Shadow enhancers: Frequently asked questions about distributed *cis*-regulatory information and enhancer redundancy

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This paper, in the form of a frequently asked questions page (FAQ), addresses outstanding questions about ''shadow enhancers'', quasi-redundant *cis*-regulatory elements, and their proposed roles in transcriptional control. Questions include: What exactly are shadow enhancers? How many genes have shadow/redundant/distributed enhancers? How redundant are these elements? What is the function of distributed enhancers? How modular are enhancers? Is it useful to study a single enhancer in isolation? In addition, a revised definition of ''shadow enhancers'' is proposed, and possible mechanisms of shadow enhancer function and evolution are discussed.

Keywords:
cis-regulatory elements; enhancers; evolution; genomics; transcriptional regulation

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Abbreviations:
BAC, bacterial artificial chromosome; **ChIP,** chromatin immunoprecipitation; **TF,** transcription factor.

Introduction

Q: What exactly are shadow enhancers?

A: Enhancers are *cis*-regulatory elements that control the spatiotemporal patterns and quantitative levels of gene transcription. They are composed of clusters of binding sites for transcription factors (TFs), each of which contributes to either the activation or repression of transcription. In multicellular organisms, the combination of TFs bound to an enhancer largely determines its target gene's response to developmental, physiological, or environmental signals. The functional definition of an enhancer will be discussed further below; for a more in-depth examination of enhancers in the context of animal development, see [1].

The phrase ''shadow enhancers'' was coined by Mike Levine and colleagues in a 2008 *Science* Brevia article describing the discovery of remote enhancers of two *Drosophila* genes, *brinker* and *sog* [2]. These enhancers, which drive gene expression in the presumptive neurogenic ectoderm of the early embryo, were identified by chromatin immunoprecipitation (ChIP)-chip assays [3] and validated with transgenic reporters. They share several features: (a) they each drive a pattern of transcription resembling that of a previously identified ''primary'' enhancer that is more proximal to the promoter being regulated; (b) they bind the same TFs as the primary enhancer, suggesting a similar regulatory logic; and (c) they are located either within an intron of, or on the far side of, a neighboring gene (Fig. 1). In that paper, the term *shadow enhancer* is proposed for ''remote secondary enhancers mapping far from the target gene and mediating activities overlapping the primary enhancer'' [2].

Q: Do I have to use that term?

A: As the phrase *shadow enhancers* has swept the field of *cis*-regulation [4–13] and the pop-science blogosphere, some in the field have complained about this new term. One objection I

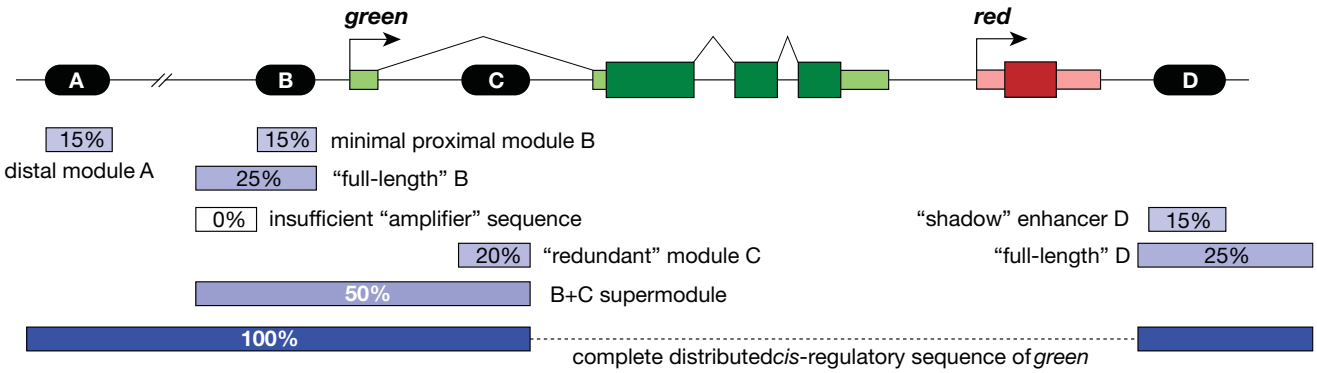

Figure 1. Diagram of a hypothetical gene locus, illustrating how multiple distributed *cis*-regulatory modules can contribute to total expression of the *green* gene. For the purposes of this diagram, patterning is ignored, and output is considered purely quantitatively. Only regulatory elements of *green* are shown; although ''shadow'' enhancer D is nearer to the *red* gene, it specifically regulates *green*. Percentages refer to the amount of gene expression driven by each element or combination of elements, relative to the entire gene locus.

