Unveiling dynamic enhancer-promoter interactions in *Drosophila melanogaster*

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Abstract

Proper enhancer-promoter interactions are essential to maintaining specific transcriptional patterns and preventing ectopic gene expression. *Drosophila* is an ideal model organism to study transcriptional regulation due to extensively characterized regulatory regions and the ease of implementing new genetic and molecular techniques for quantitative analysis. The mechanisms of enhancer-promoter interactions have been investigated over a range of length scales. At a DNA level, compositions of both enhancer and promoter sequences affect transcriptional dynamics, including duration, amplitude, and frequency of transcriptional bursting. 3D chromatin topology is also important for proper enhancer-promoter contacts. By working competitively or cooperatively with one another, multiple, simultaneous enhancer-enhancer, enhancer-promoter, and promoter-promoter interactions often occur to maintain appropriate levels of mRNAs. For some long-range enhancer-promoter interactions, extra regulatory elements like insulators and tethering elements are required to promote proper interactions while blocking aberrant ones. This review provides an overview of our current understanding of the mechanism of enhancer-promoter interactions and how perturbations of such interactions affect transcription and subsequent physiological outcomes.

Introduction

Transcription mediates RNA production and subsequent development of organisms. Gene expression in the wrong tissue type at the wrong time often results in significant developmental defects [1-5]. Specific interactions between a non-coding enhancer sequence and the cognate promoter directly regulate when and where a gene should be activated [6, 7]. More evidence points out that mutations in enhancers and promoters cause misregulation of transcriptional activity and induce physiological defects [8-11]. Despite extensive studies to elucidate the mechanism of enhancer-mediated transcriptional regulation, the complex nature of the enhancer-promoter interactions makes it a challenging and exciting problem to tackle. For example, multiple transcription factors (TF) need to compete or cooperate with one another to bind to the enhancer DNAs [8, 9]. Meanwhile, since a single gene can be regulated by multiple enhancers and a single enhancer can activate multiple promoters, precise coordination of multivariate enhancer-promoter interactions is required to drive specific transcription and prevent ectopic gene expression [12-17]. In addition, 3D chromatin topology must be arranged such that enhancers and promoters that are tens and hundreds of kilobases (kb) away from one another can be in proximity to drive transcription [16, 18]. How are all such processes seamlessly regulated to result in proper gene expression? In this review, we summarize recent findings on various parameters that mediate enhancer-promoter interactions with a focus on studies that used *Drosophila* as a model system.

Enhancers determine the spatial and temporal patterns of gene expression

As non-coding DNA sequences, enhancers contain several highly tuned TF binding sites that control the spatiotemporal patterns of gene expression. Enhancers bound by transcription factors help recruit general transcription factors and RNA polymerase II (Pol II) to promoters to initiate transcription [7]. Many studies have focused on how the composition of TF binding sites in an enhancer mediates gene regulation [8, 9, 19]. However, the following questions are yet to be fully answered. Does each TF binding site contribute independently to the transcriptional activity, or do they work synergistically with each other to recruit more TFs than a single binding site can? How do perturbations of TF binding sites affect the spatial boundaries and kinetics of the gene expression? Many studies use *Drosophila* embryo as a model system to examine the role of enhancers in transcriptional regulation [8, 9, 19, 20]. Enhancers of many developmental genes are extensively characterized [21]. Moreover, visualization and analysis of the spatial boundaries of gene expression is easy because the nuclei are located on the surface as a monolayer in early embryos [22].

Although enhancers have several binding sites for the same TF with varying affinities, gene expression is significantly altered upon deletion of a single TF binding site (Figure 1A). Systematic removal of the activator Bicoid (Bcd) binding sites from the *hunchback* (*hb*) P2 enhancer-driven reporter construct in *Drosophila* embryos abolishes the sharp *hb* expression pattern and results in a graded gene expression pattern along the anterior-posterior axis [8]. As more sites are deleted, the expression gradient of *hb* becomes more gradual. Given that Bcd concentration is low at the posterior boundary of the *hb* domain, this result indicates that multiple Bcd sites are required to maintain the sharp *hb* expression in low Bcd-containing cells.

