The Variable CTCF Site from Drosophila melanogaster Ubx Gene is Redundant and Has no Insulator Activity

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Abstract—CTCF is the most thoroughly studied chromatin architectural protein and it is found in both Drosophila and mammals. CTCF preferentially binds to promoters and insulators and is thought to facilitate formation of chromatin loops. In a subset of sites, CTCF binding depends on the epigenetic status of the surrounding chromatin. One such variable CTCF site (vCTCF) was found in the intron of the Ubx gene, in close proximity to the BRE and abx enhancers. CTCF binds to the variable site in tissues where Ubx gene is active, suggesting that the vCTCF site plays a role in facilitating contacts between the Ubx promoter and its enhancers. Using CRISPR/Cas9 and attP/attB site-specific integration methods, we investigated the functional role of vCTCF and showed that it is not required for normal Drosophila development. Furthermore, a 2161-bp fragment containing vCTCF does not function as an effective insulator when substituted for the Fab-7 boundary in the Bithorax complex. Our results suggest that vCTCF function is redundant in the regulation of Ubx.

Keywords: insulators, CTCF, Bithorax complex, Ubx

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Parasegment-specific expression of the Ubx, abd-A, and Abd-B homeotic genes in the Drosophila melanogaster Bithorax complex (BX-C) is controlled by nine autonomous regulatory domains, which are separated by special elements called boundaries or insulators [1]. Boundaries ensure autonomy by blocking contacts between regulatory elements in one domain with regulatory elements in adjacent domains. Boundaries can also prevent enhancers from interacting with promoters [2–4]. In addition to insulator activity, some boundaries have an ability to specifically interact with their target gene in BX-C, enabling enhancers in distant regulatory domains to stimulate their target promoter [5]. These properties of the boundaries ensure correct parasegment-specific expression of the BX-C genes during Drosophila development. Consistent with this idea, Fab-6, Fab-7, and Fab-8 were shown to specifically interact with the promoter upstream region of Abd-B gene [6]. It is likely that this interaction determines the correct topological positioning of the corresponding regulatory domains (iab5 — iab7) with Abd-B promoter in parasegments 10—12.

Most of the BX-C boundaries contain binding sites for Drosophila CTCF (dCTCF), and these sites are important for the insulator activity of these boundaries (Fig. 1) [7]. In the intron of the Ubx gene 30 kb downstream from the promoter, a variable dCTCF binding site (vCTCF) was identified (Fig. 1) [8]. dCTCF does not occupy this site in tissues where Ubx is inactive (imaginal discs of the first pair of legs), but binds to it when the Ubx gene is transcriptionally active (imaginal discs of the third pair of legs). Moreover, dCTCF binding to vCTCF is associated with changes in the topology of the abx/bx regulatory domain: in tissues where Ubx is active an increase in the frequency of vCTCF contacts with the Ubx promoter is observed [8]. A model was proposed according to which binding of dCTCF to vCTCF facilitates tissue-specific interaction of the abx, BRE enhancers with the Ubx promoter [9, 10]. The aim of this study was to test this hypothesis.

To study vCTCF function in enhancer-promoter interactions, we used the CRISPR/Cas9 system to delete a 3408-bp DNA fragment (3R:16701239..16704646) that spans the vCTCF site and the bx PRE (polycomb response element) 1 kb downstream, and in its place we introduced an attP site (Δ3.4attP, Fig. 1). Flies homozygous for Δ3.4attP deletion show evidence of variable LOF transformations. The deletion transforms the anterior third thoracic segment toward the anterior second thoracic, a phenotype known as bithorax (bx) [11, 12]. In mutant flies the anterior third leg

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resembles the second leg, and in ~10% of flies anterior notal tissue is present on the dorsal surface of the third thoracic segment (Fig. 2). These transformations are caused by a disruption in the interactions of enhancers downstream of \( vCTCF \) with \( Ubx \) promoter. The \( \Delta 3.4^{mp} \) deletion overlaps with a previously described 9.5 kb deletion, \( bx^{34e-prv} \). Like \( \Delta 3.4^{mp} \), it also has a variable \( bx \) phenotype which is caused by a decrease in \( Ubx \)
expression in the imaginal discs of segment T3 [11]. Next, we used attP site in Δ3.4int as an integration platform to find minimal element that can rescue the mutant phenotype. We carried out attP-attB mediated integration of the 831-bp bx PRE fragment (PRE831, 3R:16702487..16703317) into Δ3.4int deletion and discovered that PRE831 completely reverts bx phenotype to wild type. This finding suggests that vCTCF is redundant, while bx PRE may play a role in facilitation of enhancer-promoter interaction.

In order to test vCTCF insulator activity we used Fab-7intP50 replacement platform (Fig. 1). In this platform, Fab-7 boundary is removed, resulting in the fusion of iab-6 and iab-7 regulatory domains. This leads to ectopic activation of the iab-7 regulatory domain in PS11, which in turn results in the loss of the sixth abdominal segment in adult males [13–15]. It was demonstrated previously that PREs are often located in close proximity to insulators and contribute to the formation of a functional boundary [16, 17]. Therefore, a fragment containing both bx PRE and vCTCF in reverse orientation, vCTCF+PRE (2161-bp, 3R:16702487..16704647) was tested in Fab-7intP50. We found that the 6th abdominal segment is still missing in males carrying vCTCF+PRE insertion. This finding indicates that the vCTCF+PRE sequence does not have insulator activity.

Altogether, our data do not support a model in which vCTCF is a necessary mediator of enhancer-promoter interactions in abx/bx domain. Moreover, the data suggest that the bx PRE may play that role. However, further research is needed to explore the functions of this element in Ubx regulation. Since the loss of the bx PRE leads only to a variable LOF phenotype, it can be assumed that, in contrast to the Abd-B enhancers, Ubx enhancers are much more autonomous and less dependent on other regulatory elements to form appropriate promoter contacts.

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COMPLIANCE WITH ETHICAL STANDARDS

Conflict of interest. The authors declare that they have no conflicts of interest.

Statement on the welfare of animals. All applicable international, national, and/or institutional guidelines for the care and use of animals were followed.

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