have heard is that the concept itself is nothing new. It is certainly true that apparently redundant enhancers have long been known to regulate certain genes, as I will discuss in more detail below. However, recent work from a number of groups [5, 8, 14–18], most notably those led by Levine and David Stern, moves beyond simply documenting apparent redundancy by testing specific ideas about the functional significance of such a *cis*-regulatory arrangement. Because the concept of shadow enhancers is so tightly associated in the literature with these exciting proposed regulatory and evolutionary mechanisms (as described below), I maintain that the term is not a mere repackaging of the idea of redundancy.

Others have balked, not at the idea of shadow enhancers per se, but at the designation of one element as the *primary enhancer* and one (or more) as the *shadow*, with the difference in regulatory significance that those terms seem to imply. Levine recently stated, at the Keystone Symposium on Evolutionary Developmental Biology [19], that the term was meant to refer specifically to elements that reside within or on the far side of a neighboring gene (i.e. ''in the shadow'' of another gene). Still, because of the perception that *primary* connotes a higher degree of importance, and the potential nomenclatural difficulties of cases in which two enhancers are discovered simultaneously, are equidistant from the promoter, etc., I submit that the idea of designating an individual enhancer as the *primary* or the *shadow* is unsatisfactory and too narrowly applicable. I believe that the term *shadow enhancers* is a useful and memorable way to describe genes containing multiple separable enhancers driving similar patterns of expression. I therefore propose to slightly adjust the definition to describe a regulatory arrangement – a type of gene structure – without assigning the labels *primary* and *shadow* to individual elements. By this revised definition, the neuro-ectodermal enhancers of *sog* could both be considered shadow enhancers: that is, components of a multi-enhancer system regulating the same gene in the same tissue. The individual enhancers could be referred to as proximal and distal, 5′ and 3′, or in many other ways. Ideally, nothing would be implied about the relative importance or functional redundancy of any of the elements.

For researchers who strongly dislike the term and do not accept the above proposal, I offer *distributed enhancers* as a possible substitute. This phrase has the merit of accuracy, as the gene's *cis*-regulatory information (concerning a specific aspect of its expression pattern) is distributed among multiple modules. However, it cannot be used to refer to an individual enhancer, and it lacks the air of mystery conveyed by *shadow enhancers*.

Main text

Q: How many genes have shadow/redundant/distributed enhancers?

A: Of course, we do not know exactly. Even if we had genome-wide TF binding data from all organisms, for all TFs, in all tissue types, at all stages and under all relevant conditions, enhancers must be defined functionally, and such tests have been applied to only a tiny fraction of all possible enhancer sequences. For many loci that have been searched for enhancers, once an element has been found that is largely sufficient to explain a given expression pattern, researchers understandably stop searching.

Nevertheless, there is an extensive literature in vertebrate and invertebrate systems, going back over 20 years, of separable, apparently redundant enhancers (or semi-redundant enhancers, or enhancers capable of driving similar or overlapping patterns) within a single gene (e.g. [3, 6, 9, 12, 20–40]). New evidence indicates that the embryonic patterns of all of the *Drosophila* gap genes are encoded by multiple elements [17]. Still, as discussed below, many gene expression patterns are known to depend on single, non-redundant enhancers.

