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Review

Mechanisms of enhancer-promoter interactions in higher eukaryotes

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Abstract In higher eukaryotes, enhancers determine the activation of developmental gene transcription in specific cell types and stages of embryogenesis. Enhancers transform the signals produced by various transcription factors within a given cell, activating the transcription of the targeted genes. Often, developmental genes can be associated with dozens of enhancers, some of which are located at large distances from the promoters that they regulate. Currently, the mechanisms that underly the specific distance interactions between enhancers and promoters remain unknown. This review describes the properties and activities of enhancers and discusses the mechanisms of distance interactions and potential proteins involved in this process.

1. Introduction

In higher eukaryotes, the regulation of gene expression is complicated as a consequence of cell differentiation during embryonic development [1–3]. Cell specialization is determined by differences in transcription factor (TF) repertoires, and the genes responsible for cell differentiation and organismal development are typically regulated by multiple independent enhancers, each of which stimulates a promoter in a limited population of cells during a specific time interval.

Enhancers were first described nearly 40 years ago when 72-bp tandem sequences from the SV40 virus were found to enhance gene expression when integrated at large distances from the promoter and in any orientation relative to the regulated gene [4,5]. Two years later, the first cellular enhancer was identified [6]. Currently, the human genome is predicted to encode 300,000 enhancers [7].

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Enhancers are regions of DNA, typically 100 to 1,000 bp in size, that contain TF-binding sites that stimulate the initiation and elongation of transcription from promoters [1,8–10]. In most housekeeping genes, enhancers are located in close proximity to promoters, and distinguishing the borders between these regulatory elements can be difficult. In contrast, developmental genes typically feature complex regulatory regions that can consist of dozens of enhancers located at variable distances from the regulated promoter.

During transcription activation, enhancers interact directly with gene promoters [8,11]. Currently, how chromatin loops are formed between enhancers and promoters and which architectural proteins are involved in this process remain unknown. This review provides an overview of the currently available information in mammals and *Drosophila* regarding enhancers and known architectural proteins that have been shown to support distance interactions between regulatory elements.

2. Enhancer properties and functions

Enhancers consist of combinations of short, comparative, degenerate sites, 6-12 bp in length, that are recognized by DNA-binding TFs, which determine enhancer activity [1,9]. The combination of DNA-binding TFs on a given enhancer creates a platform that attracts co-activators and co-repressors that determine the enhancer activity in each specific group of cells. In some cases, a single TF can act as both a positive and a negative regulator of the same enhancer, depending on the presence of other TFs. Thus, the ability of an enhancer to stimulate transcription depends on the combination of TF sites that positively or negatively affect enhancer activity and the relative concentrations of enhancer-binding TFs within the nuclei of a given group of cells.

Recently, super-enhancers have been identified, representing a special class of regulatory elements, characterized by large sizes, sometimes reaching tens of thousands of bp, with a high degree of TF and co-activator enrichment [12,13]. Super-enhancers are often located adjacent to genes known to be critical for cell differentiation [14]. A more detailed study of super-enhancers has shown that they often consist of separate domains that can either function together to enhance the overall activity of each domain or play independent roles during the simultaneous activation of a large number of promoters [15–17].

During the process of transcription activation (Figure 1), enhancers attract complexes that possess acetyltransferase (p300/CBP) and methyltransferase (Mll3/Mll4/COMPASS) activities

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[18]. The proteins Mll3 and Mll4 both contain a C-terminal SET (suppressor of variegation, enhancer of zeste, trithorax) domain, which is responsible for the monomethylation of lysine 4 of histone H3 (H3K4me1) [19,20]. The complexes formed by Mll3 and Mll4 have partially overlapping and insufficiently studied functions in the regulation of enhancer activity. Mll3 and Mll4 are also known to be involved in the recruitment of the p300/CBP co-activator, which is responsible for the acetylation of histone H3 at lysine 27 (H3K27ac) [21]. H3K27ac and H3K4me1 histone marks are distinctive features of active enhancers and are used to identify enhancers in genomes [22–26]. However, although mutations that inactivate the methyltransferase activity of the SET domains in the Mll3 and Mll4 proteins significantly reduce H3K4me1 quantities, they do not have strong effects on the functional activities of enhancers [27,28].

