INTRODUCTION

During the early stages of development of multicellular organisms, an individual pattern of gene expression is established in different cell types and then maintained over many cell divisions. Polycomb (PcG) and Trithorax (TrxG) group proteins that act through association with chromatin. PcG/TrxG are assembled on the DNA sequences termed PREs (Polycomb Response Elements), the activity of which can be modulated and switched from repression to activation. In this study, we analyzed the influence of transcriptional read-through on PRE activity switch mediated by the yeast activator GAL4. We show that a transcription terminator inserted between the promoter and PRE doesn’t prevent switching of PRE activity from repression to activation. We demonstrate that, independently of PRE orientation, high levels of transcription fail to dislodge PcG/TrxG proteins from PRE in the absence of a terminator. Thus, transcription is not the main factor required for PRE activity switch.

Role of Transcriptional Read-Through in PRE Activity in Drosophila melanogaster

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ABSTRACT

Maintenance of the individual patterns of gene expression in different cell types is required for the differentiation and development of multicellular organisms. Expression of many genes is controlled by Polycomb (PcG) and Trithorax (TrxG) group proteins that act through association with chromatin. PcG/TrxG are assembled on the DNA sequences termed PREs (Polycomb Response Elements), the activity of which can be modulated and switched from repression to activation. In this study, we analyzed the influence of transcriptional read-through on PRE activity switch mediated by the yeast activator GAL4. We show that a transcription terminator inserted between the promoter and PRE doesn’t prevent switching of PRE activity from repression to activation. We demonstrate that, independently of PRE orientation, high levels of transcription fail to dislodge PcG/TrxG proteins from PRE in the absence of a terminator. Thus, transcription is not the main factor required for PRE activity switch.

KEYWORDS

Polycomb, Trithorax, PRE, Drosophila.

ABBREVIATIONS

PcG –Polycomb group proteins; TrxG –Trithorax group proteins; bxd – bithoraxoid.
transcriptional read-through has been also tested. It has been established that, in case of reverse orientation of bxdPRE, transcriptional read-through also does not lead to the elimination of PcG/TrxG factors.

**EXPERIMENTAL**

**Plasmid constructs design**

All constructs were made on the basis of the CaSpeR vector containing white gene with partial deletion of the first intron (encodes complete product of white gene) [25]. The enhancer of white gene (Ee) located in the genome at position −1180…−1849 bp relative to the transcription start site of white gene [26] was excised from the Ee-pBluescript SK+ plasmid [27] and inserted in forward orientation into a CaSpeR4 vector cleaved by NotI [En-White].

A fragment SmaI-Sall of 4324 bp in length from the plasmid vector CaSpeR-hs43-lacZ carrying the lacZ gene with the adh leader sequence and SV40 transcription terminator at 3′-terminus (GenBank: X81643.1) was inserted into a pBluescript SK+ vector cleaved by SmaI and Sall [LacZ-SV40-pSK].

The promoter of the hsp26 gene, 472 bp, was amplified by PCR (primers 5′-ctggaactccgctctcta-3′ and 5′-gttgaatgaacttgtttgacttgt-3′) and inserted into a pBluescript SK+ vector cleaved by EcoRV [hsp26-pSK]. A HindIII–PstI fragment of the hsp26-pSK vector was inserted into the Lacz-SV40-pSK vector at the SmaI site [hsp26-LacZ-SV40-pSK]. A fragment NotI–Sall of the hsp26-LacZ-SV40-pSK vector was incorporated into the En-white vector at the BamHI site [hsp26-LacZ-SV40-En-white].

A fragment HindIII–EcoRI containing a minimal promoter of the hsp70 gene and five GAL4 sites at 5′ terminus was excised from the pUAST vector [28] and inserted into a pBluescript SK+-sce2 vector at the EcoRV site [sce(UAS)]. The coding region of a gfp gene of 717 bp was amplified by PCR (primers 5′-atggcagcaagggcaggagct-3′ and 5′-tggtagaagctctctctctctg-3′) and cloned into the pBluescript SK+ vector cleaved by EcoRV [hsp26-pSK]. A HindIII–NotI fragment of the hsp26-pSK vector was inserted into the LacZ-SV40-pSK vector at the SmaI site [hsp26-LacZ-SV40-pSK]. A fragment NotI–Sall of the hsp26-LacZ-SV40-pSK vector was incorporated into the En-white vector at the BamHI site [hsp26-LacZ-SV40-En-white].