As interest in *cis*-regulation has grown, especially in the fields of development and evolution, and as tools for genomic manipulation have improved, reports of distributed enhancers have increased over the last few years. Two noteworthy examples, both from *Drosophila*, are the aforementioned *brinker* gene, which, apart from the "shadow" enhancer,

contains multiple 5′ enhancers driving similar expression patterns in the embryo and developing wing [35, 41], and the *shavenbaby* gene, which contains six 5′ enhancers driving overlapping expression patterns in the embryonic epidermis [8, 16, 32]. Reports like these comprise a small proportion of the enhancer-identification literature, but as mentioned above, very few loci have been comprehensively scoured to account for all possible *cis*-regulatory inputs. The relatively high frequency of insulator elements between genes [42] might suggest that most genes do not harbor their neighbors' enhancers, but this remains to be tested. Bioinformatic TF binding site predictions and genome-wide ChIP data, which brought the shadow enhancers of *brinker* and *sog* to light, will be increasingly informative on this question.

Q: How redundant are these distributed enhancers?

A: This depends on how strictly redundancy is defined, which in turn depends on how stringently it is tested experimentally. Enhancer redundancy has been claimed in the absence of in vivo phenotypic assays, in cases where multiple modules are sufficient to drive similar or overlapping patterns of gene expression, usually in transgenic reporter experiments (e.g. [24, 26, 29, 43, 44]). To a geneticist, this would not be considered sufficient proof of redundancy, but from the perspective of sufficiency (as opposed to necessity), two sequences that activate gene expression in the same cell under the same conditions could be considered ''informationally'' redundant.

In the field of developmental gene regulation, a higher standard for in vivo enhancer redundancy is the demonstration that the loss of one module has no significant observable effect on viability, organ morphology, or some other relevant metric (e.g. [8, 15, 28, 36–38, 45, 46]). For example, Frankel et al. [8] generated a chromosomal deficiency in *Drosophila* that deleted three of six epidermal enhancers of the *shavenbaby* gene; this mutation did not produce a significant loss-of-function phenotype under normal lab conditions. Similarly, Perry et al. [15] removed one of two mesodermal enhancers of the *snail* gene in the context of a bacterial artificial chromosome (BAC) rescue transgene; under normal lab conditions, this mutation had no significant effect on the pattern of gene expression or on the ability of the BAC to rescue gastrulation defects in *snail* mutant embryos. (But more on the redundancy of these shadow enhancers later.) In the mouse, targeted deletion of an enhancer of *Prx1* that is sufficient to drive limb expression caused no developmental limb phenotype [33, 47].

By the same token, it is important to note that many enhancers have been found to be *non*-redundant – i.e. individually required for normal function – by this functional standard. This category includes evidence from in vivo functional tests in genetic model systems [48–57], as well as cases of mutant phenotypes being traced to induced or spontaneous mutations in enhancers (e.g. [51, 58, 59]). There is also a growing number of documented cases of what could be called ''enhancer-opathies'', human genetic diseases caused by mutations in *cis*-regulatory elements (e.g. [12, 50, 60–62]); these enhancers are obviously functionally non-redundant.

Interest in the ''shadow enhancer'' phenomenon, and a reluctance to accept the idea of a regulatory element whose function is evolutionarily well conserved yet utterly irrelevant to fitness, have recently inspired more stringent tests of the functional requirements for ''redundant'' enhancers. To return to the cases of *snail* and *shavenbaby*, shadow enhancers in both genes that seemed to be functionally unnecessary in a normal laboratory setting were subsequently shown to be required for normal gene expression and function when fly embryos were placed in sub-optimal conditions, such as high temperatures or a sensitized genetic background [8, 15]. Thus, it is easy to speculate that any report of a functionally redundant enhancer may have overlooked the critical environmental stress, culture condition, behavioral assay, selection criterion, etc., rendering true redundancy essentially unprovable. A new report suggests that the two ventral enhancers of the *snail* gene are functionally non-redundant even under normal culture conditions [18]. Proposed roles for distributed enhancers in maintaining robustness of gene expression in the face of environmental noise will be discussed in a later section.

It is difficult to estimate the ratio of functionally redundant to non-redundant enhancers because of the varying standards of proof mentioned above, and also because of likely publication bias against negative results [63].

Q: What is the function of distributed enhancers?

