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# PRIMARY AND SHADOW ENHANCERS OF DORSAL - VENTRAL PATTERNING GENES IN *DROSOPHILA MELANOGASTER*

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**ABSTRACT**

**Recent studies have revealed that multiple enhancers can activate the transcription of a gene. They can be classified as primary or shadow enhancers. Earlier studies suggested that many dorsal target genes of *Drosophila melanogaster* might be regulated by these shadow enhancers. Therefore, primary and shadow enhancers of five different dorsal targeting genes viz., *zen, sim, vnd, bun,* and *rho* were predicted in this study. In addition, novel cluster motifs with high strand scores have been predicted and are referred to as primary enhancers, whereas similar sequences within 5kb are referred to as shadow enhancers. The information generated from this study may lead to the evolutionary innovation of enhancer mechanisms.**

**Key words:** *Drosophila melanogaster*, enhancers, transcription factors, in-silico, gene expression, activator, repressor, flanking sequence, cluster motifs

In the world of insects, *Drosophila* has been studied widely for genetic research as an ideal model for understanding how these variations and regulations contribute to its development. The gene regulatory mechanism of controlling dorsal-ventral axis formation in insects has undergone drastic changes in the field of evolution (Kapil and Kaur, 2021). This process in the *Drosophila* is mainly regulated by the dorsal protein which establishes different expression patterns in dorsal-ventral patterning by binding or activating different transcription factors. Dorsal activates twist protein in the mesodermal region and these two maternal factors further activate snail that limits the expression of dorsal in the mesodermal region. (Jiang et al., 1991). These three binding factors bind in the cluster to activate different sets of the gene (Ip et al., 1992; Zeitlinger et al., 2007). The Suppressor of hairless (Su (H)) and Zelda (vfl) also play important role in the expression of the DV patterning genes (Jiang and Levine, 1993). Both the transcription factors control the positioning of the dorsal gene boundary of the DV axis (Ozdemir et al., 2014).

Earlier, studies observed that in addition to a primary enhancer that is well defined, many genes have secondary elements with very similar transcription factor binding clusters (Hong et al., 2008; Scholes et al., 2019; Waymack et al., 2020). These secondary factors or elements are known as shadow enhancers. Shadow enhancers overlap the activity of primary enhancers and arise from duplication and separation of protein-coding sequences. Genetic repetition has long been observed as a prospective source of evolutionary alteration (Krischner and Grehart, 1998). These provide sturdiness genetic variation of a population, allow them to develop undisturbed (Cannavo et al., 2015). In vitro studies have revealed that many dorsal target genes are regulated by primary and shadow enhancers even when they are located far away from the target genes (Hong et al., 2008; Guerrero et al., 2010; Small and Arnosti, 2020). However, only a few of the shadow enhancers were predicted to be located on the flanking regions of the genes. Indeed, it is necessary to study these primary and shadow enhancers in flanking regions of target genes for understanding evolving novel expression patterns. Hence the current study was designed to predict the locations of primary and shadow enhancers of dorsal-ventral patterning genes (*Zerknullt, Single-minded, Ventral nervous system defective, Bunched* and *Rhomboid*) of *Drosophila* and the clusters of TFs (dorsal, twist, snail, Zelda, and Suppressor of hairless) by the use of computational methods to understand the evolution in enhancers mechanism. This study attempts to look into the primary and shadow enhancers in the 20 kb flanking regions of the target genes using in silico approach.

## MATERIALS AND METHODS

Gene Sequences were obtained in FASTA format from Flybase version FB2019_06. Genes are symbolized by their FlyBase ID as shown in Table. These sequences were obtained and modified in the year of 2019-2020. These sequences are modified in 20kb flanking regions through BLAST tool. The binding motif sequences of

dorsal, twist, snail, Zelda, and Suppressor of hairless were obtained from the JASPAR database. It is an open-access database that contains transcription factor binding profiles as position frequency matrices (PFMs) and TF flexible models (TFFMs) for TFs across multiple species in six taxonomic groups (Khan et al., 2018). Flanking gene sequences and motifs were uploaded to the Cluster- Buster software to predict binding sites and binding site clusters. The nucleotide sequence patterns, motifs preferentially bound by different transcription factors have been collected in this database (Martin et al., 2003). This is used for finding different clusters for pre-specified motifs in DNA sequences. In this database, the gene and motif sequences were submitted and results showed the location of the gene, size of predicted enhancer (primary and shadow), and binding sites. We arbitrarily refer to the newly identified enhancer as the shadow enhancer having similar binding sites but with a low strand score as the primary enhancer with a strong strand score of transcription factor binding clusters.

