# *In situ* dissection of a Polycomb Response Element (PRE) in *Drosophila melanogaster*

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**Summary of Ph.D. thesis**

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## INTRODUCTION AND OBJECTIVES

The main process in development is the division and specialization of cells that make up an organism. Specialization can be best characterized by the differential gene expression profiles established during patterning processes. Cells possess an inherent "cellular memory" which provides the transmission of specific gene expression configurations to daughter cells, enabling them to maintain developmental programs determined early in embryogenesis for the rest of development. *Drosophila melanogaster* is an ideal model organism to study the molecular mechanisms uderlying "cellular memory", since several of its genes affecting different developmental states have been characterized. Among these are the conserved homeotic genes and their chromatin regulators, the Polycomb (*PcG*) and trithorax Group (*trxG*) of genes. Mutations in these genes cause the transformation of a given segment into the identity of another one. PcG proteins are negative regulators: they bind to Polycomb Response Elements to maintain their target genes in a repressed transcription state. TrxG proteins are positive regulators: they act through Trithorax Response Elements to keep their target genes in an active state. Both groups of proteins form large multiprotein complexes, which use various biochemical activities to alter higher order chromatin structure and, thus, regulate transcription.

Our studies focus on the most extensively studied silencer region in the homeotic bithorax complex of *Drosophila melanogaster*, called *bithoraxoid* Polycomb Response Element (*bxd* PRE). Although PREs are known to interact with each other over long distances, all previous experiments used mobile element constructs with various reporter genes; no *in situ* dissection of this region has been performed. In different tests, PREs often proved to be of varying size; moreover, results were sometimes even contradictory. As PREs function cooperatively and behave differently in different chromosomal contexts, we decided to study the *bxd* PRE *in situ*. For this purpose, we devised a novel P element mediated gene conversion method. To analyze how PREs function in their natural chromosomal context, and, first, to determine their sequence requirements in terms of binding sites, we have generated *in situ* deletions within a 3-kb region of the *bxd* PRE/TRE, and examined their effect. Our technique also enabled us to replace the *bxd* PRE with previously defined PRE sequences to test if they can compensate for the loss of the *bxd* PRE. We also placed the marker gene *Gal4-VP16* into different modified versions of the *bxd* PRE/TRE region. Combined with the *UAS-eGFP* system, we could study the higher order local cromatin structure of the *bxd* PRE.

## MATERIALS AND METHODS

### Recombinant DNA techniques

- Polymerase chain-reaction (PCR)
- Restriction digestion
- Agarose gel electrophoresis
- Isolation of plasmids and DNA fragments
- Ligation of DNA fragments
- Transformation of bacteria (*E. coli*)
- Directed mutagenesis of restriction sites
- Southern blotting

### Injection of *Drosophila* embryos

### *Drosophila* crosses

- Identification of convertatnts
- Deletion generation by FRT/Flip recombination and *I-SceI* restriction endonuclease
- Recombination between chromosome arms by FRT/Flip recombination

### Phenotypic analysis

- Quantification of adult phenotypes
- Preparation and photography of larval cuticles

### Immunostaining of embryos and larvae

### Computational sequence analysis

**RESULTS AND CONCLUSIONS**

**1.** To carry out the deletional analysis of the *bxd* PRE, we have devised a novel strategy for gene conversion in *Drosophila*. We designed two different types of conversion constructs, which allowed us to generate small deletions of either pre-determined or random size. Templates for conversion included FRT-sites flanking small genomic sequences or restriction sites for the yeast *I-Sce*I enzyme, which permit generating small deficiencies by FLP/FRT recombination or by the *I-Sce*I induced double strand DNA breaks, respectively. Compared to the previously existing gene conversion methods, the use of marker genes in our constructs enabled us to easily identify conversion events and deletion-carrying animals, thus, increasing efficiecy. The FRT sites remaining in the deletion chromosomes could also be used to merge different deletions or create duplications.

**2.** Previously, 3 sub-elements with potential PRE-activities were identified by chromatin immuno-precipitation in the 3-kb region of the *bxd* PRE/TRE. However, we have found that only the removal of the central one (Δ1-2, 665 bp) decreases *bxd* PRE function to a detectable degree. Flies carrying this deletion showed partial posteriorly directed transformations, such as wing into haltere and/or 3rd thoracic segment into 1st abdominal segment, with a penetrance of ~64 % when heterozygous. The observed mutant phenotypes correspond to the transformation of parasegment 5 (PS5) to parasegment 6 (PS6). Consistent with our findings, no increase in the penetrance was observed when we extended the size of the deleted sequence to the whole 3-kb region (Δ7-13).

**3.** Relying on previous transgenic results, the 3-kb Δ7-13 deletion removes not only PREs, but also all known TREs from the *bxd* region. Although from the loss of TREs one would expect a reduction in the expression of the target gene (*Ubx)*, in Δ7-13 homozygotes, we saw no anteriorly directed transformations, which would have indicated such a change. In agreement with the suggestions from transgenic data by other groups, we propose that TREs function to counteract PREs inactivating effect in those segments, where the regulatory region harbouring them is supposed to be active. Thus, we further confirm that TREs do not act as activators, as widely thought previously, but rather as antirepressors.