A: Three functions have been proposed:

1. *Robustness*. Gene expression levels naturally fluctuate between individuals in a population and between cells in a tissue. Genetic variation and environmental instability can exacerbate these differences. If the expression of a developmental regulator (say, a TF or signaling pathway component) dips below a minimum threshold level, cell fate specification or differentiation will be affected, which can result in a morphological defect. Developmental programs can be buffered against minor variations by maintaining super-threshold expression levels of important regulators (as long as these levels remain below the threshold for triggering gain-of-function defects) [8, 10, 15].

This argument, which provides a mechanism for Waddington's phenomenon of ''canalization of development'' [64], is perfectly sound – in fact, given what we know of loss-of-function genetics, it must be correct. For example, diploid multicellular organisms need only one copy of most genes for near-normal viability and fertility, at least under lab conditions, but these heterozygous mutants are more vulnerable to poor culture conditions, and they are far more sensitive to partial loss-of-function of other genes (this is the basis of enhancer-suppressor screens). The question is not whether super-threshold levels of gene expression increase developmental robustness – rather, the question is, why use two (or more) separate enhancers to do the job? One could imagine that one extra-strength enhancer would be just as effective for the purpose, and to me at least this seems like a neater, simpler solution: just add a couple of activator binding sites here, or remove a nucleosome positioning sequence there. Of course, evolution allows any solution that is not forbidden, whether it appeals to human intuition or not, and perhaps genes do not care whether their *cis*-regulatory information is organized in a way that seems tidy to us. But there may be

