two Dorsal sites, two Giant sites and five Gal4 binding sites was introduced into the *Eco*RI site of the C4PLZ vector in both orientations. The C4PLZ vector lies between two divergently transcribed genes, the TATA-less *white (w)* gene and the *lacZ* gene. The *lacZ* gene is driven by the TATA containing P element *transposase* basal promoter (Fig. 2). Two additional Giant binding sites were introduced at the *Sph*I site in the M2g5u-lacZ vector between the five Gal4 binding sites and the *Hsp70* TATA box. The resulting vector was further modified by introducing oligos containing two Twist and two Dorsal binding sites (Szymanski and Levine, 1995) at the *Not*I site upstream of the Giant sites resulting in the **2twi.dl-M2g5u2g-lacZ** (Fig. 3).

P-element transformation, crosses to reporter genes and whole-mount in situ hybridization of embryos

P-element transformation vectors were introduced into the *Drosophila* germline by injection of yw^{67} embryos as described previously (Small et al., 1992). Embryos were collected either directly from each transgenic reporter line or from a cross between a reporter line and a line expressing the Gal4 activator in the ventral regions of the embryo. The embryos were fixed and stained using digoxigenin-UTP labeled antisense RNA probes to either *lacZ* or *w* as described previously (Small et al., 1992).

Results

Limited ability of short-range repressors to block activators

The activity of developmental cis-regulatory elements has been studied mostly in the context of complex endogenous enhancers (Arnosti et al., 1996a; Gray et al., 1994; Kosman and Small, 1997; Small et al., 1993; Small et al., 1992). This approach is complicated by the functional complexity of many cis-regulatory elements where the identity and/or the stoichiometry of transacting factors is not always well defined. To analyze enhancer function in a setting in which activator-repressor stoichiometry and spacing can be exactly defined, we constructed chromosomally integrated, compact regulatory elements containing binding sites for endogenous short-range repressors Giant or Knirps, endogenous activators Twist and Dorsal, and chimeric Gal4 activators. The space between repressor and activator sites on these elements is less than 100 bp, a distance over which short-range repressors have been previously shown to be effective (Arnosti et al., 1996b; Gray et al., 1994; Hewitt et al., 1999; Keller et al., 2000). Twist and Dorsal drive gene expression in a ventral swathe approximately 22-24 cells in width, while the Gal4 activator protein, expressed under the control of the *twist* enhancer, drives reporter gene expression in a narrower 18- to 20-cell wide pattern. The protein product of the gap gene *giant* is present in broad anterior and posterior stripes, while the Knirps protein is present in a broad posterior stripe and in more anterior regions in the early embryo. As anticipated, Giant and Knirps mediate repression of adjacent Dorsal and Twist activators, eliminating expression of the *lacZ* reporter gene in portions of the embryo where these repressor proteins are localized (Fig. 1A,B). Strikingly however, Giant and Knirps are unable to

repress an element containing five Gal4 activator sites, although these proteins also bind within 100 bp of the repressors, revealing a hitherto unknown limitation of short-range repressors (Fig. 1C,D). This lack of repression is not due to an inherent resistance of the Gal4 activation domain to repression, for Knirps and Giant can effectively repress an element containing only three Gal4 binding sites (M.M.K., unpublished data).

Simultaneous repression and activation

When Gal4 activators are combined with Dorsal and Twist activators on a composite element, strongly enhanced staining is noted in the central regions of the embryo, indicative of additive or synergistic activation. In the regions of the embryo containing the repressors Giant or Knirps, the width of the area in which the nuclei are stained (a 18-20 swathe of nuclei) is the same as the pattern of staining driven by the Gal4 protein alone. We conclude that in nuclei containing Giant and Knirps protein, the pattern of staining directed by Dorsal and Twist is being selectively repressed by the short-range repressors, while transcription driven by Gal4 (a narrower 18-20 nuclei swathe) is unimpeded (Fig. 1E,F). The pattern of gene expression indicates that, in nuclei where the activators and repressors are co-expressed, transcription is driven by one cluster of activators within the compact regulatory element, while at the same time other activators within the same element are being actively repressed by Giant or Knirps. This compact regulatory element therefore, has subelements that represent both 'active' and 'inactive' states simultaneously, unlike the binary switch activity observed for many enhancers, where it appears that a single signal to activate or repress is present.

