



Functional analysis of the boundary region between forum domains in the band 84D in the *Drosophila melanogaster* genome.

Moiseeva, Evgenia D., and Nikolai A. Tchurikov. Engelhardt Institute of Molecular Biology Russian Academy of Sciences, Laboratory of Genome Organization, Vavilov str. 32, Moscow, 119991, Russia. Author for correspondence:

Moiseeva, E.D.; e-mail address: edmois@gmail.com.

Abstract

The eukaryotic genome consists of forum domains, which are structural-functional units 50-200kb long, containing various genes or large genetic loci that are concertedly silenced or activated. In order to investigate the possible functions of the boundary region between forum domains located in the band 84D, we used the luciferase reporter gene and transient transfection assays in Schneider-2 cell line. The 84D fragment was cloned in both orientations. We found that this fragment reveals properties of a silencer. Chromatin immunoprecipitation revealed that this region is enriched with H3K27me₃, the mark specific for PRE/TRE. We conclude that the boundary region 84D may serve as PRE/TRE *in vivo*.

Introduction

Forum domains are 50-200 kb long units of coordinated silencing or activation of genes (Tchurikov *et al.*, 2004). The evidence of existence of such domains was independently provided by Tolhuis *et al.* (2006), who called them Pc-domains. The polycomb/trithorax response elements (PRE/TRE) located at the boundaries separating neighboring forum domains maintain the transcriptional state of genes associated with them. The existence of such a region in the *cut* locus in *Drosophila* was demonstrated for the Sau10 silencer (Sosin *et al.*, 2006), a boundary element between forum domains. To continue studying the DNA sequences located at the boundaries of forum domains, we selected the boundary region between forum domains of the band 84D in the *Drosophila* genome. The fragment 84D was obtained earlier using end-labeled forum DNA probes (Tchurikov *et al.*, 1998). The region involved is under-replicated in polytene chromosomes and the SuUR protein specifically binds with the fragment 84D (Tchurikov *et al.*, 2004).

Materials and Methods

The functional analysis of the fragment 84D was performed using the procedure of transient expression of mini-chromosome constructs in *Drosophila* cells, which was developed earlier (Kretova and Tchurikov, 2005; Sosin *et al.*, 2006; Tchurikov *et al.*, 2008). The fragment concerned was inserted in both orientations into the base construct (Figure 1, construct 1), which contained the firefly luciferase gene under the control of the *Hsp70* promoter and the 3'-end maturation region from the actine 5C gene (3'-*act5C*), into *HpaI* site at a distance of 3.2kb from the promoter. The fragment 84D (2283..3173, AC U89926) was obtained by PCR using oligonucleotides containing artificial *XhoI* sites (5'-cccctcgagTGATTATTAAGTAATTATATTGCTAATT-3'; 5'-cccctcgagTTATTTATCTGATGCATGGCACT-3'). During cloning, the recessed 5' ends were filled in using the Klenow fragment (Fermentas, Lithuania). The enhancer from the *copia* transposable element was inserted into some genetic constructs (Thurikov *et al.*, 2008).