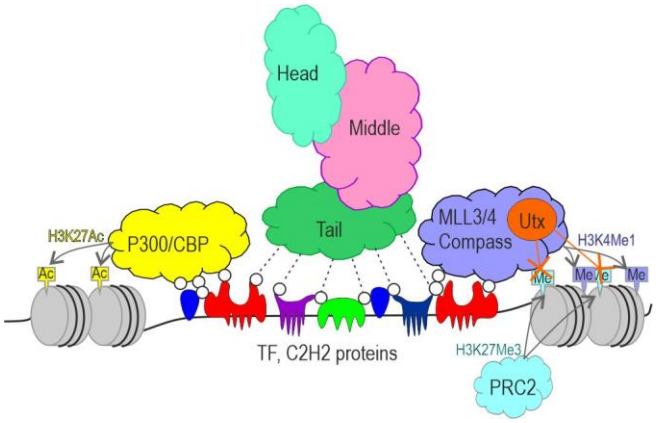

Figure 1. Schematic representation of the transcriptional complexes involved in enhancer activity. Various transcription factors (TFs) bind to enhancers sites and recruit complexes involved in transcription stimulation. p300/CBP possesses acetyltransferase activity and is responsible for H3K27 acetylation. The Mll3/4 complexes induce H3K4 monomethylation and recruit the UTX demethylase, which can remove the H3K27me3 associated with repressed chromatin. p300/CBP, Mll3/4, and UTX are thought to regulate transcription and enhancer activity through the modification of currently unknown components of primary transcriptional complexes. The subunits of the mediator complex form three main modules: head, middle, and tail. The mediator complex is recruited to the enhancer via multiple interactions between subunits of the tail module and the intrinsically disordered regions of TFs.

The H3K27ac and H3K4me1 modifications of histone H3 are thought to reduce the stability of nucleosomes [29], resulting in the formation of open chromatin and the stabilization

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of TF binding to enhancers. The Mll3/4 and p300/CBP complexes antagonize two Polycomb gene (PcG) complexes, Polycomb-repressive complexes 1 and 2 (PRC1 and PRC2, respectively), which are involved in the repression of enhancers and promoters [30]. PRC1 and PRC2 have ubiquitin transferase and methyltransferase activities, respectively, resulting in transcriptional repression [31]. The best-known activity of the PRC2 complex is the trimethylation of lysine 27 in histone H3 (H3K27me3), which is a characteristic marker of transcriptionally repressed chromatin regions. The Mll3 and Mll4 complexes are associated with UTX demethylase, which can remove the PcG-deposited H3K27me3 [18], allowing the lysine 27 in H3 to be acetylated by p300/CBP, preventing the trimethylation (H3K27me3) by PRC2 and stabilizing the active chromatin in the enhancer region. In addition to the acetylation of H3K27, p300/CBP may play an important but poorly understood role in the acetylation of transcription factors and serve as a component of the pre-initiation complex during transcription activation [32,33].

A highly conserved mediator complex, consisting of 25 subunits in yeast and 30 subunits in humans, plays a primary role in the enhancer-mediated activation of transcription [34]. The subunits found in the mediator complex form three main modules: head, middle, and tail. The head and middle modules determine the primary functions of the complex during transcription activation, whereas the tail module is responsible for interaction with the TFs bound to enhancers and gene promoters [35–39]. Together with general transcription factors assembled on the promoter, the mediator complex facilitates the assembly of a pre-initiation complex and is involved in the recruitment of RNA polymerase II to promoters [40,41]. However, many of the processes that involve the mediator complex during the activation of transcription remain insufficiently studied [42].