## RESULTS AND DISCUSSION

In this study, the location of primary and shadow enhancers, size of the enhancer, binding sites clusters of different transcription factors have been analyzed. In order to predict primary and shadow enhancers, similar clusters of transcription factor binding sites (TFBS) on target genes were identified using Cluster-Buster tool. The target gene sequences were collected in FASTA format from Flybase version FB2019. Using the BLAST tool, these gene sequences were modified into a 20kb flanking region. PWM, transcription factor binding motifs format were derived from JASPAR. These 20kb flanking sequences and PWM for motifs were uploaded into the Cluster-Buster software version of year 2019-2020 for the identification of TFBS. The generated output data is summarized in Table 1. In this study, the *zen* gene showed the binding sites for twi, snail, and dl factors. Both the primary and shadow enhancers were located downstream to the TSS of the gene as depicted in Fig. 1. The size of the predicted primary enhancer was found to be 487 bp and that of the shadow enhancer was 181 bp. The *sim* gene showed binding sites for dl, twi, vfl, Su (H), and snail. The size of the predicted primary enhancer was 340 bp and that of the shadow enhancer was 245 bp. Both the primary and shadow enhancers were located downstream to the TSS of the gene as shown in Fig. 2. Apart from the three known binding sites (dl, twi, sna), this study has identified two more binding sites *viz*. Su (H) and vfl which further controls the expression of the *sim* gene. The binding of Su(H) up-regulates sim expression in the mesectoderm and prevents the uprooted expression of the sim gene dorsally in the neuroectodermal region (Morel and Schwiesguth, 2000). *Vnd* gene showed the binding sites for dl, snail, and twi transcription factors. The size of the predicted primary enhancer was 756bp and that of the shadow enhancer was 187 bp.

The primary enhancer was located downstream of the TSS whereas the shadow enhancer was lying downstream just near to the TSS (Fig. 3). *Bun* gene showed the binding sites for dl, twist, and snail. The size of the primary enhancer was 400 bp and the size of the secondary enhancer was 412 bp. Both the enhancers were lying downstream of the TSS (Fig. 4). In the current study, the *Rho* gene showed binding sites for dl, twi, snail, and vfl. The primary enhancer was 544 bp in size and the secondary enhancer was 150 bp in size. Both predicted enhancers located upstream of the TSS as shown in Fig. 5. This study has identified primary and shadow enhancers of five different dorsal targeting genes viz. *zen, vnd, rho, sim*, and *bun*. To validate this prediction, primary enhancer sequences were aligned with verified enhancers in the Redfly

Table 1. Gene sequences, Flybase IDs, used for enhancer prediction, and size of the enhancer

| Gene | Gene Sequence | Flybase ID | TSS | Location of enhancer from TSS of the gene | | Size of the enhancer | | Figure no. |
|---|---|---|---|---|---|---|---|---|
| | | | | Primary | Shadow | Primary | Shadow | |
| *Zerknullt (zen)* | 6752864-6754194 | FBgn004053 | 6752864 | + | + | 487bp | 181bp | 1 |
| *Single minded (sim)* | 13057755-13078124 | FBgn0004666 | 13057755 | + | + | 340bp | 245bp | 2 |
| *Ventral nervous system defective (vnd)* | 582121-598151 | FBgn0261930 | 582121 | + | + | 756bp | 187bp | 3 |
| *Bunched (bun)* | 12433118-12528775 | FBgn0259176 | 12455540 | – | – | 400bp | 412bp | 4 |
| *Rhomboid (rho)* | 1463811-1468944 | FBgn0004635 | 1463811 | – | – | 544bp | 150bp | 5 |

The enhancers' locations denoted by "+" (downstream to the TSS) and "-" (upstream to the TSS); Table also depicts figure numbers in which results generated by cluster buster software are summarized in form of 2-D diagrams.