**UDTPD construct.** A XbaI–XbaI fragment of the (UAS)sce-eGFP-lox(SV40) vector was incorporated into the vector linker1828-SV40s-pSK at the EcoRV site [(UAS)sce-eGFP-lox(SV40)-linker1828-SV40s-pSK]. A fragment XbaI–XbaI containing bxdPRE of 656 bp (3R:16764122..16764777) was excised from the frt(PRE) vector [29] and incorporated into the vector (UAS)sce-eGFP-lox(SV40)-linker1828-SV40s-pSK at the AorI site in forward [(UAS)sce-eGFP-lox(SV40)-linker1828-SV40s-pSK] or reverse [(UAS)sce-eGFP-lox(SV40)-linker1828-SV40s-pSK] orientation.

**UDTPR construct.** A XbaI–XbaI fragment of the (UAS)sce-eGFP-lox(SV40) vector was incorporated into the hsp26-LacZ-SV40-En-white vector at the BamHI site.

All details of the constructs design are available upon request.

**Transformation of Drosophila melanogaster embryos and phenotypic analysis of yellow and white expression in transgenic lines**

DNA constructs and a P element with defective inverter repeats P25.7wc, which served as a source of transposase [30], were injected into a y\(^{w1118}\) line at the stage of preblastodermal embryo according to [31, 32]. The surviving flies were crossed with the y\(^{w1118}\) line. Transgenic flies were selected based on phenotypic manifestation of white expression. The number of copies was determined by Southern blot hybridization with a white gene fragment. Lines containing a single copy of the construct per genome were selected.

For in vivo deletion of the DNA fragment, flies carrying the construct were crossed with transgenic flies expressing Flp (w\(^{1118}\); S2CyO, hsFLP, ISA/Sco; +) or Cre (y\(^{w}\); Cyo, P[w+,cre]/Sco; +) recombinase [33, 34]. Accuracy of fragment removal was confirmed by PCR.

Line y\(^{w1118}\); P[w, tubGAL4]117/TM3,Sb, a derivative of the Bloomington Stock Center #5138 line with deletion of the mini-white marker gene [35], was used for expression of GALA under the control of a tubulin promoter.
The expression of white gene was determined by visual evaluation of eye pigmentation using the standard scale: red color is the pigmentation of eyes in wild-type flies (white expression in case of complete stimulation by a tissue-specific enhancer), white the color of the eyes is observed in the absence of pigmentation (complete inactivation of white gene). Various degrees of mosaic phenotype are observed in case of repression.

In order to analyze the phenotype of transgenic flies, 3- to 5-day-old males developed at 25°C were used. The details of all crosses conducted for the genetic analysis and excision of functional elements can be provided upon request.

**Chromatin immunoprecipitation (X-ChIP)**

A total of 150–200 mg of adult flies was collected for each experiment. Chromatin immunoprecipitation was performed according to the technique described previously [21].

### Antibodies

Antibodies to the PH protein [to fragment 86–520 aa, ph-p-PA]; dSfmt [to fragment 1–348 aa, Sfmt-PB] [27]; PC [to fragment 191–354 aa, Pc-PA]; TRX-N [to fragment 8–351 aa, trx-PA]; and GAF [1–519 aa, Trl-PB] [21] were obtained in rabbits. Antibodies to H3K27me3: Abcam (ab6002, ChIP Grade).

### Real-time PCR with Hot-Start Taq DNA polymerase

Real-time PCR was conducted using C1000™ ThermalCycler (Bio-Rad) in a 25 μl volume according to the following protocol (per one reaction): 2.5 μl of 10× buffer (0.5 M Tris–HCl, pH 8.8, 0.5 M KCl, 15 mM MgCl₂, 1% Tween 20), 2 μl of 25 mM MgCl₂, 0.5 μl of 10 mM dNTPs, 1.5 μl of each primer (at a concentration of 5 pmol/μl), 0.25 μl of SYBR Green100× (Sigma), 0.3 μl of Hot-Start Taq DNA polymerase (SibEnzyme), 11.45 μl of mQ, 5 μl of sample. Data were assessed using the Bio-Rad CFX Manager software and Microsoft Excel.