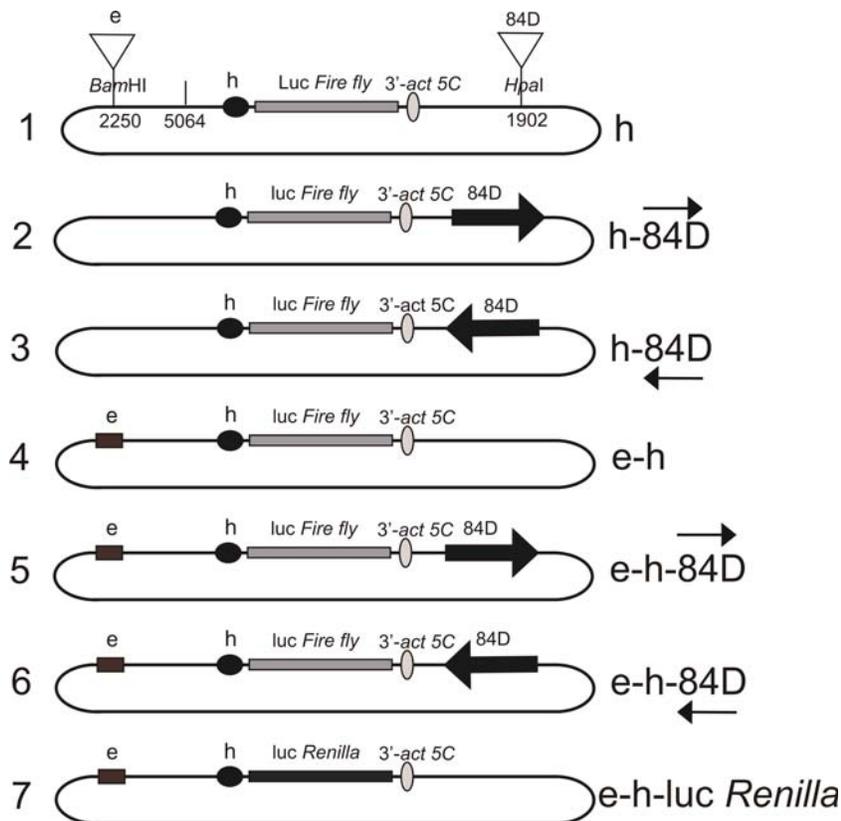


Figure 1. Genetic constructs used for cotransfection (without scaling). The sites used for inserting the 84D fragment and the *copia* enhancer are indicated in the base construct (1). The enumeration is given according to the initial vector pGL3-Enhancer (Promega, AC U47297). Abbreviated names of constructs are shown to the right. Designations: e, *copia* enhancer; h, *Hsp70* gene promoter; luc *Fire fly*, fire fly luciferase; luc *Renilla*, Renilla luciferase; 3'-act 5C, the 3'-end maturation region from the actin 5C gene. The orientation of the 84D fragment is shown with arrows. The correctness of the genetic constructs was corroborated by sequencing.

Obtained constructs were used for cotransfection of Schneider-2 cells together with the control construct (Figure 1, construct 7), which contained the Renilla luciferase gene under the control of the *Hsp70* promoter, the 3'-act 5C region, and the enhancer. Cotransfection was performed and luminescence was measured using the reagent kits from Promega (United States) as described earlier (Tchurikov *et al.*, 2008). Data were normalized by the amount of DNA by analyzing the *Bam*HI restriction fragments of the DNA constructs fractionated in 0.8% agarose gels using the QuantityOne software (Bio-Rad, United States).

Chromatin immunoprecipitation (ChIP) with cross-linked chromatin from *Drosophila* cultured cells Schneider-2 was performed using the reagent kit from Diagenode (Belgium) according to the protocol provided by the manufacturer. We used antibodies against modified histones: histone H3 trimethylated at Lys-9 (H3K9me3, Upstate, United Kingdom), or Lys-27 (H3K27me3, Upstate,

United Kingdom), or Lys-4 (H3K4me3, Diagenode, Belgium), and histone H4 trimethylated at Lys-20 (H4K20me3, Upstate, United Kingdom). The obtained DNA was analyzed by semiquantitative PCR using the primers corresponding to the 84D region (5'-TGGTCGAACTGAGCATTCTGGA-3'; 5'-AGGGACATGACAAATTACACCGA-3'). The 300-bp amplification products were fractionated in 2% agarose gels.

Results and Discussion

The functional activity of the 84D fragment was assessed by its effect on the expression of the firefly luciferase reporter gene. Figure 2 shows the results of functional analysis of this element. One can see that the fragment concerned suppresses firefly luciferase expression. Thus the 84D fragment, similarly to the Sau10 element (Sosin *et al.*, 2006), exhibits the properties of a silencer in either orientation.