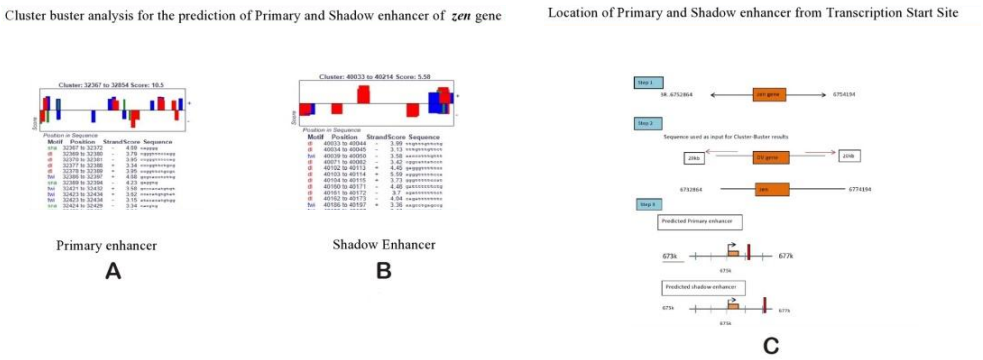


Fig. 1. Screenshot of Cluster – Buster output and 2- D construct for the location of Primary and shadow enhancers of the *zen* gene. Both enhancers were located downstream to the TSS

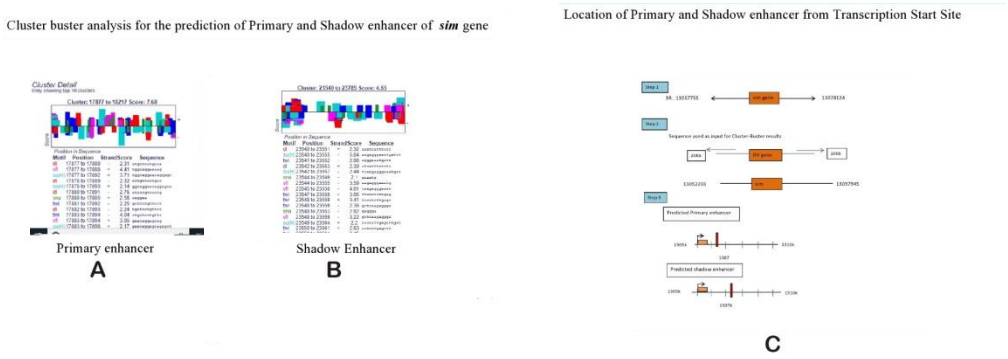


Fig. 2. Location of primary and shadow enhancers of the *sim* gene. Both the primary and shadow enhancers were located downstream to the TSS

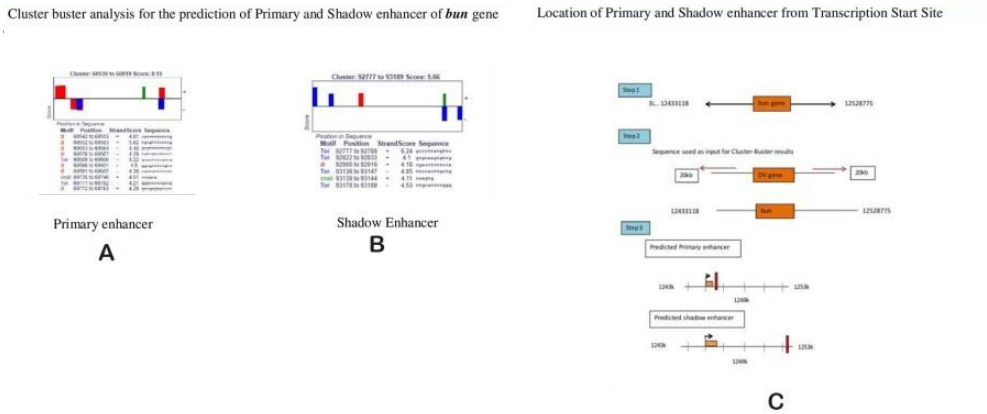


Fig. 3. Cluster- buster output and construct for the *vnd* gene shows that the primary enhancer was located downstream of the TSS whereas the shadow enhancer was lying downstream and just near the TSS