We make this conclusion based on the activity of the elements when only one set of activators is present (Fig. 1A-D), and on the characteristic narrower pattern driven by the Gal4 activators. Consistent with this conclusion, a similar pattern of exclusive repression of the Dorsal and Twist activators is seen when expression of Gal4 is driven in a ubiquitous pattern using the *nanos* promoter (Tracey et al., 2000). Here, we can compare promoter activity with Gal4 alone or in combination with Dorsal/Twist (Fig. 1G). In dorsal regions of the embryo, the only activator on the element is Gal4, and no repression by Giant is visible. In the ventral regions where Dorsal and Twist are present, but the repressor is absent, more intense staining is seen, consistent with synergistic or additive activation. Importantly, in the ventral regions also containing the Giant repressor (Fig. 1G, arrows), *lacZ* expression is similar to that observed in the dorsal regions of the embryo. This result indicates that Dorsal and Twist are not working together with Gal4, but are functionally independent and selectively repressed in the regulatory element.

Compact element functions in a distance-, orientation-, promoter-independent manner

To further evaluate the properties of this element, we tested whether it possessed classical characteristics of a transcriptional enhancer, namely, acting in a distance- and orientation-independent manner (Banerji et al., 1981). The element containing Giant binding sites was placed in either orientation between the divergently transcribed *white* (at –265