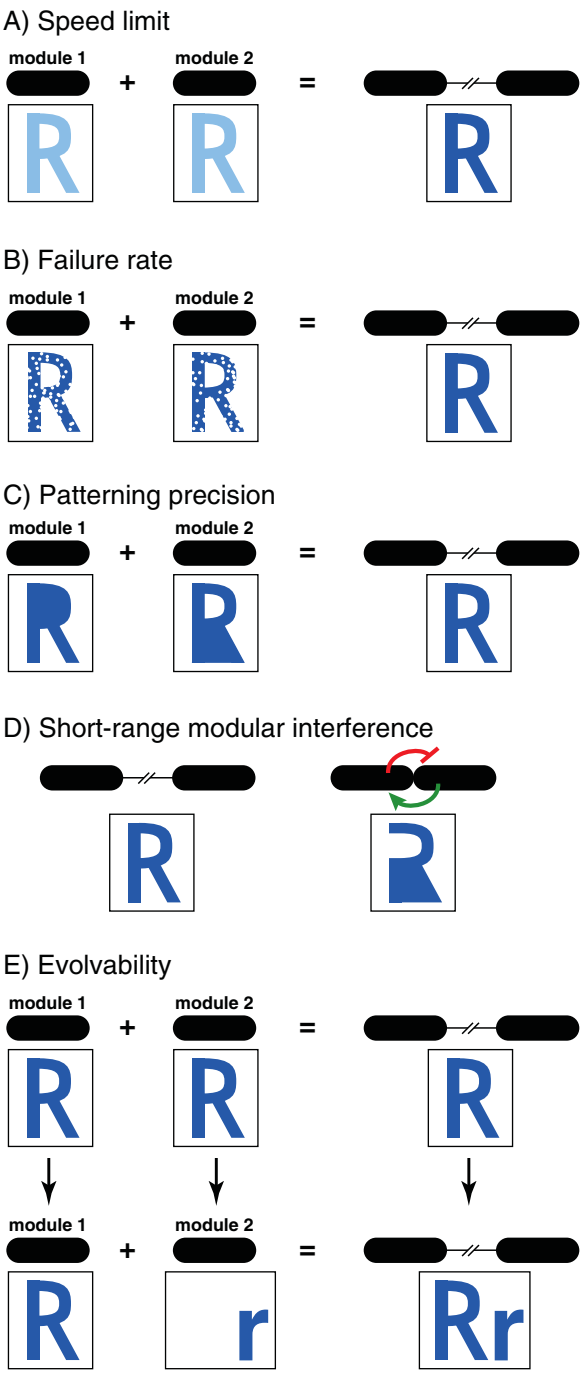

Figure 2. Possible mechanisms for the function and evolution of distributed enhancers. **A:** ''Speed limit'': if a single module drives a sub-optimal rate of transcription, multiple modules may be required. **B:** ''Failure rate'': if a given enhancer fails to activate transcription in a fraction of cells, a second, independently acting enhancer may significantly improve fidelity of gene expression. **C:** Patterning precision: two modules with overlapping patterns, but different regulatory logic, combine to produce a novel, refined gene expression pattern. **D:** Short-range modular interference: two enhancer modules may require a physical separation to prevent undesirable short-range positive and negative TF interactions between the two modules. **E:** Evolvability: enhancer redundancy may create opportunities for the generation of novel patterns of gene expression. See text for a fuller discussion of these concepts.

reasons why two weak enhancers might be preferable to one strong one.

For example, imagine that a given enhancer has a maximum level of activation activity, or a ''speed limit'', resulting from biochemical or biophysical constraints. The only way to boost the transcription rate of the gene, outside of adjusting the promoter itself, would be to acquire more enhancers that drive activation in the same pattern (Fig. 2A). This scenario requires that enhancers have a maximum activation level that is below the maximum firing rate of the promoter.

A plausible idea, developed by Levine and coworkers in recent papers [15, 17], is that enhancers have an inherent ''failure rate'', the probability that, in a given cell within the domain of expression, a sufficient rate of transcription will not be reached. This was demonstrated at the *snail* and *hunchback* loci of *Drosophila*: BAC reporter genes lacking the ''primary'' or ''shadow'' embryonic enhancer show an increased number of ''holes'' in the gene expression domain (i.e. nuclei with gene expression below a given threshold). If there is no way to reduce the failure rate of a single enhancer to an acceptably low level, adding a second enhancer can solve the problem (Fig. 2B): if one enhancer has a failure rate of 3%, two copies will sufficiently activate the gene in 99.91% of cells.

This model of robustness, or at least the simplest form of this model, seems to make a testable prediction: that boosting the quantitative activity of an enhancer by adding activator binding sites will not reduce the inherent failure rate. If this is not the case, then we return to the original question – why acquire two separate weak enhancers, rather than one sufficiently strong one?

2. *Patterning precision*. There is also strong evidence that, at least in some cases, quasi-redundant enhancers make important individual patterning contributions, such that the final gene pattern of gene expression depends on multiple *cis* elements (Fig. 2C). Levine's group demonstrates in a recent report [17] that, for the gap genes *hunchback*, *Krüppel*, and *knirps*, two enhancers are required to draw sharp, consistent stripe boundaries of embryonic gene expression, and also to properly position those boundaries along the anterior-posterior axis. The latter finding implies that, for these genes, the two enhancers employ slightly different *cis*-regulatory logic: this is confirmed by the expression patterns of single-enhancer reporter genes [17]. A similarly multi-modular regulatory circuitry applies to *snail* [18].

Again we can ask, somewhat teleologically: would it not be simpler to ''evolve'' a single enhancer that drives the correct pattern? Leaving aside the robustness arguments detailed above, there may be good reasons for such a seemingly complicated arrangement. In vivo reporter experiments have demonstrated that, when two enhancers are brought into close proximity, short-range repressors that set expression boundaries for one enhancer can interfere with the activity of the nearby enhancer, disrupting the final pattern of gene expression to which both enhancers contribute [65–67]. Thus, it is possible that the shadow enhancers of the *Drosophila* gap genes must be kept separate to avoid inappropriate short-range interactions among activators and repressors (Fig. 2D). This model is fairly easily testable.