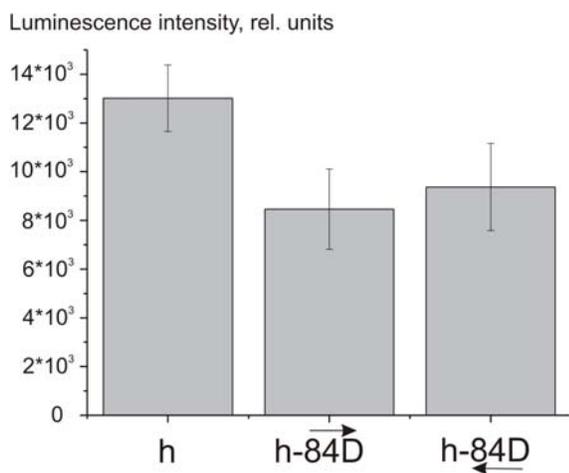


Figure 2. Functional analysis of the 84D fragment. For designations of constructs, see Figure 1.

The effect of the enhancer that was active in Schneider-2 cells on the properties of the 84D silencer was assessed using the constructs containing the enhancer from the transposable element *copia* (Figure 1, constructs 5 and 6). As evident from Figure 3, under these conditions the 84D fragment also functioned as a nonpolar silencer and suppressed the reporter gene expression. Thus the active enhancer has no effect on this silencer.

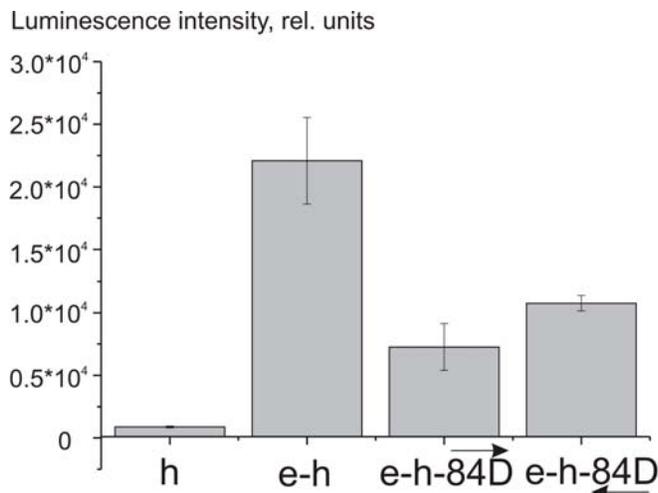


Figure 3. Analysis of the effect of the enhancer on the function of the 84D silencer. For designations of constructs, see Figure 1.

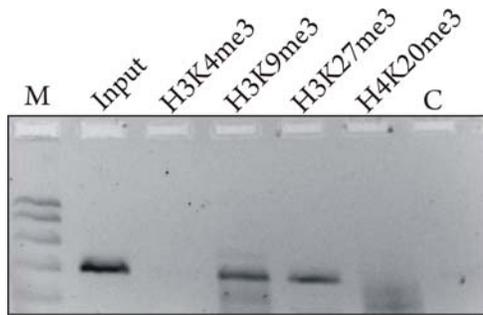


Figure 4. Modified histones pattern at the 84D boundary region of forum domains. Designations: M, molecular mass marker (1000bp, 750bp, 500bp, 300bp, 150bp); Input, 15% of the initial chromatin used for immunoprecipitation; C, negative control (ChIP with nonspecific control antibodies).