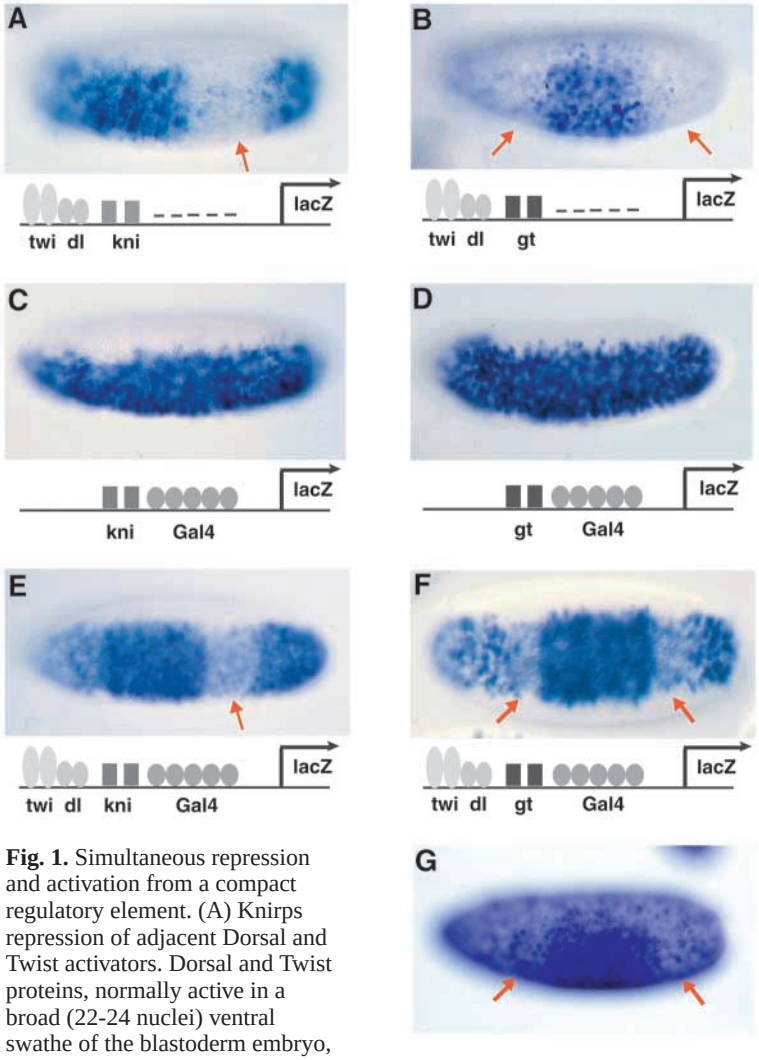

Fig. 1. Simultaneous repression and activation from a compact regulatory element. (A) Knirps repression of adjacent Dorsal and Twist activators. Dorsal and Twist proteins, normally active in a broad (22-24 nuclei) ventral swathe of the blastoderm embryo, fail to activate a linked *Hsp70 lacZ* transgene in regions containing Knirps (kni) protein (arrow). (B) Giant repression of Dorsal and Twist. Repression is seen in anterior and posterior regions where the Giant (gt) repressor is expressed (arrows). (C,D) Gal4 activators, expressed in a narrower (18-20 nuclei) ventral swathe, are not inhibited by Knirps and Giant. (E) A composite element containing Dorsal, Twist and Gal4 activators exhibits repression of Dorsal and Twist by Knirps, while the narrower Gal4-driven expression pattern is unaffected. (F) A composite element with Dorsal, Twist and Gal4 activators, and Giant repressor, exhibits a similar complex expression pattern (arrows). (G) A similar pattern of selective repression of the Dorsal and Twist activators within the composite element used in F is seen when the activator Gal4 is driven throughout the embryo under the control of the *nanos* promoter (NGT40, Bloomington Stock no. 4442). In the central regions of the embryo more intense staining is visible, indicative of additive or synergistic gene activation by Dorsal, Twist and Gal4. In the regions of the embryo where the repressor Giant is expressed (arrows), the intensity of *lacZ* staining is the same as in the dorsal regions of the embryo where activation is driven by Gal4 alone. The difference in *lacZ* staining intensity between cells containing or lacking Giant or Knirps is due to a difference in intensity in each cell, not the number of cells stained. Patterns of gene expression were visualized in 2-4 hour embryos by in situ hybridization with digU-labeled antisense *lacZ* probes. Embryos are oriented anterior to left; ventrolateral views (A-E,G) and ventral view (F) are shown.