3. Distance interactions between enhancers and promoters

Most enhancers are located in close proximity to the promoters that they activate. However, even in the compact *Drosophila* genome, approximately 20% of enhancers are located at a distance of 50–100 kb from their associated promoters and can often be isolated by other genes [43,44]. In mammals, the distances between enhancers and promoters are typically even larger [8].

Many distance interactions between enhancers and promoters have been shown to be stable throughout *Drosophila* embryogenesis [44]. Multiple interactions have been observed between various enhancers and promoters with similar expression profiles. Similar results were obtained in mammalian cells, in which stable promoter-promoter and promoter-enhancer

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interactions were also identified [45,46]. Interactions between enhancers and promoters can be formed both before and simultaneous with transcription activation [44,47,48]. Super-enhancers have been shown to interact with a large number of promoters during cell differentiation [49]. Recently, the mediator complex was found not to be involved in the organization of distance interactions between enhancers and promoters [50], which is consistent with the finding that the mediator interacts only transiently with an enhancer [51].

The study of *β-globin* gene activation by a strong locus control region (LCR) enhancer in real time showed that enhancers activate transcription by increasing the frequency of intermittent transcriptional bursts [52]. Improving the stability of the interaction between the LCR and the β-globin gene promoter resulted in the increased frequency of transcription bursts. A similar study was performed on reporter genes in living transgenic *Drosophila* embryos [52], which revealed that strong and weak enhancers induce transcription bursts of equal strength, but the enhancer strength determines the burst frequency. Moreover, one enhancer can simultaneously, and in a coordinated manner, activate the promoters of two reporter genes but with different activation amplitudes [53].

These results are consistent with a model in which the stable interaction between an enhancer and one or more promoters allows the TFs bound to these regulatory elements to form a platform to which the mediator complex and other transcriptional co-activators can transiently bind (Figure 2). The unstructured acidic domains of TFs can interact with various subunits of the mediator complex, most of which are located in the tail module [54,55]. The efficiency of mediator recruitment is directly correlated with the number of TFs associated with enhancer-promoter sequences. During the transient binding with the enhancer-promoter complex, the mediator manages to induce only a short transcriptional pulse. The strength of transcription is determined by the burst frequency, which is directly dependent on the number of TFs that attract the mediator to the protein platform formed by the interaction between the enhancer and promoter.

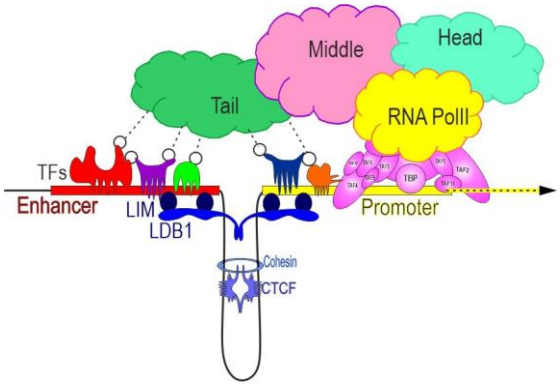

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Figure 2. Model showing the remote activation of a promoter by an enhancer. The interaction between an enhancer and a promoter can be stabilized by CCCTC-binding factor (CTCF), in cooperation with the cohesin complex and the dimerization of LOB domain-containing protein 1 (LBD1), which is recruited to chromatin through interactions with LIM proteins. The TFs bind to the enhancer and a promoter to form a platform for the transient recruitment of the mediator complex. The mediator complex transfers RNA polymerase II to the promoter transcription factor IID (TFIID) complex and accelerates further transcription initiation steps to induce a short transcriptional pulse (burst). The enhancer strength is directly correlated with the efficiency of mediator recruitment to chromatin.

4. Distance interaction mechanisms between enhancers and promoters in mammalian genomes

The genome is spatially organized into compartments and topologically associated domains (TADs), which are important for proper enhancer-promoter interactions in the nucleus [56]. All eukaryotic chromosomes are organized into TADs, which are formed by boundaries, and contacts between TADs occur with relatively high frequency [57–59]. TAD boundaries are defined based on preferred interactions, and no known physical barriers restrict trans-interactions between regulatory elements located in neighboring TADs [57,59–61]. However, the TAD architecture can facilitate interactions between regulatory elements located within the TAD by reducing the physical distance between these elements.