### Primers for real-time PCR analysis of the material obtained by chromatin immunoprecipitation

<table>
<thead>
<tr>
<th>Primer Type</th>
<th>Forward Primer</th>
<th>Reverse Primer</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-ChIP forward</td>
<td>5’-gagaactctgaatagggaattgg-3’</td>
<td></td>
</tr>
<tr>
<td>1-ChIP reverse</td>
<td>5’-agetctcetgctctgtgctacat-3’</td>
<td></td>
</tr>
<tr>
<td>2-ChIP forward</td>
<td>5’-ccgaccactaccaacagae-3’</td>
<td></td>
</tr>
<tr>
<td>2-ChIP reverse</td>
<td>5’-gtcactgccagagtgatcc-3’</td>
<td></td>
</tr>
<tr>
<td>3-ChIP forward</td>
<td>5’-tctcgaggtatcagaatacgcttg-3’</td>
<td></td>
</tr>
<tr>
<td>3-ChIP reverse</td>
<td>5’-cataatgctgcgcctaatcag-3’</td>
<td></td>
</tr>
<tr>
<td>4-ChIP forward</td>
<td>5’-ggtagaatctgatgagtggtg-3’</td>
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</tr>
<tr>
<td>4-ChIP reverse</td>
<td>5’-cagttteacaccgcagcagata-3’</td>
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</tr>
<tr>
<td>5-ChIP forward</td>
<td>5’-aaaaattttctcagctgctlc-3’</td>
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<td>5-ChIP reverse</td>
<td>5’-ggtattagccctgcattga-3’</td>
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</tr>
<tr>
<td>6-ChIP forward</td>
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<td>6-ChIP reverse</td>
<td>5’-tggaacagagagggcgacaca-3’</td>
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<td>Ras64B forward</td>
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<td>Ras64B reverse</td>
<td>5’-gtgcaagttcagctgcact-3’</td>
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</table>

### RESULTS

**Model system for studying the impact of transcription on the recruitment of Polycomb and Trithorax group proteins to PRE**

The influence of transcriptional read-through on PRE activity was studied using transgenic constructs integrated into the D. melanogaster genome by microinjection of embryos with plasmid DNA due to the 5’ and 3’ termini of the P element flanking the transgene. The 660 bp bxdPRE element from the regulatory region of the Ubx gene was used [36, 37]. This PRE element is well studied and has binding sites for various PcG and TrxG proteins [15, 21, 36, 37].

Two constructs were created containing bxdPRE inserted between the UAS promoter and the reporter genes: lacZ under the control of the hsp26 gene promoter and white gene. The marker white gene is responsible for eye pigmentation. Increased level of white gene expression in the eyes of flies was obtained by insertion of a tissue-specific enhancer directly upstream of the white promoter. The UAS promoter used for induction of transcription through bxdPRE is the minimal promoter of the hsp70 gene with five upstream binding sites for yeast GAL4 activator. A high level of transcription is achieved upon induction of the UAS promoter (by crossing transgenic lines with a line carrying the GAL4 gene under the control of the tubulin promoter). In both constructs, the UAS promoter is di-
Fig. 1. Schematic representation of transgenic constructs and phenotype analysis of flies. A – UDTPD transgene. Minimal promoter of hsp70 gene under the control of binding sites for activator protein GAL4 (UAS) triggers transcription through eGFP and bxdPRE “T” – transcription terminator; hsp26-lacZ and white – reporter genes; “E” – white gene enhancer. Phenotypes of the obtained lines are shown below. P/P – homozygous line; P/+ – heterozygous line; P/+ tubGAL4 – heterozygous line expressing GAL4; PΔlox/PΔlox – homozygous line carrying a deletion of the transcription terminator; PΔlox/+ – heterozygous line with deletion of the transcription terminator; PΔlox/+ tubGAL4 – heterozygous line expressing the GAL4 protein with deletion of the terminator; PΔfrt/PΔfrt – homozygous line with deletion of bxdPRE; PΔfrt/+ – heterozygous line with deletion of PRE; PΔfrt/+ tubGAL4 – heterozygous line expressing GAL4 with deletion of bxdPRE. Scale of eye pigmentation depending on the level of white expression: R+ – dark red (wild-type); R – red; BrR – brownish-red; Br – brown; dOr – dark orange; Or – orange; dY – dark yellow; Y – pale yellow; pY – very light yellow; w – white. Mosaic pigmentation of eyes is indicated as mosaic. B – UDTPR transgene.
rected towards bxdPRE. However, the first construct (UDTPD) carries bxdPRE in forward orientation, while in the second construct (UDTPR) bxdPRE is located in reverse orientation relative to the UAS promoter (Fig. 1). In order to suppress internal transcripts of the transgene, two SV40 terminators were used: upstream of the hsp26-lacZ gene and upstream of the white gene enhancer. An additional transcription terminator, SV40, was inserted at the 5’ side of bxdPRE in order to block transcription from the UAS promoter.