Interestingly, removal of a high- or low-affinity Bcd site has similar effects on transcription, such that all binding sites contribute comparably to the *hb* expression [8].

Live imaging techniques, such as the MS2/MCP system, which enable visualization of nascent transcripts, allow analysis of changes in temporal dynamics of target gene upon perturbation. For the MS2/MCP technique, 12-24 repeats of MS2 sequences derived from bacteriophage are inserted proximal to the gene of interest (5'UTR, introns, 3'UTR) [23]. Each MS2 sequence forms an RNA stem loop upon transcription, and each loop is detected by two copies of the MS2 coat protein (MCP) tagged with fluorescent proteins. In this way, nascent transcripts can be visualized in *vivo* over time at single-cell resolution [9, 24, 25]. This live imaging technique was first developed in yeast and has been implemented in many other species including *Drosophila* [26-28]. Using the MS2/MCP-based live imaging, Keller et al. demonstrates that deletion of a high- or low-affinity Dorsal (Dl) activator site reduces the target gene *t48* mRNA production by 90% or 50%, respectively, mainly by delaying the transcriptional initiation (Figure 1A) [9]. Upon deletion, transcriptional initiation is delayed, and the expression domain is narrowed, affecting both the temporal dynamics and spatial pattern of *t48*. Here, the absence of a single site has a more significant impact than expected, implying that TF binding sites work synergistically in the *t48* enhancer [9].

Unlike the studies above that demonstrate the importance of a single TF binding site in an enhancer, another recent publication suggests that multiple sites work as a buffer, such that the effect of mutations in one site can be minimized by other nearby binding sites [19]. When a binding site for the repressor Giant is deleted from reporter constructs with the minimal *even-skipped* (*eve*) stripe 2 enhancer or the full *eve* stripe 2 enhancer, only the mutated minimal enhancer leads to expansion of the *eve* stripe 2 domain. The full *eve2* enhancer with the Giant site mutation maintains the same gene expression as the wildtype enhancer because the full enhancer contains other Giant binding sites that can buffer the effect of the mutation [19]. While the contribution of TF binding sites on transcription is different for each enhancer, these studies demonstrate that the arrangement of TF binding sites within an enhancer determines when, where, and how much a gene is expressed.

With technological advancements in microscopy, recent studies have focused on probing the dynamics of TF binding to target DNA and elucidating the mechanism of enhancer-mediated transcriptional regulation. It is suggested that the clustering ability of TFs facilitates their binding to enhancers (Figure 1C) [29-37]. Initially observed for proteins with strong intrinsically disordered regions (IDRs), protein clustering occurs in mammalian cells for many TFs and transcriptional machineries such as RNA Pol II, Mediators, and BRD4, all of which contain low complexity IDRs [32]. In *Drosophila*, a pioneer factor Zelda (Zld) and a pioneer-like factor GAGA Factor (GAF) form clusters with varying sizes and persistence times in the nucleus [33-35]. Interestingly, most Zld hubs do not colocalize with active transcription loci of the target genes like *snail* and *hb* but make frequent transient contacts with the target gene [33, 34].

Clustering of Zld facilitates binding of other TFs to low-affinity binding sites within enhancers [33, 34, 36, 37]. For example, despite the low concentration of a morphogen Bcd at the posterior side of a *Drosophila* embryo, substantial Bcd binding events occur in posterior nuclei because