In mammals, most TAD boundaries contain binding sites for CCCTC-binding factor (CTCF) [62]. A characteristic feature of CTCF is the presence of a cluster, consisting of 11 C2H2-type zinc finger domains [63]. Five C2H2 domains in CTCF specifically bind to an extended DNA motif that is conserved in most animals [64]. The N-termini of CTCF homologs from representative bilaterian species feature unstructured domains that are capable of homodimerization [65]. A motif that interacts with the cohesin SA2-SCC1 sub-complex was identified between the N-terminal homodimerization domain and the C2H2 cluster [66].

Together with the cohesin complex, CTCF defines the boundaries of most TADs [11,59,67]. The CTCF/cohesin complex is also involved in organizing interactions between enhancers and promoters within TADs [68,69]. CTCF inactivation resulted in the re-localization of cohesin complexes from the CTCF binding sites to the promoters of active genes, which was

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accompanied by the partial disruption of the TADs [70]. Mutations in CTCF that disrupt the interaction with the cohesin complex also result in the loss of some distance interactions and a decrease in the efficiency of TAD formation [66,71].

Only a small fraction of CTCF sites located in the opposite convergent orientation are involved in TAD organization [72,73]. To explain the preferable formation of chromatin loops between CTCF sites located in a convergent orientation, a model was proposed in which the cohesin complex binds to the chromosome and initiates the extrusion of DNA, resulting in the formation of a chromatin loop. CTCF can block the progression of the cohesin complex when the interaction between its N-terminal domain and the SA2-SCC1 sub-complex [66] is oriented correctly relative to the moving cohesin complex. The dimerization between the N-terminal domains of the CTCF protein is thought to stabilize the formation of chromatin loops [65].

The inactivation of either cohesin or CTCF typically results in only the partial disruption of chromatin loops [70,74], and some interactions between enhancers and promoters have been found to occur independently of cohesin and CTCF [75,76]. The existence of other architectural proteins has been proposed, which may also be involved in the organization of distance interactions, together with CTCF. To date, only a few proteins have been described that can potentially participate in the organization of chromosome architecture in mammals [11]. Two of these proteins, zinc finger protein 143 (ZNF143) and Yin Yang1 (YY1), bind DNA using C2H2 domains.

ZNF143 shares similarity with CTCF and the central region of ZNF143 contains a cluster that consists of seven C2H2 domains, three of which bind to a specific CCCAGCAG motif [77]. The N-terminal domain contains three 15-aa repeats with unknown functions, and the C-terminal domain is enriched in acidic amino acids, which is typical of transcription activators. ZNF143 is essential for embryonic development in mammals [78]. ZNF143 functions in the promoter region by participating in the formation of open chromatin regions and the recruitment of complexes that activate transcription [79]. Genome-wide studies have shown that ZNF143 can participate in the formation of chromatin loops between enhancers and promoters [80–82]. In human HEK293T cells, ZNF143 functions together with CTCF to form chromatin loops at several genomic sites [82]. However, in contrast to CTCF, no experimental evidence has suggested that ZNF143 participates in the localization of the cohesin complex on chromatin. Thus, how ZNF143 supports specific distance interactions remains unknown.

The mammalian YY1 protein, which consists of only 414 amino acids, belongs to a multifunctional, evolutionarily conserved family of mammalian transcription factors and contains 4 C2H2 domains at the C-terminus [83]. *Drosophila* expresses two homologs of the YY1 protein, PHO and PHOL, which are involved in the recruitment of Polycomb proteins [84]. The N-terminal region of YY1 has been implicated in transcriptional activation, whereas the domain between 201-226 amino acids is involved in the recruitment of PcG proteins that are responsible for repression [85–87].

