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Regulated Gene Expression as a Tool for Analysis of Heterochromatin Position Effect in *Drosophila*

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Abstract—Position effect variegation (PEV) is a perturbation of genes expression resulting from the changes in their chromatin organization due to the abnormal juxtaposition with heterochromatin. The exact molecular mechanisms of PEV remain enigmatic in spite of the long history of PEV studies. Here, we developed a genetic model consisting of PEV-inducing chromosome rearrangement and a reporter gene under control of the UAS regulatory element. Expression of the reporter gene could be regulated by adjustment of the GAL4 transactivator activity. Two UAS-based systems of expression control were tested – with thermosensitive GAL4 repressor GAL80^{ts} and GAL4-based artificial transactivator GeneSwitch. Both systems were able to regulate the expression of the UAS-controlled reporter gene over a wide range, but GAL80^{ts} repressed the reporter gene more efficiently. Measurements of the heterochromatin-mediated repression of the reporter gene in the GAL4+GAL80^{ts} system point to the existence of a threshold level of expression, above which no PEV is observed.

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Position effects are disruptions of gene expression resulting from the gene transfer to another genomic region without changes in its DNA sequence. The existence of the effects of this type indicates that the chromosomal environment influences the level of gene expression. Position effect variegation (PEV) is a particular case of position effect manifested as perturbations in the expression of euchromatin genes after their relocation close to the heterochromatin as a result of the chromosomal inversions, translocations, or transpositions. Heterochromatin here is a type of chromatin organization in eukaryotes; it consists of different types of DNA repeats and is characterized by a specific set of non-histone proteins, histone modifications, and a high level of compaction during interphase [1]. Chromosome regions close to centromeres (pericentromeric heterochromatin) and telomeres are mainly composed of heterochromatin, as well as chromosomes Y and 4 in *Drosophila*. Heterochromatin contains a rather small number of genes, compared to euchromatin.

PEV in *Drosophila melanogaster* was discovered more than 60 years ago [2] and has been actively studied since then [1, 3, 4]. PEV-associated perturbations in the expression of euchromatin genes are caused by changes in the chromatin structure of the gene environment [5], the so-called heterochromatinization (the term introduced by Prokofieva-Belgovskaya [6]). Searches for the genes that affect PEV revealed a number of non-histone chromatin proteins and demonstrated the role of histone modifications in the processes of gene repression and activation [7]. It was found that the mechanisms of chromatin structure formation are quite similar among eukaryotes, non-histone proteins are conserved, and histone modifications have the same effects in yeast to mammals [8]. The most interesting aspect of the PEV is variegated repression – a situation when a gene is active in certain cells and repressed in others. This mosaic expression indicates the ability of the repressed/active state of chromatin to be inherited during mitosis (epigenetic inheritance).

Earlier, we have created and studied the PEV-causing chromosomal rearrangement *In(2)A4* [9-11]. This rearrangement is an inversion of a fragment of the chromosome 2 left arm with the breakpoints in the *Mcm10* gene (region 39B) and in the left arm pericentromeric

Abbreviations: PEV, position effect variegation; *UAS-eGFP-Ret*, insertion of the reporter gene *UAS-eGFP* in the *Ret* gene.

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heterochromatin region containing the $(AATAACATAG)_n$ satellite (Fig. 1a). The *In(2)A4* rearrangement affects genes located near the breakpoint (*cis*-effect), as well as the reporter transgenes located on the normal chromosome in regions homologous to those that were relocated closer to the heterochromatin in the inversion (*trans*-inactivation) (Fig. 1, a and b).