Key elements, bxdPRE and the SV40 terminator at the 5’ side of bxdPRE in both constructs, were flanked by the LOX or FRT site for site-specific recombinases Cre or Flp, respectively. This approach allows one to excise in vivo the selected DNA fragments and to compare the expression of the marker gene and functional changes in the system in the presence or absence of key elements at the same genome position (sites of transgene integration).

As a result of construct transformation, four independent transgenic lines for UDTPD (Fig. 1A) and three lines for UDTPR (Fig. 1B) were obtained with bxdPRE in repressed state. Repression of the white gene was enhanced in homozygous flies. This effect is characteristic of PRE elements and called PSS (Pairing Sensitive Silencing) [38]. The phenotypes of the UDTPD and UDTPR transgenes were similar; i.e., the effects were independent of bxdPRE orientation. Deletion of the transcription terminator located between the UAS promoter and PRE did not result in any phenotypic changes. However, the induction of the UAS promoter by GAL4 led to derepression of the white gene both in the case of terminator deletion and in intact lines. Thus, GAL4 inactivates bxdPRE in the studied system regardless of orientation and presence of a terminator between the UAS promoter and bxdPRE.

Transcription through bxdPRE does not lead to elimination of Polycmb and Trithorax group factors from bxdPRE

We have previously shown that even robust transcription does not lead to complete elimination of PcG/TrxG complexes from bxdPRE if it is oriented forward in the transgene. We tested the influence of transcriptional read-through in the case of reverse orientation of bxdPRE. For this purpose, we conducted immunoprecipitation of chromatin isolated from adult homozygous flies in the presence or absence of GAL4 (Fig. 2). Immunoprecipitation was carried out using samples obtained from the transgenic line UDTPR (№ 2) with a deleted SV40 transcription terminator. Six areas of the construct were used for PCR analysis: 1 – UAS promoter, 2 – eGFP gene coding region, 3 – bxdPRE, 4 – LacZ gene coding region, 5 – white gene enhancer, and 6 – white gene promoter. As a positive control, we used the genomic region of bxdPRE adjacent to the element utilized in transgenic constructs, while the coding region of the Ras64B gene was used as a negative control (Fig. 2).

It has been shown that the peak of the PH (PRC1 complex, Fig. 2A) and dSfmbt (PhoRC complex, Fig. 2B) factors recruitment corresponds to bxdPRE in the transgene. Localization of these factors is consistent with the data according to which PH and dSfmbt are found predominantly in PRE elements but not in other regions of the repressed domain [14, 15, 21, 39, 40].

The level of recruitment of these factors decreases upon induction of transcription through bxdPRE, but they are not eliminated completely. A similar result was obtained when analyzing the impact of transcription on the recruitment of the PH and dSfmbt factors to bxdPRE located in transgene in forward orientation relative to the UAS promoter [21].