YY1 is predominantly associated with gene enhancers and promoters, which is consistent with a potential role in distance interactions [88,89]. The inactivation of YY1 results in a marked decrease in the number of distance interactions *in vitro* [88]. According to the proposed model, YY1 forms homodimers, which can bring the associated gene enhancers and promoters closer together. A cluster of C2H2 domains and an adjacent unstructured domain are responsible for the dimerization and oligomerization of YY1 [90,91]. Moreover, the dimerization and subsequent oligomerization of YY1 results in non-specific binding to DNA, especially with guanine quadruplexes (G4). Some experimental evidence has suggested that the dimerization of YY1 and the subsequent binding to G4 structures contribute to the YY1-mediated formation of long DNA loops [91]. YY1 may also participate in the organization of distance interactions through the regulation of proteins that are directly involved in the formation of chromatin loops. For example, YY1, together with Oct4, participates in the recruitment of the BAF remodeling complex to promoters and super-enhancers [92]. BAF can improve TF binding and stabilize the chromatin loops formed by CTCF/cohesin [89]. Therefore, YY1 appears likely to regulate enhancer activity and enhancer-promoter interactions through epigenetic mechanisms [93].

The role of the small protein LIM domain-binding factor 1 (LDB1) in the maintenance of distance interactions between enhancers and promoters has been studied in detail [94]. Unlike C2H2 proteins, LDB1 binds to enhancers and promoters through the interaction between its C-terminal domain and DNA-binding TFs that belong to the LIM family [94]. Through interactions with various LIM partners, LDB1 plays roles in several regulatory processes during embryonic development and cell differentiation, including erythropoiesis. Initially, the N-terminal domain of LDB1 was shown to be involved in the organization of interactions between a strong enhancer (LCR) and the promoters of the *beta-globin* locus [95,96]. Structural analysis showed that the N-terminal domain of LBD1 contains both alpha helices and beta sheets, which form a stable homodimer [97,98]. TFs in the LIM family predominantly bind to gene enhancers and promoters, facilitating the recruitment of LDB1 to these regulatory elements. According to the

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model, specific interaction between the N-terminal domains of LBD1 molecules associated with enhancer and promoter elements can stabilize distance interactions between these regulatory elements. Interestingly, LDB1 not only homodimerizes but can also interact with CTCF, which can promote the organization of contacts between enhancers and promoters associated with a large group of erythroid genes [99]. A small domain in LDB1, located near the N-terminal dimerization domain, interacts with an unidentified C2H2 domain in the CTCF protein. However, whether the interaction between the LBD1 domain and the C2H2 domain in CTCF is capable of forming a sufficiently stable and specific interaction between enhancers and promoters to regulate gene expression remains unknown. Recently, mutations in LDB1 that disrupt dimerization were shown to have no effect on the transcriptional activation of *beta-globin* genes [98], which suggested the existence of additional mechanisms to support the interaction between the LCR enhancer and *beta-globin* promoters. Therefore, LBD1 likely acts in cooperation with other unidentified proteins to support distance interactions.

5. Specific activation of olfactory receptor genes in mammals is supported by super-long-distance interactions between enhancers and promoters

The most interesting models for understanding the mechanisms of distance interactions are proven examples of interactions between enhancers and promoters that are separated by megabase distances. In mammals, expression mechanisms associated with a large family of genes encoding olfactory receptors have been well-studied [100]. In the mouse genome, approximately 1,100 genes encoding olfactory receptors (ORs) have been identified [101,102]. These genes are located in 40 clusters that are scattered throughout the mouse genome. Olfactory sensory neurons (OSNs) are derived from progenitor cells, in which all *OR* genes are very weakly co-expressed. Constitutive heterochromatin is formed on inactive *OR* genes, which are enriched in H3K9me3 and H4K20me3 histone modifications [103]. During OSN maturation, the transcription of one *OR* gene is activated randomly, while all others remain completely repressed [104]. A negative feedback loop likely exists, in which the strong expression of one OR receptor results in the complete inactivation of all other *OR* genes [105–107]. However, the exact mechanism associated with this process remains poorly understood.