An example of *cis*-acting heterochromatin position effect is repression of the *mini-white* gene within the P-element located 55 kb from the eu-/heterochromatin boundary in *In(2)A4* (Fig. 1a). The repression is manifested as the eye color variegation and demonstrates the specific features of heterochromatin position effect, such as the sensitivity to PEV modifier mutations and temperature [9, 11]. The *trans*-acting position effect (*trans*-inactivation) caused by the *In(2)A4* inversion is manifested as an impaired expression of the *mini-white* reporter genes located in the homologous normal chromosome. *Trans*-inactivation is observed in the 500-kb region of the normal chromosome homologous to the region around the breakpoint in *In(2)A4*. *Trans*-inactivation demonstrates a complex pattern of spreading; the genomic regions with no repression are surrounded by *trans*-inactivated regions (Fig. 1b). Only a few examples of *trans*-acting position effect have been reported in the literature so far; the *In(2)A4* inversion represents one of the two thoroughly studied cases (the first is the $brown^{Dominant}$ mutation caused by the insertion of about 1.5 Mb of the AAGAG satellite into the coding region of the *brown* gene) [12-15]. *Trans*-inactivation results from the somatic conjugation (pairing) between the normal and rearranged chromosomes and translocation of a region of coupled chromosomes into the heterochromatin compartment of the nucleus via the sticking of heterochromatin blocks. According to our data, *trans*-inactivation of the reporter genes is a result of their *de novo* heterochromatinization, since the reporter gene on the normal chromosome can be inactivated, whereas the same region in the chromosome with the inversion remains transcriptionally active and does not contain heterochromatin marks [9].

Studies of molecular mechanisms of heterochromatin interaction with the gene transcription machinery require a genetic system in which a reporter gene of a known structure and with the adjustable expression level could be exposed to the position effect. Such system could be based on *trans*-inactivation-inducing *In(2)A4* rearrangement; the reporter construction could be integrated into a normal chromosome which would be then combined with the rearranged chromosome in the same genome in order to study the position effect. In our work, transgene constructions bearing the *UAS-eGFP* and *mini-white* reporter genes were inserted into the region undergoing *trans*-inactivation using the phiC31 integrase-based site-specific integration system.

Here, we studied two transcription control systems based on the yeast GAL4 transactivator. The GAL4+$GAL80^{ts}$-based system includes genomic sources of the GAL4 and the thermosensitive variant of the GAL80 protein (GAL4 inhibitor) [16, 17]. At low temperatures, $GAL80^{ts}$ inhibits GAL4 transactivation activity, whereas at high temperatures the $GAL80^{ts}$ degrades, allowing GAL4 to activate transcription. In the GeneSwitch system, transcription is activated by synthetic steroid hormone RU486. GeneSwitch is an artificial protein consisting of the GAL4 DNA-binding domain (recognizes UAS), the hormone-binding fragment of the human progesterone receptor, and the transactivation domain of the NF-κB protein [18, 19]. It was shown that both systems can activate the reporter gene expression hundred times. The GAL4+$GAL80^{ts}$ combination displays low background expression and is efficient in the situations when the low levels of the reporter gene expression are required.

Analysis of the influence of the *In(2)A4* rearrangement on the level of the reporter genes transcription showed that high expression levels and the presence of additional regulatory elements prevented *trans*-inactivation. The use of the transcription regulation system allowed us to tweak the level of the reporter gene expression up to the threshold at which the heterochromatin repression is abolished. In the described system, the reporter gene expression should be 100 times higher than the background expression to suppress the PEV.

The aim of this work was to develop a system allowing to regulate the expression of the reporter gene affected by the heterochromatin position effect, and to use this system to study the relationship between the gene transcription and its repression in the heterochromatin environment.

MATERIALS AND METHODS

Fly stocks and transgenic constructions. Inversion *In(2)A4* was produced from the fly strain carrying the *mini-white* reporter gene in the region 39B (5′-UTR of the *Hr39* gene) of the chromosome 2. Inversion *In(2)A4* reduces the viability of the flies and is maintained over the balancer chromosome *Cy* (*In(2)A4/Cy*).

The following transgenic constructions in the chromosome 3 were used (Bloomington collection, http://flystocks.bio.indiana.edu): the *P[w[+mC]=Act5C(−FRT)GAL4.Switch.PR]3* construction (hereafter *pAct(GS)*) containing the *GeneSwitch* gene under the Actin5C gene promoter (stock 9431); the *P[tubP-GAL80^{ts}]7* transgenic construction containing the *Gal80^{ts}* gene under the α-tubulin gene promoter (stock 7018); and the *P[tubP-GAL4]LL7* construction containing the *Gal4* gene also under the α-tubulin gene promoter (stock 5138). The Actin5C and α-tubulin gene promoters provide the high level of gene expression in most of the tissues at all developmental stages.