To analyze the state of chromatin in the studied boundary region of forum domains *in vivo* we perform ChIP with antibodies against modified histones H3K9me3, H3K27me3, and H4K20me3, which are present in PRE/TRE regions (Papp and Müller, 2006), and with the antibodies against H3K4me3, which occurs in promoters of actively transcribed genes (Mikkelsen *et al.*, 2006) as a negative control. As seen in Figure 4, the 84D region is enriched in the histone form H3K27me3, which is produced by proteins of the Polycomb group – the histone methyltransferase E(z) and the polycomblike protein (Nekrasov *et al.*, 2007). This fact indicates that the complexes containing these proteins may bind to the studied region *in vivo*. The H3K9me3 form, which is also present at the 84D region, is characteristic of some set of PRE/TRE regions (Ringrose *et al.*, 2004). It is believed that this form accelerates the initial binding to the proteins of the Polycomb group. The H4K20me3 modification, which usually occurs in pericentromeric heterochromatin regions and is present at the known PRE/TRE region bxd from the *bithorax* locus of *Drosophila* (Papp and Müller, 2006), is not characteristic of the studied region. This modification is not always associated with silencing processes (Pesavento *et al.*, 2008). Thus this analysis of the state of chromatin of the 84D region allowed us to assume the presence of PRE/TRE region in it.

The results of this study provide convincing evidence for the functional activity of the 84D fragment studied. This element reveals properties of a nonpolar silencer in our functional assay and bears the histone marks characteristic for PRE/TRE. So this region may serve as PRE/TRE *in vivo*. Further studies of this and other boundary regions of forum domains are required to determine the nature of these DNA sequences.

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Pupal expression pattern of an Ocellar specific *P-GAL4* enhancer trap strain of *Drosophila melanogaster*.

Venkatesh, C.R., and B.V. Shyamala. Department of Studies in Zoology, University of Mysore, Mysore 570 006 India. E-mail shyamalabv@yahoo.com.

Drosophila adult brain is a highly complex system, consisting of millions of neurons, organized into specific functional modules with intricate and highly specific internal and external connectivity. The genetic pathways which regulate the developmental program of the construction of this remarkable structure have scarcely been understood. A *P-GAL4* enhancer trap screen (Brand and Perrimon, 1993) was done as an attempt to identify genes with an expression in the brain during development (Shyamala and Chopra, 1999). We here report the expression pattern of an ocellar specific strain, in the brain, during its pupal development.

The F1 embryos of the cross between *P-GAL4* strain SG19.1 and the UAS *Lac-Z* strain were raised at 22°C. Formation of white pupa was taken as zero hour, and pupae were staged accordingly as the number of hours After Puparium Formation (APF). The staged pupae were dissected out, and the brain along with ventral ganglion was stained for β -galactosidase activity using the standard protocol (VijayRaghavan *et al.*, 1986).

The insertion of reporter gene in the strain SG19.1 is in the third chromosome, and the homozygous individuals are viable. At 0 hr APF (Figure 1a) the expression of reporter gene is seen in a pair of cells in the brain located in the Interhemispheric junction (IJ) (Hanesch *et al.*, 1989), a pair of doublets in the Suboesophageal ganglion (SOG) (Truman, 1990), (Figure 1a) along with two cells present towards the posterior abdominal neuromeres of Ventral ganglion. At 14 hr stage the expression gets restricted to the cells at IJ and SOG with a marked enhancement in the expression levels (Figure 1b-c). Expression in the ventral ganglion cells is withdrawn. A pattern similar to that of 14 hr stage is observed through 24, 37, 48, 72 till 82 hr APF (Figures 1d and 1e: 37 and 48 hr APF, respectively). In 99 hr APF and in the pharate brain (Figure 1f), the reporter gene expression is consistently restricted to only the pair of cells situated in the cortical region present anterior to the superior medial Protocerebrum (SMP) (Ref: Flybrain). These cells are equivalent to the cells seen in the Interhemispheric junction of brain during the earlier pupal stages.

Earlier studies have shown that the adult head sections of this strain have reporter gene expression in the three Ocellar neurons present on the forehead, ocellar nerve, and in the cells situated in the frontal brain (Shyamala and Chopra, 1999). The ocellar specific expression of the reporter gene during pupal stages strongly suggests that the native gene at the site of *P-GAL4* insertion may have an important role in the development and differentiation of the ocellar neurons.

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