Each cluster of *OR* genes has a nearby enhancer, which is involved in the selection of one gene that will be actively transcribed in a particular OSN [108,109]. A total of 14 specific enhancers have been identified, and the *in vivo* deletion of three of these enhancers has been

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shown to result in the complete inability to activate any of the *OR* genes encoded in the corresponding cluster [108,110–112]. A number of studies have shown that enhancers form a single cluster in the nucleus, which regulates the activation of a single selected *OR* gene [112], and all other genes form heterochromatin. Thus, active chromatin appears to be generated by the interactions between enhancers and the promoter of a single *OR* gene that is encoded in close proximity to clusters of *OR* genes to organize heterochromatin regions.

The mechanisms that underly the physical separation and stable balance between regions containing both active and repressed chromatin remain poorly understood. *OR* gene promoters and enhancers contain binding sites for Ebf and Lhx2, which are specific TFs expressed in neurons [20,109,112]. According to the model [100], the formation of a cluster of enhancers can increase the efficiency of Ebf and Lhx2 recruitment, resulting in the significant enrichment of transcription activators associated with the active *OR* promotor compared with the promotors associated with repressed *OR* genes (Monahan et al., 2017). A high concentration of activators can also prevent the spread of heterochromatin to active promotor regions associated with enhancers.

The most important aim is the identification of proteins that can support the specific super-long-distance interactions between enhancers that form a single cluster. CTCF and cohesin were not identified in the regulatory regions of *OR* genes. However, Lhx2 has been shown to recruit LDB1 to *OR* enhancers, and the inactivation of LBD1 has been shown to result in the partial disruption of *OR* enhancer colocalization in the nucleus [113]. These results suggest a potential role for LBD1 in the organization of super-long-distance interactions among *OR* enhancers. However, the most likely scenario is that several unknown architectural proteins are involved in the initiation and maintenance of distance interactions and that LBD1 facilitates their functions.

6. Super-long-distance interactions in the *Drosophila* genome and the role of architectural proteins during this process

In *Drosophila*, super-long-distance interactions between regulatory elements were first described when studying the effects of an insulator, found in the *gypsy* retrotransposon, on reporter gene repression induced by a Polycomb-dependent silencer [Polycomb response element (PRE)] [114]. This study used a 660-bp PRE, found in the regulatory *bxd* region of the homeotic *Ubx* gene of the *bithorax* locus (*bxd* PRE). Typically, pairing two PRE-containing transgenes results in the increased repression of the reporter, which is likely associated with an increase in

the recruitment efficiency of PcG complexes [31]. The combination of two transgenes that both contained PREs and the *gypsy* insulator resulted in the significant repression of reporter genes [114], despite these genes being separated by several megabases or even located on different chromosomes.

The interaction between *gypsy* insulators can facilitate a functional interaction between an enhancer and a promoter that are located in association with various transgenes that are integrated into genomic sites separated by several megabases [115]. These results strongly suggested that interactions between *gypsy* insulators can support stable interactions between genome regions separated by very large distances.

A similar study was performed for transgenes containing the Miscadestral pigmentation (*Mcp*) boundary, which separates the regulatory domains that control the expression of the homeotic genes *abd-A* and *Abd-B* of the *bithorax* complex [116]. The *Mcp* boundary consists of an insulator, which is flanked on both sides by Polycomb-dependent silencers. The functional interaction between transgenes, which are inserted in different regions of the third chromosome, was investigated. The results indicated that transgenes located at a distance of several megabases, and in some cases on different arms of the same chromosome, can induce the mutual enhancement of repression, which suggested that they were able to interact physically. In a similar study, the *bxd* PRE, in combination with the 210-bp core of the *Mcp* insulator, was able to support repression between two transgenes located at super-long-distances [117]. The co-repression of reporters and their colocalization in the nucleus were observed only in the presence of the *Mcp* insulators for both tested transgenes [117–119].