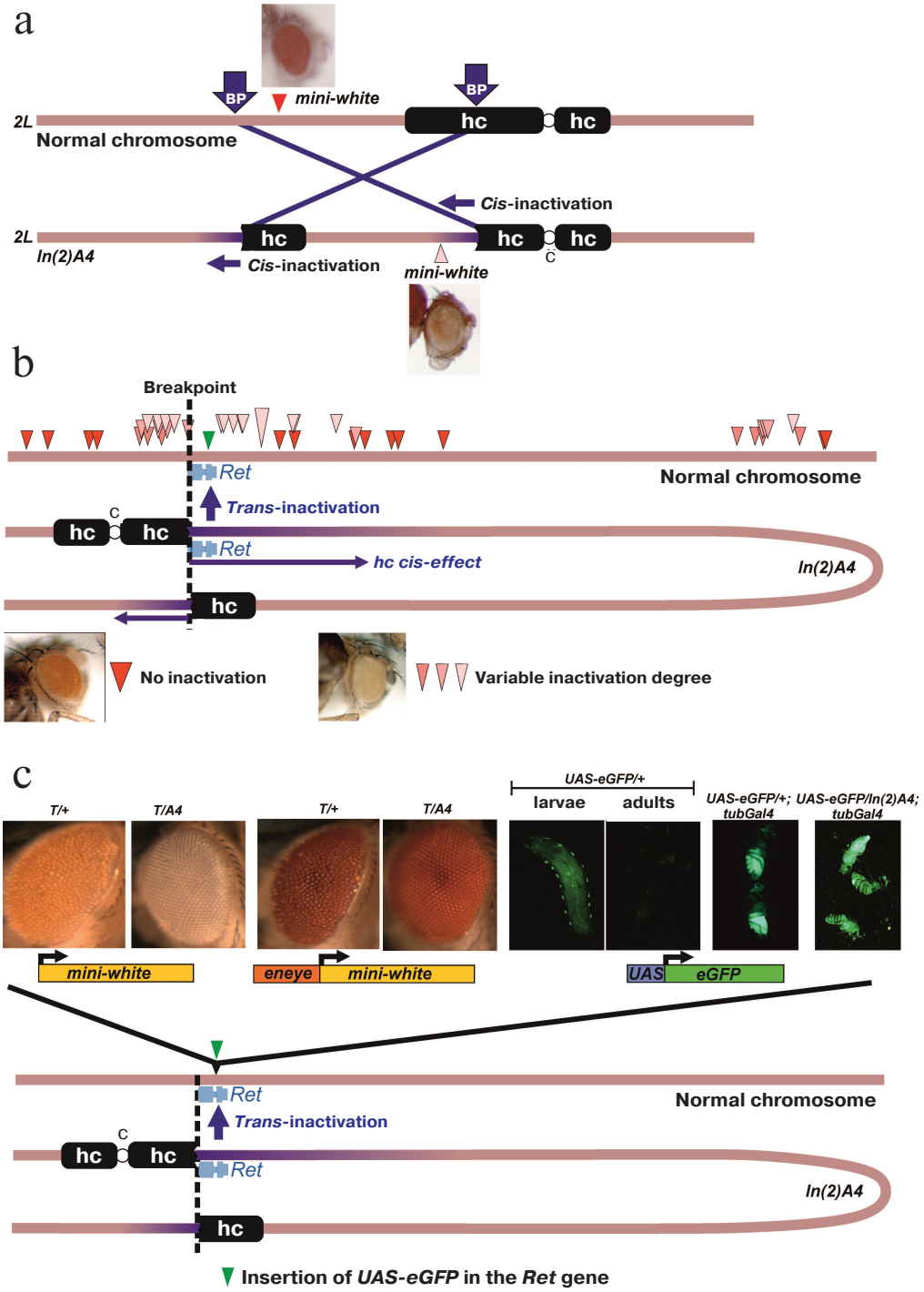

Fig. 1. Structure of the *In(2)A4* inversion and manifestation of the reporter genes *trans*-inactivation. a) Structure of the *In(2)A4* rearrangement. Rearrangement results from the breaks in euchromatin and pericentromeric heterochromatin (BP, breakpoints) and leads to the formation of two new euchromatin/heterochromatin borders. The reporter gene (*mini-white*) relocated to heterochromatin demonstrates the position effect (*cis*-inactivation). b) *Trans*-inactivation caused by the *In(2)A4* inversion. The inversion causes *trans*-inactivation of the reporter genes in the homologous non-rearranged chromosome in heterozygous flies. Positions of the *trans*-inactivated *mini-white* reporter genes are shown with triangles of different color according to the degree of their repression: from the total absence (red) to strong repression (light pink) [9]. The inversion is represented as a loop so that regions of the normal chromosome and the respective regions of *In(2)A4* would have the same orientation. c) Reporter constructions in the region of *trans*-inactivation caused by the *In(2)A4* inversion. All constructions were site-specifically integrated into the *Ret* gene closest to the euchromatin/heterochromatin border. Constructions from left to right: *mini-white* reporter gene is strongly repressed in *In(2)A4*; *mini-white* gene under control of the eye-specific enhancer (*eneye*) is strongly expressed and its repression is not observed (T-reporter transgene *mini-white* or *eneye-mini-white*, respectively). The *UAS-eGFP* reporter is weakly expressed in larvae and is not expressed in the adults. Activation by the source of GAL4 under the tubulin promotor (*tubGal4*) results in the high level of *eGFP* expression, whereby the repression in *In(2)A4* is not observed. Abbreviations: hc, heterochromatin; c, chromosome 2 centromere.

To insert the reporter genes into specific genomic regions, we used the system of site-specific integration based on phiC31 integrase and *Drosophila* strains bearing transgenic MiMIC constructions [20-22]. The MiMIC construction is a landing site for specific integration of transgenes into the genome by phiC31 integrase. The *vasdPhiC31*strain bearing the *phiC31* gene under the control of the *vasa* gene promoter on the X chromosome [20] was used as an integrase source. The efficiency of integration in our system was approximately 70%.