Bcd forms dynamic hubs with Zld [37]. Images taken on a lattice light-sheet microscope demonstrate that Bcd and Zld form transient subnuclear clusters. The local concentration of Bcd within the hubs is higher than the overall nuclear Bcd level at the posterior side, allowing significant DNA binding. As a result, sharp *hbP2>MS2-lacZ* expression boundary is formed despite graded Bcd expression [33]. Similarly, addition of Zld binding sites to the *snail* enhancer accelerates the activation of the target reporter gene *MS2-lacZ*, as transient Zld clustering reduces the time required for pre-initiation steps [34]. Meanwhile, removal of Zld sites from the *short gastrulation* (*sog*) enhancer results in a narrower target gene expression domain, consistent with previous studies [36, 38]. Interestingly, the wild type *sog* enhancer, which contains three Zld binding sites, drives uniform gene expression, even if its activator Dl's nuclear concentration is graded along the dorsoventral axis of an embryo (Figure 1C). It is suggested that Zld recruits more Dl to bind to the enhancer, such that the nuclei with different concentrations of nuclear Dl can achieve a similar degree of Dl binding to the DNA [36]. Another study also shows that the recruitment of TFs like Twist and Dl to the *single-minded (sim)* enhancer allows stable and robust transcriptional activation, by "priming" the enhancer, even if the two proteins alone cannot directly activate *sim* [39]. Though most of the published studies show how clustering of Zld helps recruit other TFs, it is possible that more TFs will show the ability to cluster independently of Zld or GAF since many TFs contain low complexity IDRs [40].

Promoter specificity affects the kinetics of transcription

Independent of the TF binding site arrangement within enhancers, promoters also affect transcriptional properties. Recent papers using the MS2/MCP live imaging system have emphasized the role of promoter motifs in modulating "transcriptional bursting", a term that refers to the discontinuous nature of transcriptional activity involving intermittent active and inactive phases [41]. Transcriptional bursting is regarded as a common feature of transcription, having been observed across multiple species [42-44]. Altering core promoter motifs within the same enhancer and reporter gene construct induces significant changes in transcription bursting kinetics [10, 11]. For example, TATA-containing promoters produce stable transcription with high amplitude, while INR-containing promoters drive more intermittent and shorter transcriptional activity (Figure 1B) [10]. Constructs with different classes of promoters can be fitted into different mathematical models of transcriptional bursting; a two-state model for TATA- and a three-state model for INR-containing promoters. An additional inactive state related to Pol II pausing (multiple Pol II being stalled near promoters) is needed for INR-motifs, indicating that promoter motifs can modulate transcriptional initiation [10]. Similarly, mutations in the motif ten elements (MTE) and downstream promoter element (DPE) result in fewer transcriptional bursts, whereas GAGA site mutations induce moderate decreases in bursting amplitude and duration, supporting the distinct roles of each motif in transcriptional regulation [11]. Indeed, endogenous *fushi tarazu* (*ftz*) requires both the TATA and DPE motif for proper expression [11]. Taken together, core promoter elements affect distinct parameters of transcriptional bursting during *Drosophila* embryonic development by regulating different parts of the transcriptional machinery.

Enhancer-promoter interaction is a multivariate process

Enhancer-promoter interaction is a multivariate process that involves multiple enhancers and promoters (Figure 2). Each gene is typically regulated by multiple enhancers, ranging from 2 to 5 in *Drosophila*, and 5 to 20 in mammalian systems [45, 46]. For example, the *Drosophila eve* gene has 5 stripe-specific enhancers, each of which works independently, yet with similar kinetics, to drive the formation of the seven *eve* stripes [24, 47]. Similarly, early patterning genes like *snail*, *Krüppel*, and *knirps* have multiple enhancers that contribute to the final expression of the target gene [12-14]. Some of these enhancers have redundant functions [12-14]. Bothma et al. uses MS2/MCP live imaging to demonstrate that two "weak" *knirps* enhancers work synergistically with each other to produce more mRNAs than the summation of mRNAs produced from each enhancer, while "stronger" enhancers (*hb, snail*) work additively or even sub-additively [48]. Through smFISH, low-affinity *svb* enhancers are also shown to work cooperatively with each other by forming local transcriptional hubs [49, 50].