Factor PC of the PRC1 complex specifically interacts with histone 3 trimethylated at lysine 27 (H3K27me3) [41, 42], a modification characteristic of PcG-repressed chromatin [16, 40]. Recruitment of the PC factor, as well as H3K27me3, contrary to other core components of PcG complexes, is not limited to PRE and covers a wider area subjected to repression [16, 21, 40, 43]. In agreement with this, a wider profile of distribution of the PC factor (Fig. 2C) and H3K27me3 modification (Fig. 2D) has been found in the derivative of the UDTPR transgene. Introduction of a GAL4 activator did not lead to complete elimination of PC and H3K27me3, but there was a significant decrease in the level of their recruitment to bxdPRE and the surrounding areas of the transgene.

We also analyzed the recruitment of the TrxG factors Trx (Fig. 2E) and GAP (Fig. 2F). It was established that the induction of transcription through bxdPRE leads to a 2-fold increase in the recruitment of both factors to bxdPRE.

Thus, transcription through PRE leads to a change in the level of PcG/TrxG factors recruitment but not to complete displacement of these proteins from DNA.

CONCLUSION

The repression/activation of various Drosophila genes requires PcG/TrxG proteins [1–4] that bind to the DNA elements termed PREs [5, 6]. A series of studies has shown that a lack of PRE-mediated repression correlates with the presence of non-coding transcripts [24, 44]. On this basis, a model was proposed according to which transcriptional read-through physically dislodges PRE-associated factors and replaces repressive histone modifications with active ones [24]. Despite its apparent clarity, this hypothesis has not been tested directly.
On the other hand, according to other data, non-coding RNAs from Ubx locus (lncRNA-bxd and lncRNA iab-8) are associated with the domain subjected to repression [45, 46]. Moreover, in spite of scrupulous studies, non-coding RNAs have not been detected in the regions of PRE elements of several loci (invected, engrailed), which indicates the absence of a key role for transcription, at least in the functioning of several PRE elements [47].

Previously, we tested the effect of transcription on GAL4-mediated activity switch of PRE [21]. As a result, we found that even robust transcription through bxdPRE does not lead to complete elimination of PcG/TrxG factors but changes the ratio in the binding of these proteins: recruitment of PcG decreases, while the recruitment of TrxG increases. The transcriptional effect was analyzed in detail for bxdPRE incorporated into transgene in direct orientation [21]. At the same time, active and inactive states of PRE in vg locus correlate with transcriptional read-through from different DNA strands [48]. Therefore, the direction of transcription through PRE can potentially be crucial for the activity of PRE. We have tested this possibility and found that alteration of bxdPRE orientation does not lead to a
change in the transcriptional read-through effect. Recruitment of PcG/TrxG factors is not abolished upon transcription. However, the recruitment of the TrxG proteins Trx and GAF increases, while the recruitment of PcG proteins (PH, dSfmbt, PC) decreases.

The presence of the strong terminator SV40 between the UAS promoter and bzdPRE also does not prevent abolition of repression. Apparently, the GAL4-binding sites themselves are capable of neutralizing PRE-mediated repression and transcription though PRE does not play a crucial role in this process.

PRE elements regulate genes the expression of which is changed during differentiation and development. Thus, a particular gene must be expressed in certain cells at a certain stage of development, and then its expression should be suppressed. Apparently, the recruitment of repressor factors to PRE in activating state could be required for the quick PRE activity switch to the repressing state and to abort the expression of the target gene at a certain moment in time. A logically similar mechanism has been described for many eukaryotic promoters: pausing of RNA polymerase II. In this case, RNA polymerase II binds to a transcriptionally inactive promoter and, if necessary, quickly triggers transcription.

The mechanism that allows the recruitment of proteins to PRE during transcriptional read-through is unclear. A series of DNA-binding factors with zinc finger motifs are known to be associated with PREs. It is possible that transcription does not interfere with direct DNA–protein contacts. On the other hand, there is a possibility that retention of complexes at PRE during transcriptional read-through is mediated by the contacts between the PcG/TrxG factors and histone proteins. In accordance, PcG proteins contain domains capable of interacting directly with nucleosomes (for example, the MBT domains of dSfmbt and Scm) [14, 49, 50] and transcription does not result in complete dissociation of nucleosomes [51]. However, the details of these processes are currently unclear and require further investigation.

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REFERENCES