Plasmids *attBs-Eneye-whited700-MCS-attBsrev-pSK=aeca* containing the *mini-white* gene under control of the eye-specific *eneye* enhancer (*eneye-mini-white*) and *attB* recombination sites and *pCaspew15-good-1RI* with the *mini-white* gene without an enhancer were obtained from the Laboratory of Regulation of Genetic Processes, Institute of Gene Biology, Russian Academy of Sciences. To generate the transgene construction with *mini-white* reporter gene, the *eneye-mini-white* sequence in the *attBs-Eneye-whited700-MCS-attBsrev-pSK=aeca* plasmid was replaced by the *mini-white* gene sequence from *pCaspew15* keeping the *attB* recombination sites intact. The construction containing the *UAS-eGFP* reporter gene flanked by the *attB* recombination sites (*attBs-UASeGFP-attBsrev-pSK*) was obtained by cloning the *UAS-eGFP* fragment into the vector with *attB* sites (*attBs-Eneye-whited700-MCS-attBsrev-pSK=aeca*). This construction was integrated into the genome by recombination with the MiMIC transgene located in the *Ret* gene (*Drosophila* stock 43099 from the Bloomington collection, genotype *y[1]w[*]; Mi[y[+mDint2]=MIC]Ret[MI07200]/SM6a*). The exact position, orientation, and structure of the *UAS-eGFP* transgene insertion (below referred as *eGFP*) are shown in Fig. 2a.

Regulation of the reporter gene expression in the GeneSwitch system. The synthetic hormone RU486 (Sigma, USA) binds the GeneSwitch protein [18, 19] and transforms it into a strong transcription activator. RU486 penetrates the cell membrane, so experimental organisms can be treated simply by adding RU486 to the food or culturing medium or by incubation in RU486-containing solution. The concentrations of the hormone in the medium could vary from 2 to 200 μg/ml without effects on viability. Preliminary experiments showed that the maximal level of the *eGFP* expression was achieved at the RU486 concentration of 50 μg/ml and remained constant upon further increase of the hormone concentration (Fig. 2b). Therefore, we used 50 μg/ml RU486 in all our experiments.