Although not as frequently observed as multi-enhancer-single-promoter interactions, in some cases, a single enhancer regulates multiple promoters [15-17]. A shared enhancer can activate two *cis*-linked promoters separated by a large distance (>15 kb). The two reporter genes tagged with MS2 stem loops show coordinated transcriptional trajectories, suggesting that the enhancer and promoters form a "transcription hub" [15]. One enhancer can co-activate two target promoters in an endogenous context as well. A shared enhancer co-activates *knirps related* & *knirps* and *scylla* & *charybde*, which are located about 20 kb and 250 kb away from each other, respectively [16]. More single-enhancer-multi-promoter contacts have been observed in other loci as well [17]. It is suggested that promoter-proximal tethering elements are responsible for the co-regulation (see later sections).

In some cases, interactions between two homologous alleles are found to affect transcriptional output. When driven by a strong enhancer, mRNA production of the target gene from homozygous embryos is significantly less than twice that of hemizygous embryos [51, 52]. In other words, the homologous alleles compete with each other and result in decreased transcriptional activity. It is suggested that the local concentration of TFs [51] or general regulatory elements like RNA Pol II or pre-initiation complex [52] within each transcription hub may work as limiting factors that induce allelic competition. These studies emphasize the complexity of elucidating all the parameters that contribute to the precise control of transcription.

Regulatory elements facilitate long-range enhancer-promoter interactions

Although many enhancers are located immediately upstream of the target gene, some enhancers interact with a distal target promoter [45]. For example, the *sex comb reduced* (*Scr*) and *scyl* enhancers are located 35 kb and 175 kb away from the target promoter, respectively [17]. Such long-range enhancer-promoter interactions are possible because the genome adopts a specific 3D conformation that allows the two regions to be in physical proximity despite their long linear distance [53]. Chromatin capture assays like 4C, Hi-C, and 5C reveal that chromatin is folded within a nucleus in specific structures known as topologically associating domains (TADs) [17, 54]. Chromatin interactions occur more frequently within TADs, whereas inter-TADs interactions are not as frequent, even if the two regions are close to each other in the linear genome. Through this 3D architecture, enhancer-promoter interactions within TADs are fostered, while ectopic promoter activations by enhancers outside the TADs are suppressed [55].

Chromatin is folded in a specific structure with the help of boundary elements that facilitate long-range chromatin interactions [56, 57]. *Drosophila* TAD boundaries and enhancer-promoter loops contain binding sites for many architectural proteins such as CCCTC-binding factor (CTCF), suppressor of hairy wing (SuHw), boundary element-associated factor of 32kD (BEAF32), Centrosomal protein 190kDa (Cp190) and Rad21. While the majority of architectural proteins are enriched in all enhancer-promoter loops, CP190 and BEAF32 sites are preferably observed in housekeeping genes, and more Fs(1)h and Rad21 sites are found in developmental genes [56]. Interestingly, CTCF, the most enriched boundary element in mammalian cells, is only found in around 8% of the domain boundaries in *Drosophila* larvae. Subsequently, the loss of CTCF has little effect on overall chromatin structure [57].

The effect of chromatin loops on transcription has been debated across species [58-60]. In *Drosophila*, most of the gene expression does not change significantly in highly rearranged balancer chromosomes, where TAD boundaries are rearranged and disrupted, indicating that transcriptional activities of many genes are impervious to topological changes [61]. Similarly, Micro-C on *Drosophila* embryos demonstrates that the 3D chromatin organization is maintained across different tissue types, despite tissue-specific chromatin states and gene expression [62]. These results suggest an indirect role of chromatin loops as a scaffold that facilitates enhancer-promoter interactions rather than directly driving transcriptional activation.