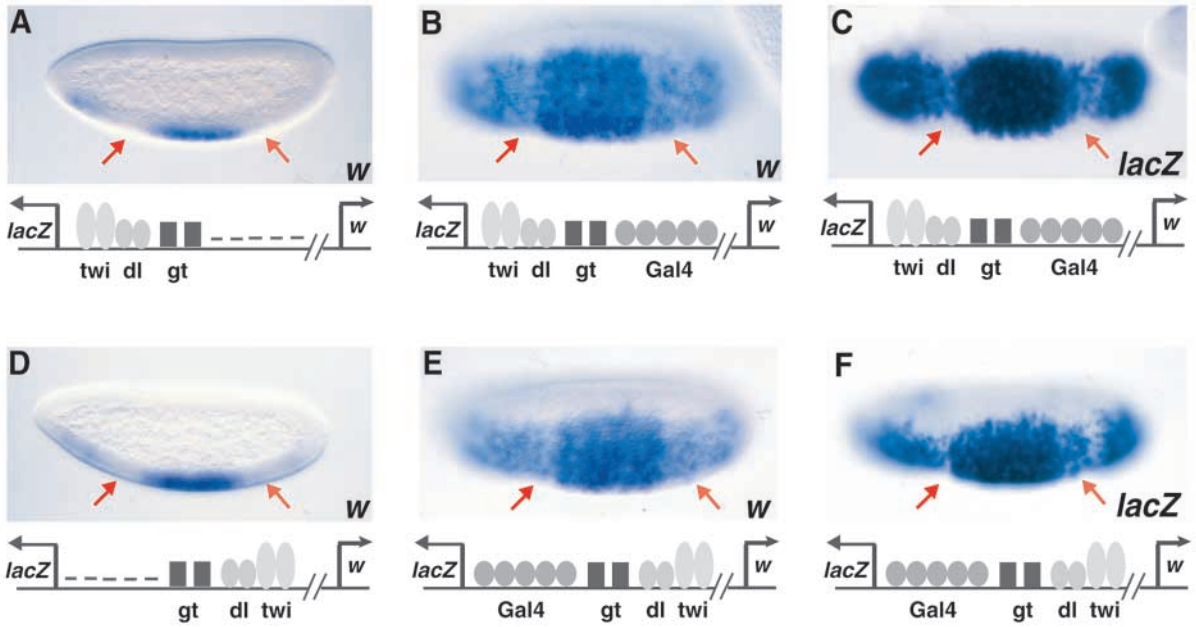

Fig. 2. Compact regulatory element displays enhancer-like properties of distance and orientation independence. (A,D) The regulatory element shown in Fig. 1F was inserted in either orientation into a vector containing divergently transcribed *white* and *transposase lacZ* reporter genes. When situated at –265 bp, Dorsal/Twist (dl/twi) activators within the element drive expression of the *white* reporter gene. Repression by Giant (gt) is evident in anterior and posterior regions (arrows). (B,E) In the presence of Dorsal, Twist, and Gal4 activators, a composite pattern of gene regulation is seen as in Fig. 1F with inhibition of Dorsal/Twist and activation by Gal4. (C,F) A similar expression pattern is observed with the divergently transcribed *transposase lacZ* promoter, with repression by Giant of Dorsal/Twist and activation by Gal4. Embryos are oriented anterior to left; lateral views (A,D) and ventrolateral views (B,C,E,F) are shown.

bp) and *lacZ* (at –130 bp) genes. In both orientations tested, this element directed *white* expression from –265 bp in a manner closely resembling that seen for the *Hsp70 lacZ* reporter; Giant efficiently repressed Dorsal and Twist, while Gal4 activated transcription in a continuous ventral swathe (Fig. 1B,F, Fig. 2A,B,D,E). A similar pattern of repression and activation is seen with the *transposase lacZ* gene (Fig. 2C,F). The identical results observed in Fig. 1F and Fig. 2B,C,E,F indicate that the specific patterns of activation and repression are not dependent on the particular promoter context or orientation of activators and repressors.

Conversion of enhancer output to a binary on/off state

The compact regulatory element assayed in Figs 1 and 2 fits the classic definition of an enhancer, functioning in a distance- and orientation-independent manner. In addition, the size of this element resembles that of naturally occurring enhancers ~200-800 bp in length. However, the element does not function in the biphasic either ‘on or off’ mode, normally thought to be a characteristic feature of enhancers. We are unaware of documented cases where a single enhancer displays two different states at the same time and in the same nucleus, thus this dual activity appears to be unusual. It is possible that rather than being an inherent functional property of enhancers, the uniform output of enhancers might reflect evolutionary pressure to arrange repressor and activator binding sites to optimize a consistent output. To simulate this situation, two additional Giant repressor binding sites were introduced into this element 3′ of the Gal4 binding sites. Now, complete loss of staining is evident in nuclei containing the Giant protein (arrows) yielding a classic biphasic ‘on or off’ state (Fig. 3A-D).

Discussion

Redundancy in enhancer function

If an enhancer were an indivisible unit of transcriptional regulation, the functional independence of adjacent binding sites within the composite element (Fig. 1E,F) would suggest that this compact element is in fact two separate enhancers. However, this element is of similar size to natural enhancers and does conform to the classic definition of an enhancer, namely a compact element that functions to regulate transcription in a position- and orientation-independent manner (Banerji et al., 1981).

Functional analyses of cis-regulatory regions provide evidence for redundancy and hence divisibility, of natural enhancers, suggesting that they can also contain multiple, independently acting subelements. In the viral setting, the well-studied SV40 enhancer comprises two independently acting subelements that can be separately assessed (Herr and Clarke, 1986). In *Drosophila*, recent evidence suggests that *eve* enhancers possess redundant activities. Deletion of the entire 480 bp *eve* stripe 2 element within the *eve* locus fails to completely abrogate stripe 2 expression (M. Kreitman, personal communication) indicating the presence of redundant regulatory sequences in the locus. Furthermore, tissue-specific expression of the yolk protein genes *yp1* and *yp2*, is supported by flanking sequences after deletion of the 125 bp yolk protein