Activation of the *eGFP* expression was studied in the *eGFP/+*; *pAct(GS)/+* flies versus the control e*GFP/+*; *+/+*. The expression was activated at 25°C in starving adult flies (incubated overnight in tubes with agarose prepared on MilliQ water) by adding the RU486-containing yeast suspension as a food. The activation of *eGFP* expression was observed after 1 h of feeding (Fig. 2b). Addition of RU486 at the early developmental stages was lethal.

Regulation of the reporter gene expression in GAL4+GAL80^{ts} system. To test the ability of the thermosensitive form of GAL80 protein (GAL80^{ts}) to suppress activation of the *UAS-eGFP* reporter gene by GAL4 (encoded by *tubGal4*), flies with the following genotypes were used: *eGFP/+*; *tubGal4/tubGal80^{ts}* (experiment), *eGFP/+*; *tubGal4/+* and *eGFP/+*; *+/tubGal80^{ts}* (positive and negative controls, respectively).

Drosophila flies were grown at 18, 25, and 30°C. No *eGFP* expression was visually observed at 18°C, while at 25°C, eGFP fluorescence was detected in almost all tissue types. Fluorescence in flies incubated at 30°C was very bright, however, a strong decrease in viability and fertility was observed. For further experiments, *eGFP/+*; *tubGal4/tubGal80^{ts}* flies were grown at 18°C; adult flies were then incubated for different periods of time at 30°C for inactivation of GAL80^{ts} and activation of GAL4 (Fig. 2b).

Regulation of the *eGFP* reporter gene transcription using the GAL4+GAL80^{ts} and GeneSwitch systems. To analyze the profile of *eGFP* expression activation, the amounts of *eGFP* mRNA were measured in adult flies containing the *eGFP* reporter gene and the GeneSwitch or GAL4+GAL80^{ts} sources. When using the GAL4+GAL80^{ts} system, *eGFP/+*; *tubGal4/tubGal80^{ts}* (experiment), *eGFP/+*; *tubGal4/+* (positive control, contains only *Gal4*), and *eGFP/+*; *tubGal80^{ts}/+* (negative control, no *Gal4*) flies were grown at 18°C; 1- to 2-day-old adult flies were then incubated at 30°C for different times. mRNA was isolated from the flies before treatment and after incubation for 1, 3, and 24 h at high temperature (Fig. 2c). To determine the established level of *eGFP* expression at different temperatures, *eGFP/+*; *tubGal4/tubGal80^{ts}* flies were grown at 18, 25, and 30°C during the entire period of development, and then mRNA was isolated from 1-2-day-old adults. Incubation at 30°C resulted in high lethality; however, some flies survived and were used for analysis (Fig. 2e).

When using the GeneSwitch system, mRNA was isolated from the *eGFP/+*; *pAct(GS)/+* flies before and 1, 3, 8, 24 h after feeding (during 1 h) with the RU486-containing yeast paste. *eGFP/+* flies (without *GeneSwitch* or *Gal4*) were used as a negative control (Fig. 2c).

Quantitative PCR. RNA from adult flies was isolated using Trizol reagent according to a standard protocol and treated with DNase (Thermo Fisher Scientific, USA) to eliminate the traces of DNA. Reverse transcription was performed using random hexanucleotides (Silex, Russia) and MINT reverse transcriptase (Eurogene, Russia). Relative quantities (ΔCq) of *eGFP* mRNA were estimated by real-time PCR with a DT-96 amplifier (DNA Technology, Russia); obtained mRNA values were normalized to the *Rpl32* gene mRNA amount. The *Rpl32* gene encodes one of the ribosomal proteins and is char-