Yet, some genes, especially those near TAD boundaries, are significantly affected upon disruption of chromatin loops. These TAD boundaries are established by insulator elements, which are DNA sequences where insulator proteins like CTCF, Su(Hw), and Cp190 bind to. The insulator elements play an important role in allowing or blocking such long-range enhancer-promoter interactions [63-65]. Single-cell analysis with the imaging-based chromatin reconstruction assay, ORCA, reveals many aberrant enhancer-promoter interactions upon deletion of the CTCF- and Rad21- enriched *Front-Ultraabdominal* and *Frontabdominal-7* insulator elements (Figure 3A). Both deletions result in the fusion of the two neighboring TADs and subsequent ectopic gene expression and development defects [63]. Similarly, deletion of the SF1 boundary element between the *Scr* and *fushi tarazu* (*ftz*) loci results in weak ectopic *Scr* expression that overlaps with the *ftz* expression domain, indicating that the SF1 insulator normally blocks aberrant interactions between the *ftz* enhancer and the closely located *Scr* promoter [64]. It is postulated that a pairing between SF1 and a nearby boundary element SF2 is required to ensure proper *Scr* enhancer-promoter interactions [64, 66]. However, according to a recent study, endogenous *Scr* expression is not affected upon SF2 deletion, suggesting that SF1-SF2 pairing is dispensable for endogenous *Scr* expression [65].

Meanwhile, it is suggested that insulators mainly divide TAD boundaries and prevent ectopic enhancer-promoter interactions, while another regulatory sequence, distal tethering elements (DTE), mostly promote specific long-range enhancer-promoter interactions within TADs (Figure 3). DTE sequences are enriched with pioneer factors like Trithorax-like, Grainyhead, and Zld, and show minimal physical overlap with insulators [18]. DTE deletions at several genes abolish enhancer-promoter interactions, yet TAD structure is maintained (Figure 3B). On the other hand, insulator deletions result in the fusion of two neighboring TADs and induce non-specific enhancer-promoter interactions, similar to what is shown in other insulator deletion studies [63]. However, specific interactions between the enhancer and target gene are preserved despite TAD fusions. This study emphasizes distinct roles of insulators and tethering elements in genome organization and transcriptional regulation [18]. A recent study on a canonical insulator protein Cp190 also supports the view that enhancer-blocking and enhancer-pairing functions of boundary elements are independent of each other. Cp190 does not promote specific long-range enhancer promoter interactions but is important for blocking aberrant interactions [65]. It will be of future interest to examine the specific roles played by each boundary element on enhancer-promoter interactions and transcriptional regulation.

While not directly affecting the 3D genome topology, relatively "short-range" enhancer-promoter interactions occasionally require extra regulatory elements: "promoter proximal elements (PPE)", DNA sequences located close to the promoter and bound by specific proteins to mediate enhancer-promoter interactions [67, 68]. For example, early embryonic expression of *brinker* (*brk*) is determined by the 5' and 3' enhancers, located 10 kb upstream and 8 kb downstream of the promoter, respectively [67, 68]. Both enhancers need PPE to interact with the target promoter. While the PPE alone does not activate transcription, deletion of the PPE abolishes *brk* expression, indicating that PPE mediates enhancer-promoter interactions. PPE is thought to be required only for "intermediate-range" enhancer-promoter interactions since transgenic constructs with a *brk* enhancer right upstream of the promoter can drive the gene expression without PPE [67, 68]. Recently, it has been shown that the PPE is also required between the brkB enhancer and the *brk* promoter in *Drosophila* ovaries, where the brkB enhancer is located 8 kb away from the promoter [68].

Close enhancer-promoter proximity may not be required for transcriptional activation

As seen in the studies above, enhancer-promoter proximity has long been thought of as the key to transcriptional activation [17, 55, 63-65]. To examine the enhancer-promoter distance required for active transcription, Chen et al. inserts an insulator *homie* and a *PP7-lacZ* reporter gene 142 kb away from the endogenous *eve* locus (PP7 is another stem loop that works similarly with the MS2) [44, 69]. The exogenous *homie* interacts with the endogenous *homie* near the *eve* locus to foster *eve* enhancer's long-range interaction with the *PP7-lacZ* reporter gene. Enhancer-promoter proximity and transcriptional activity are monitored using the DNA and RNA labeling. The average enhancer-promoter distance of actively transcribing nuclei is about 350 nm compared to about 700 nm in non-active nuclei. This indicates that there may be a proximity threshold is required for transcriptional activation [69]. Similarly, an enhancer is able to *trans*-activate the target gene on the homologous allele when the two alleles are tightly associated within 200-300 nm from each other [70, 71]. This distance, however, is still considered larger than what is expected for traditional enhancer-promoter looping models [72].