Super-long-distance interactions can be also be maintained by the *Frontadominal-7* (*Fab-7*) boundary, which separates the regulatory domains responsible for the activation of the *Abd-B* gene in the *bithorax* complex [120]. At various genomic sites, a 3.6-kb DNA fragment that included the *Fab-7* boundary and an adjacent PRE functionally interacted with endogenous *Fab-7* [120]. The pairing between the 1,250-bp *Fab-7* insulators was also able to support super-long-distance interactions between transgenes in the *Drosophila* genome [117,121].

Two insulators, *Nhomie* (*Neighbor of Homie*) and *Homie* (*Homing* insulator at *eve*), were identified at the 16-kb boundaries of the regulatory region of the *even-skipped* (*eve*) gene (Fujioka et al. 1999; 2009; 2016). *Nhomie* and *Homie* interact with each other and can also maintain super-long-distance interactions between the transgene and the endogenous *eve* locus,

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which allows endogenous enhancers to activate the reporter gene promoter in the transgene [122,123].

The interaction specificity between identical regulatory elements is also manifested in the "homing" phenomenon, in which the *P*-transposon, which contains either an insulator or a promoter region, is inserted with high frequency into the genomic region where this regulatory element is located. For example, the *P*-transposon that contains the promoter region of *engrailed* or *linotte* was predominantly inserted (20%–30%) into the corresponding genes [124,125]. Similar results were obtained upon integration into the genome of the *P*-transposon containing the *Fub* boundary, which separates the regulatory regions of the *Ubx* and *abd-A* genes in the *bithorax* complex [126]. The *Fub* boundary between two TADs formed from the architecture of the 300,000-bp *bithorax* locus [127]. The "homing" effect occurs due to the interaction between architectural proteins, which is associated with two identical regions and directs the integration of the *P* transposon into the region of the corresponding gene.

In all of these examples, super-long-distance interactions were supported by a pair of identical regulatory elements that were unique to the genome. The *gypsy* insulator consists of 12 sites that bind the Su(Hw) protein [128–130]. In the genome of most *Drosophila* lines, the *gypsy* retrotransposon is only found in heterochromatin [131]. Only a small portion of the several thousand proteins with identified Su(Hw) binding regions contain 2-3 binding sites [132,133]. Thus, the *gypsy* insulator is unique due to a large number of Su(Hw) binding sites. According to ModEncode, the *homie* insulator contains binding sites for the *Drosophila* homolog of CTCF (dCTCF), Su(Hw), and GAF proteins [134]. The *Mcp* boundary contains binding sites for dCTCF and Pita [135]. Similar to dCTCF, Su(Hw) and Pita contain clusters of C2H2 domains, some of which facilitate the specific binding of these proteins with long DNA motifs [132,136,137]. The *Fub* boundary contains binding sites for CTCF, Su(Hw), and Pita [135,138,139] (Bender and Lucas 2013, Kyrchanova et al. 2017; 2020). Using *in vivo* deletion analysis, additional unknown proteins are also determined to be involved in determining the boundary function of *Mcp* and *Fub* [139].

Finally, the *Fab-7* boundary has been subdivided into three domains, which coincide with mapped DNase 1 hypersensitivity sites [140–142]. HS2 contains two Pita sites [135], whereas the central HS1 domain was found to contain six sites for the GAF protein that overlap with long, degenerate binding sites for the newly identified late boundary complex (LBC) DNA-binding complex [143], which is likely to be involved in the regulation of distance interactions between enhancers and promoters [135,144]. Thus, several proteins likely function together to

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organize active boundaries/insulators that are capable of supporting super-long-distance interactions.