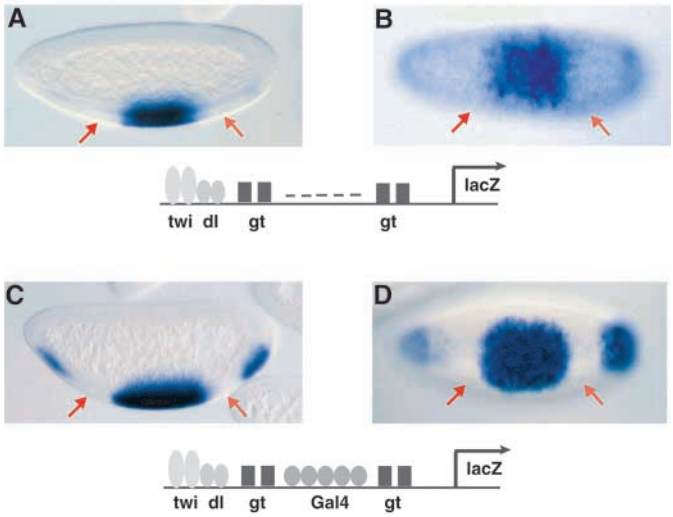

Fig. 3. Conversion of a multiple state element to a binary on/off switch. Two additional Giant (gt) binding sites were introduced at the 3′ end of the Gal4 activator cluster. (A,B) As observed previously, Dorsal/Twist (dl/twi) activators are repressed in anterior and posterior regions of Giant expression (arrows). (C,D) In the presence of Dorsal/Twist and Gal4 activators, complete repression of transcription is observed in areas of Giant expression (arrows). Embryos are oriented anterior to the left. Lateral (A,C) and ventral (B,D) views are shown.

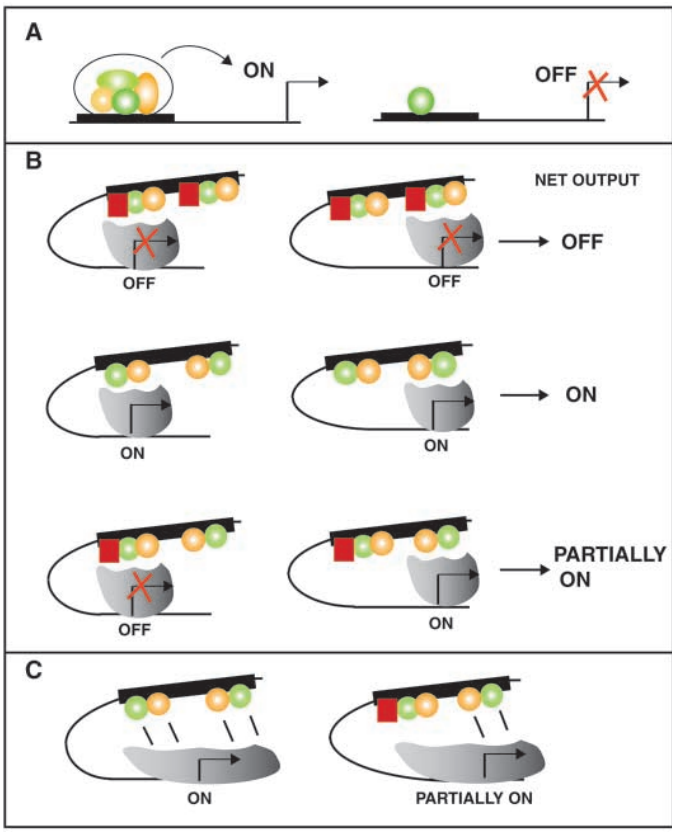

Fig. 4. Enhanceosome versus Information Display enhancer models. (A) In the enhanceosome model, the enhancer serves as an information processing center, receiving inputs from multiple transcription factors that bind it. A highly structured complex or enhanceosome, creates a stereospecific interface for docking with and recruiting the basal transcription machinery. Here the enhancer serves as a molecular computer, resolves multiple inputs and provides a single output to the basal transcription machinery. With such an enhancer, the target gene would be activated only upon the assembly of a complex, providing a precise on/off binary transcriptional switch in response to the appropriate stimulus. Graded responses from such an element could be achieved by varying the stability of the entire complex, possibly in response to activator concentrations. (B,C) Information Display or "Billboard' enhancer. Rather than acting as a central processing unit, subelements can display contrasting information, which is then interpreted by basal transcription machinery. In this model, the basal machinery 'samples' discrete regions of the enhancer each composed of a small number of transcription factor binding sites, either iteratively (B) or simultaneously (C). Successive/multiple interactions with the basal machinery, and the biochemical consequence of these interactions, would dictate the overall output of the enhancer.

enhancer (Piano et al., 1999). The resilience of natural enhancers to loss of single binding sites further supports the notion that these elements are built of redundantly acting sequences (Arnosti, 2003).