Indeed, more studies show that while enhancer-promoter interactions are required for transcription, they do not need to be in close physical proximity to each other. During the zygotic genome activation of *Drosophila* embryos, many Zld-mediated enhancer-promoter, enhancer-

enhancer, and promoter-promoter loops are formed but physical proximity is not correlated with active transcription. In fact, many loops are formed before the TADs formation and subsequent transcriptional activation [73]. Recent studies also show that homologous alleles compete with each other to produce a reduced amount of mRNA per allele, yet there is no correlation between allelic distance and transcriptional output [52]. Lastly, using RNA Pol II immunofluorescence and dual-color RNA FISH, Huang et al. visualize RNA Pol II speckles and measure the distance between the two nearby actively transcribing genes, where two physically proximal genes occupy discrete RNA Pol II speckles rather than sharing a single shared Pol II cluster. Furthermore, varying physical distances between the two genes does not alter transcriptional output [74]. These findings are consistent with recent results from mammalian cells, which show no apparent correlation between the enhancer-promoter distance and transcription [75, 76].

Perspectives

- Disruptions in enhancer-promoter interactions drive ectopic or reduced gene expression, resulting in developmental defects and other phenotypes. The emergence of new imaging and molecular techniques in the past decade allows significant advancement in understanding the nature of enhancer-promoter interactions. In addition to analyzing one-on-one enhancer-promoter interactions, more studies start focusing on multivariate interactions between multiple enhancers and multiple promoters.

- Long-range enhancer-promoter interactions require additional regulatory elements such as insulators and tethering elements, which are thought to have distinct functions. Specific roles of different architectural proteins on 3D genome organization and their effect on transcriptional regulation need to be further examined.

- Physical proximity between an enhancer and the target promoter is required to activate the target gene. However, conflicting results on the correlation between proximity and transcription are reported, it is still unclear whether the proximity is needed only transiently or if a stable association is needed to maintain gene expression.

Competing Interests

The authors declare that there are no competing interests associated with the manuscript.

Author Contributions

H.D., G.J., and B.L. designed and wrote the manuscript. H.D. and G.J. generated figures.

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Figure Legends

Figure 1. The composition of enhancers and promoters affect transcriptional activity. (**A**) Deletion of the high- or low-affinity Dorsal binding site reduces the mRNA production by 90% and 50% compared to the wild type, respectively. Adapted from Keller et al. 2020 [9]. (**B**) Promoters containing TATA box motif (*sna*) produce stronger and more stable transcription compared to more stochastic transcriptional activity driven by INR-containing promoters (*Kr*). Adapted from Pimmett et al. 2021 [10]. (**C**) Schematics of the *sog* enhancer, which contains Dl and Zld binding sites, in nuclei with high (Left) and low (Center) Dl concentrations. Clustering of Zld recruits Dl and allows more Dl to bind to the enhancer independently of the nuclear Dl concentration. (Right) Schematic of a *Drosophila* embryo expressing uniform *sog* expression (orange) despite the difference in Dl concentration (red). Adapted from Yamada et al. [36].

Figure 2. Multiple enhancer-promoter interactions occur simultaneously. Multiple promoters can be regulated by one enhancer, while multiple enhancers can also initiate transcription at one promoter.

Figure 3. Regulatory elements that facilitate enhancer-promoter interactions. (**A**) Deletion of an insulator between two existing TADs results in merging of the neighboring TADs. The red marks show the focal contacts of enhancer-promoter interactions. Upon insulator site deletion, enhancer-promoter1 interaction within the original TAD is not disrupted, but ectopic enhancer-promoter2 interaction occurs. (**B**) Deletion of a tethering element does not affect the structure of TADs. Enhancer-promoter interaction is abolished upon deletion of the tethering element.

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