In transgenic model systems, the pairing between two copies of repetitive binding sites for dCTCF, Su(Hw), or Pita was able to bring the yeast GAL4-dependent activator region and the reporter gene promoter in close proximity, resulting in the transcription of the reporter gene [145–147]. The dCTCF N-terminus contains an unstructured domain that can form tetrameric complexes [65,148], which can contribute to distance interactions. Similarly, Pita contains an N-terminal zinc-finger-associated domain (ZAD) capable of forming homodimers [147]. ZADs have also been identified in the N-termini of approximately one hundred *Drosophila* proteins containing clusters of C2H2 domains (ZAD-C2H2) [149,150]. An important feature of ZADs is their preferentially ability to homodimerize into an antiparallel dimer [147,151]. In addition to Pita, several other ZAD-C2H2 proteins, including ZIPIC (Zinc-finger protein interacting with CP190), Zw5 (Zeste-white 5), and ZAF1, have been shown to support distance interactions and form functional insulators [137,152–155]. Mutational analysis, *in vivo*, showed that the presence of ZADs determined the ability of these proteins to support distance interactions [154,155]. In transgenic lines, various combinations of reiterated binding sites from different ZAD-C2H2 proteins were unable to support the distance activation of the reporter gene by the GAL4 activator [145,147]. Thus, the homodimerization of ZAD regions is an important feature required to support specific distance interactions between ZAD-C2H2 proteins.

The C2H2 proteins, including Pita, dCTCF, and Su(Hw), can recruit chromatin to BTB (bric-a-brac, tramtrack, and broad complex)-containing proteins, such as Mod(mdg4) and CP190 [137,156–158]. The CP190 protein contains a classical N-terminal BTB domain that forms homodimers and is conserved among higher eukaryotes [159–161]. In contrast, the BTB domain of Mod(mdg4)-67.2 belongs to an insect-specific group [162,163]. The BTB domains in this group of proteins can form both homo- and heteromultimeric complexes [161]. Previously, both CP190 and Mod(mdg4)-67.2 were thought to be capable of participating in the formation of distance interactions between regulatory elements and Su(Hw)-dependent insulators [164,165]. Both proteins are required for the functional activity of the *gypsy* insulator [158,166–168]. However, the CP190 and Mod(mdg4)-67.2 can also interact with a large variety of DNA-binding proteins [137,148,156–158,169–171], which is inconsistent with their key role in the organization of specific distance interactions between *gypsy* insulators. Most likely, CP190 and Mod(mdg4)-67.2 participate in the stabilization of previously formed specific distance interactions that occur between other regulatory elements.

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7. Conclusion

Additional study is necessary to better understand the mechanisms of distance interactions between enhancers and promoters. The homodimerization that occurs between the N-terminal domains of LDB1, which is recruited to regulatory elements through interaction with LIM proteins, has been best-described to support the maintenance of interactions between enhancers and promoters [172]. Simultaneously, evidence has suggested that LBD1 supports remote interactions between currently unknown partners. The mammalian CTCF protein contains binding sites that would allow it to interact simultaneously with enhancers and promoters associated with a number of genes, and, together with the cohesin complex, CTCF appears to be involved in facilitating distance interactions between these regulatory elements [68,69].

In *Drosophila*, identical insulators, consisting of specific combinations of binding sites for a variety of C2H2 proteins, are able to interact at large distances. According to the current model [150], many C2H2 proteins predominantly homodimerize, typically through N-terminal domains, which ensures the specificity and stability of interactions between identical insulators that are located at large distances from each other. The identification of binding sites for currently uncharacterized human and *Drosophila* C2H2 proteins will facilitate the assessment of the true contributions of this class of proteins to the organization of the chromosomal architecture. In addition, the use of gene-editing techniques, such as the (CRISPR)/Cas9 method, will allow the role of each identified C2H2 protein in the maintenance of distance interactions to be assessed using model regulatory systems.

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Abbreviations

TF Transcription Factor

CBP CREB-binding protein

Mll3/4 Mixed Lineage Leukemia 3/4

UTX demethylase

PcG Polycomb Group

PRC1/2 Polycomb repressive complexes 1/2

CTCF CTC binding factor

ZNF143 Zinc Finger Protein 143

LBD1 LIM domain – binding factor 1

CP190 Centrosomal protein 190kD

Mod(mdg4) Modifier of mdg4

CRISPR the clustered regularly interspaced short palindromic repeats

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