3. *Evolvability*. Genetic redundancy has long been considered a potential source of evolutionary innovation [68]. It is increasingly widely recognized that much evolutionary morphological change stems from changes to *cis*-regulatory sequence [19], so it is natural that enhancer redundancy should be proposed as an important mechanism for evolving novel expression patterns (Fig. 2E) [2, 5, 16, 43, 69–72]. I wish to make only two comments on this train of thought. First (with the caveat that I am not a qualified evolutionary theorist), the idea that genes acquire redundant enhancers *as a means to achieve evolvability* seems to put the cart before the horse. However, I am assured by those who know better that this is not what is actually being proposed: rather, the idea is that traits that facilitate change enhance fitness without requiring evolution to look ahead, and apparently the math works out. Second, if shadow enhancers are not really functionally redundant (see points 1 and 2), then neither enhancer is ''free'' to modify its expression pattern. This means that for a given set of distributed enhancers, evolvability should be mutually exclusive with essential functions in transcriptional robustness or precision.

Regarding the evolutionary origins of shadow enhancers, there is currently no evidence that they arise by duplication. Therefore, they may differ dramatically from gene duplications, both in the mechanisms that create them and in the evolutionary forces that later shape them.

Q: How modular are enhancers, really?

A: The existence of shadow/redundant/those enhancers might reasonably inspire us to question the idea that enhancers are truly modular, discrete units. It is easy to imagine a gene whose *cis*-regulatory information is spread fairly evenly across the non-coding sequences of the locus, and this would be consistent with some of the findings of enhancer redundancy cited above. Could the concept of a discrete enhancer module largely be an artifact of 30 years of reporter gene experiments that have often focused on sufficiency, rather than necessity?

Those in vivo reporter experiments have led to a view of a typical enhancer module that is manageably sized (usually several hundred base pairs in length, when serious efforts at trimming are made) and sufficient to drive a pattern of gene expression resembling one or more aspects of the parent gene's expression profile. Published maps of enhancers within a gene locus nearly always show them as discrete, labeled boxes (''eye enhancer''; ''limb enhancer''; ''stripe 2 enhancer''). Generally speaking, such a box describes the ''minimal'' element (e.g. [73, 74]), typically defined as the shortest sequence that is sufficient to activate easily detectable levels of gene expression (via a short promoter sequence that is silent on its own) in a pattern that is similar or identical to that of the native gene in a given tissue. Less frequently, the minimal element is defined functionally, by its ability to drive rescue transgene expression that suppresses the loss-of-function phenotype of the relevant gene (e.g. [75, 76]).

Veteran enhancer-bashers, and those who carefully read the papers, know that ''minimal'' enhancer fragments do not always perfectly replicate the precise spatial boundaries of expression of the native gene, and that the most minimal fragment that can reproduce a given pattern often drives lower levels of gene expression than more extensive fragments (Fig. 1; [25, 74, 76–82]). Certain complex multigenic loci, such as Hox clusters and the β-globin locus, defy analysis by traditional sufficiency-based reporter gene assays [83–86]. Is individual enhancer analysis useful or appropriate for the study of genes with shadow/redundant/distributed enhancers?

Conclusions

Q: Given all of the above, is it useful to study a single enhancer in isolation?

A: It depends on the type of question one is trying to answer. If the goal is to unravel higher-order *cis*-regulation at a complex gene locus, in which multiple elements interact, synergize, or interfere with one another, the study of isolated modules is clearly insufficient. Tools for large-scale genetic manipulation, which are required to effectively tackle this type of problem, have dramatically improved in recent years [34, 85, 87–90].

Still, we owe almost all of what we know about *cis*-regulatory logic to fine-scale functional analyses of individual enhancers, mostly employing transgenic reporter assays to determine what is sufficient, at the DNA sequence level, to specify proper patterning of gene expression. If rescue BAC deletion analyses and targeted in situ enhancer mutations had been available 30 years ago, and if they were the only tools available to the field, the entire problem of transcriptional regulation might have been given up as hopelessly complex. By reducing the scale of the problem to a short sequence that is capable of driving a specific (if not always a perfectly precise or robust) pattern of gene expression in vivo, we have made remarkable advances in our understanding of the logic of transcriptional networks and their evolution, and I believe we will continue to do so, even in the era of systems biology. By simultaneously attacking the problem at the genomic scale, the gene-locus scale, and the reductionist scale of individual *cis*-regulatory modules and TF binding sites, the field can now make even faster leaps forward.

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