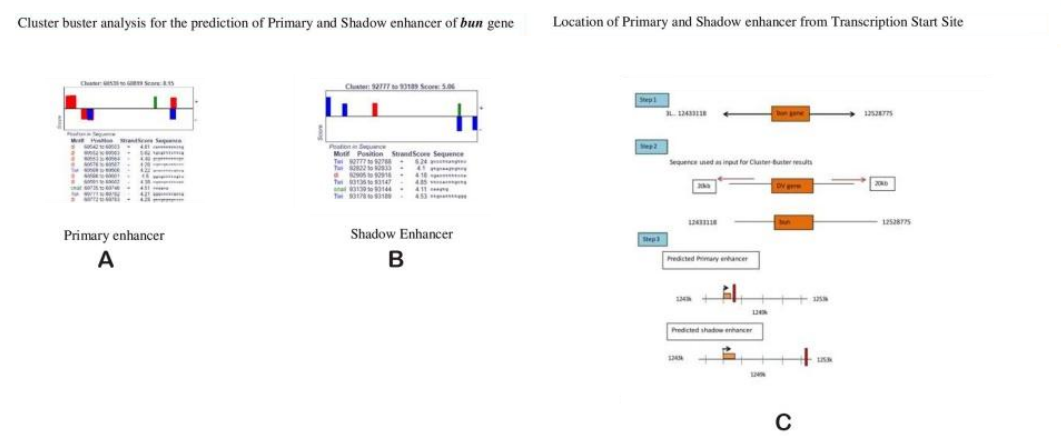


Fig. 4. 2- D construct and Cluster- Buster result for the location of Primary and shadow enhancers of *bun* gene. Both the enhancers were lying downstream of the TSS

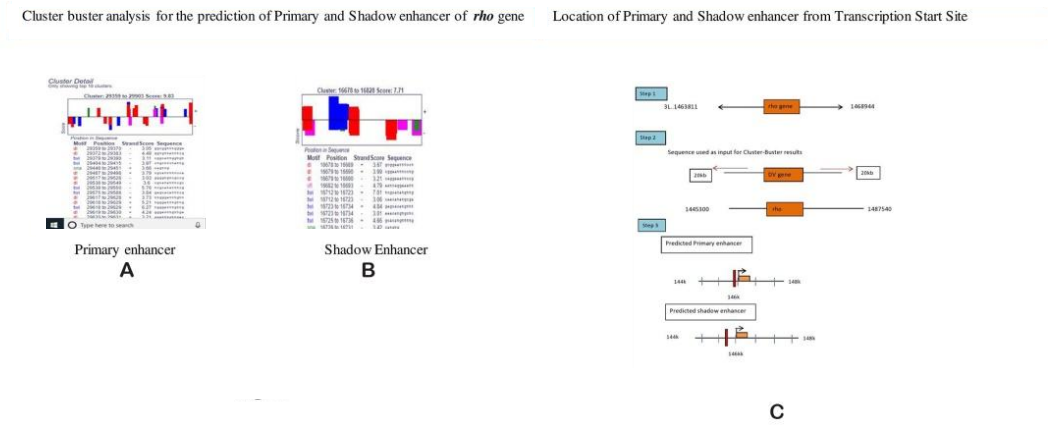


Fig. 5. This figure is showing that both predicted enhancers of the *rho* were located upstream of the TSS

database. In relation to previous studies, this study predicted multiple enhancers for all selected dorsal target genes. Apart from the prediction of previously identified multiple enhancer, this study has predicted new shadow enhancer sequences and strong primary enhancers with respect to their postion in the flanking regions of the target DV genes. Therefore, it can be concluded that gene expressions can be controlled by primary and shadow enhancers and these can be located far away from the target gene. The study of these enhancers and their evolution will further help to understand variations in regulatory elements.

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## AUTHOR CONTRIBUTION STATEMENT

TK conceived and designed research. SK has predicted data. TK and SK analyzed data. SK wrote the manuscript. Both authors read and approved the manuscript.

## CONFLICT OF INTEREST

No conflict of interest.

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