Selection for uniformity of enhancer output

A scenario of an enhancer with simultaneously displayed activation and repression states is reminiscent of the modular, autonomous pair-rule stripe enhancers, such as *even-skipped* stripe elements, where separate enhancers represent different 'states' of repression and activation in the same nucleus (Gray and Levine, 1996). An important distinction is that our findings suggest that a similar discrimination is taking place within the tight confines of a single enhancer, and that in order to establish a uniform signal output, enhancers require a proper stoichiometry or distribution of repressor and activator binding sites to ensure that all possible enhancer subelements provide the same information (Fig. 4). Indeed a distributed pattern of short-range transcriptional repressor binding sites is typical of many developmental enhancers that function in the early *Drosophila* embryo; this configuration would allow repressors to block multiple modes of enhancer-promoter interactions (La Rosee et al., 1997; Small et al., 1992; Small et al., 1996). In this study we actually measure the simultaneous independent activity of sub-elements (Figs 1, 2) and show that they can be deployed to give a unitary response (Fig. 3) as is seen with natural enhancers. Thus, the carefully designed internal organization of cis-regulatory modules can provide uniform information that closely simulates an integrative information processing capacity.

In contrast to the precision of the enhanceosome, a more flexible arrangement of regulatory proteins has been suggested to be the predominant pattern for elements that provide diverse patterns in developing systems (Struhl, 2001). Evolutionary and experimental studies of the *eve* stripe 2 enhancer suggest that this element can tolerate and has undergone considerable rearrangement, with great flexibility in the number and arrangement of individual sites (Arnosti et al., 1996a; Ludwig et al., 2000; Ludwig and Kreitman, 1995; Ludwig et al., 1998). For example, the recent acquisition of a strong Bicoid activator site appears to have been counterbalanced by the closer apposition of a nearby Giant binding site (Hewitt et al., 1999;

Ludwig et al., 1998). The plasticity of this enhancer suggests that much variation in spatial placement of individual transcription factors is possible, consistent with a model in which these factors contact the basal machinery in a flexible framework, not necessarily as a rigid complex.

With such flexibility, the transcription factors of an enhancer might still engage the transcriptional machinery in simultaneous cooperative interactions, as is suggested with enhanceosomes. However, our studies suggest that an individual enhancer is capable of representing both the state of activation and repression, suggesting that the basal machinery may 'sample' discrete regions, consisting of a small number of transcription factor binding sites, within the enhancer (Fig. 4B,C). Successive interactions with the basal machinery, and the biochemical consequence of these multiple interactions would dictate the overall output of the enhancer (Fig. 4B). Alternatively the enhancer may engage in multiple, simultaneous contacts with some or all of the enhancer bound proteins, with repressors such as Giant and Knirps preventing some of these interactions (Fig. 4C). In either case, multiple iterative sampling of the enhancer, or simultaneous readout, the enhancer would function as an information display element with computation at the level of enhancer-promoter interactions.

Our results suggest that a closer examination of enhancer classifications is warranted. The terms enhancer and enhanceosome are frequently used interchangeably to denote a complex of DNA-bound regulatory proteins, yet there appear to be important functional distinctions between enhanceosomes, as typified by the IFN-β enhancer, and other regulatory elements. In the light of the functional differences outlined above, a distinction should be made between the terms *enhanceosome*, which requires the cooperative assembly of a higher order structure within an enhancer, and other cis-regulatory elements that may or may not function in this manner. We propose a model, the information display or 'billboard' model for enhancer action, in which an enhancer, rather than acting as a central processing unit, can display contrasting information, which is then interpreted by basal transcriptional machinery (Fig. 4B,C). The binary 'on or off' decisions that appear to be transmitted by the enhancer to the basal machinery actually result from the basal machinery reading a series of redundant signals encoded within the enhancer. The model does not explicitly describe the molecular mechanisms of repression and activation, but direct contacts between the *Drosophila* activators used here and components of the basal machinery are supported by biochemical studies (Koh et al., 1998; Pham et al., 1999; Yuh et al., 1998; Zhou et al., 1998)

The billboard enhancer model appears to more accurately describe many developmentally regulated enhancers, whose internal architecture is subject to rapid evolutionary change, even as the overall output remains constant (Ludwig et al., 2000; Ludwig et al., 1998). Although studies such as those on the IFN-β gene indicate that cells may commonly use enhanceosomes to achieve regulatory precision in gene expression, it is likely that eukaryotic organisms use the 'billboard' type of enhancers to achieve diversity in gene expression patterns and evolutionary flexibility.

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