**4.** To further narrow down the size of the region essential for *bxd* PRE function, we generated three deletions internal to Δ1-2 (Δ10, 280 bp; Δ17, 185 bp; Δ12, 127 bp). Δ17 was the smallest one which still caused posterior transformations in 22 % of the flies. If we left parts of this 185-bp fragment intact in the chromosome, we observed no transformations, even if we excised the neighbouring sequences in

addition. Thus, we named the 185-bp sequence the *bxd* PRE core. Interestingly, in all stocks with the missing PRE core, the penetrance of the phenotype gradually decreased in subsequent generations when heterozygous. In homozygous Δ1-2 and Δ10 flies, the penetrance approached 100 % and was stable during stock maintenance. In the case of Δ17, the penetrance decreased even in the homozygous stock. However, the expressivity of the phenotype was stable only in homozygous Δ1-2; it decreased in flies homozygous for the two smaller deletions. From these data, we concluded that the *bxd* PRE has a modular structure. The sequences flanking the PRE core can partially replace the function of the core. Even the PRE core itself consists of redundant modules, which, probably cooperating with the neghbouring sequences, can fully compensate for the loss of other module(s) from the core. The phenotype is more serious in hemizygous Δ1-2 than in heterozygous Δ1-2 flies, which shows that the *bxd* PRE also cooperates with other sequences (probably the *bxd* PRE) on the wild type homolog.

**5.** Considering that the *bxd* and *iab-7* PREs share a similar pattern of GAGA and PHO protein binding sites, reported to be important for PRE function, and many of which found in the *bxd* PRE core, we decided to replace the *bxd* PRE core with two "foreign" PRE cores from the *iab-5* and *iab-7* regions of the BX-C. Both sequences, in either orientation, could fully replace the function of the *bxd* PRE core, and, besides, did not cause any "loss-of-function" phenotypes, showing that PREs do not carry positional information; rather, they act as simple silencers. We also searched for DNA stretches with homology and similar binding site pattern in the human genome, in which no PREs have been identified to date. We found one such sequence (H1), and then tested if it could fulfil the funcion of the *bxd* PRE core. H1 did not behave as a PRE, however, our results show that we established an efficient test system to identify sequences with (*Drosophila*) PRE function.

**6.** To examine the degree of contribution of different protein binding sites to PRE function, we mutated the arrays of three types of binding sites within the *bxd* PRE core. Mutating either GAGA or PHO motifs resulted in relatively low penetrances, however, mutating GAGA and PHO motifs together was equivalent in terms of penetrance with the removal of the whole core. Thus, these two protein binding sites essential building stones of the *bxd* PRE. In contrast, DSP1 protein binding sites, reported to be required for the activaton of *Ubx,* appeared to play little role, if any, *in situ* in the function of the *bxd* PRE. Thus, we could only partly confirm previous transgenic results about essential PcG binding sites *in situ*, and, more importantly, devised a powerful method to further define the sequence requirements of PREs.

**7.** Besides observing the phenotypes of adult flies, we have also examined the expression pattern of the *Ubx* gene controlled by the *bxd* region, using antibody staining against the UBX protein in embryonic and larval tissues. Also, the *Gal4-VP16* marker gene, cloned appropriately in certain constructs, enabled us to study, through the UAS-eGFP system, the local chromatin structure of the *bxd* regulatory region. In agreement with the adult phenotypes, the *Ubx* gene showed only a modest ectopic expression in PS5 when at least the core was removed from the *bxd* PRE. In contrast to *Ubx*, eGFP showed extreme ectopic expression even in the head segments. Another striking difference was observed in the gene expression level of these two genes: in deletion-bearing heterozygotes, the intensity of eGFP decreased, while the level of *Ub*x increased, as compared to the simple *Gal4-VP16* insertion. Very likely, these differences reflect the differences between the regulation of the two genes: *Gal4-VP16* is most likely controlled by a single neighbouring enhancer, while *Ubx* falls under a much more complex regulation that modifies the effect of *bxd* PRE deletion. Importantly, our data also imply that the *bxd* PRE binds to at least one *Ubx* enhancer and regulates its activity even in those segments, where the *bxd* region is active. Thus, besides confirming previous findings that PREs bind to the target gene promoter through proteins, we propose that in these finely tuned, large chromatin complexes, specific enhancers are also major components in those segments, where *Ubx* is turned on..

## LIST OF PUBLICATIONS

### Publications

**Kozma, G.** (2005) *In situ* dissection of the bxd PRE in *Drosophila melanogaster*. *Acta Biol Szeged* **49(3-4)**, 47

**Sipos, L., Kozma, G., Molnár, E. és Bender, W.** (2007) *In situ* dissection of a Polycomb response element in Drosophila melanogaster. *Proc Natl Acad Sci U S A* **104**, 12416-21.

**Kozma, G., Bender, W. és Sipos, L.** (2008) Replacement of a Drosophila Polycomb response element core, and *in situ* analysis of its DNA motifs. *Mol Genet Genomics* accepted, published online

### Conference presentations

**Kozma, G., Bender, W. , Sipos, L.** (2005). A *bxd* PRE *in situ* vizsgálata *Drosophila melanogaster*-ben.**VI.** *Magyar Genetikai Kongresszus* / **XIII.** *Sejt- és Fejlõdésbiológiai Napok*: E79 (lecture in Hungarian)

**Kozma, G., Sipos, L., Bender, W.** (2005). *In situ* dissection of *bxd* PRE in *Drosophila melanogaster*. *European Drosophila Research Conference* **19**: CR15 (poster).

**Kozma, G., Bender, W. , Sipos, L.** (2005). Gene conversion, site-directed mutagenesis: *In situ* dissection of a Polycomb Response Element (PRE) in *Drosophila melanogaster. Straub-napok* (lecture)

**Kozma, G., Bender, W. , Sipos, L.** (2006). *In situ* dissection of *bxd* PRE in *Drosophila melanogaster*. *Regional Drosophila Meeting* **12** (lecture)

**Kozma, G., Bender, W. , Sipos, L.** (2007). A *bxd* PRE *in situ* vizsgálata *Drosophila melanogaster*-ben. **VII.** *Magyar Genetikai Kongresszus* / **XIV.** *Sejt- és Fejlõdésbiológiai Napok*: P060 (poster in Hungarian)