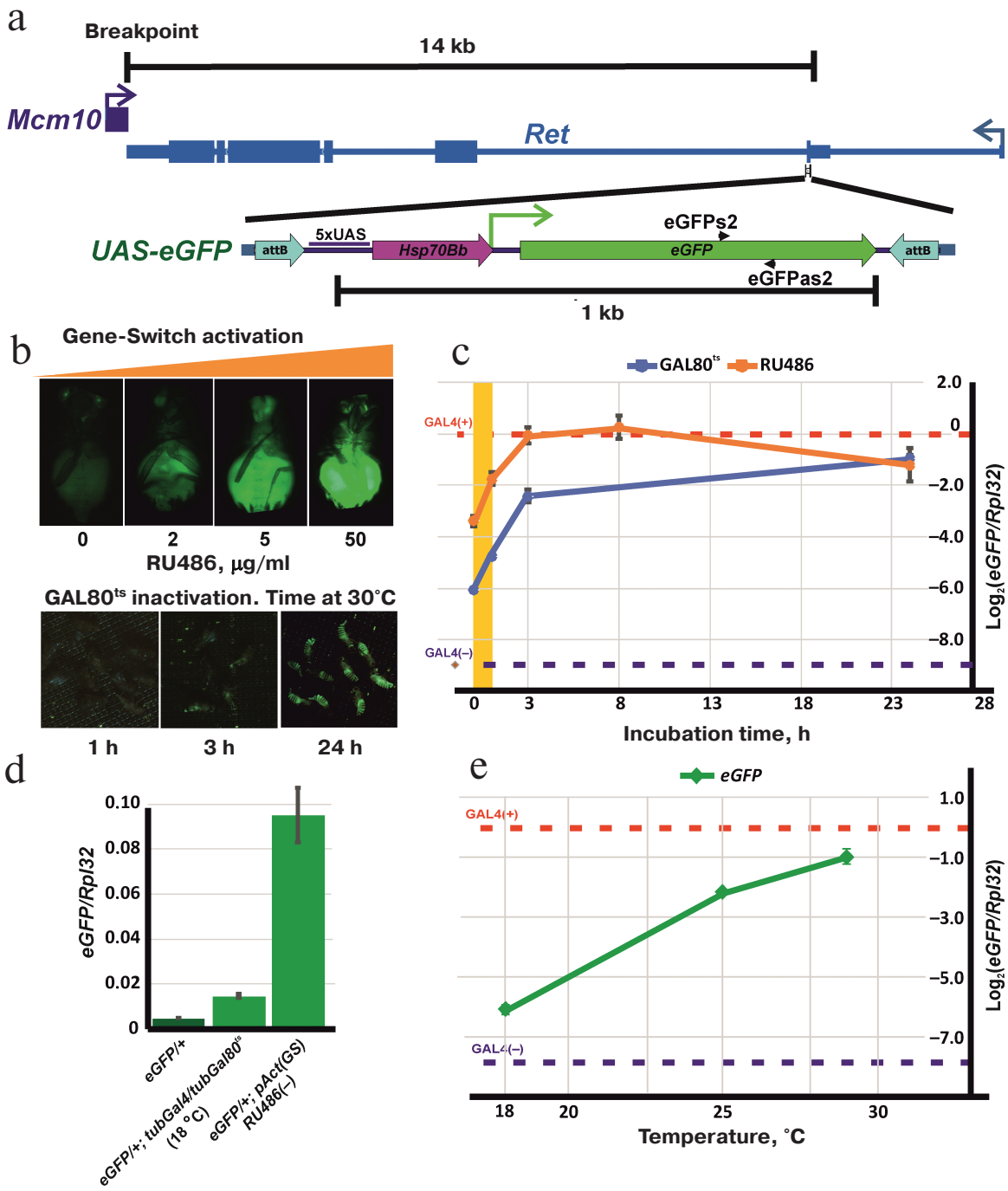

Fig. 2. Schematic representation of the transgenic *eGFP* construction and regulation of the *UAS-eGFP* expression using GAL4+GAL80^{ts} and GeneSwitch. a) Position and orientation of the transgenic *eGFP* insertion relative to the *Ret* gene and the breakpoint of *In(2)A4* inversion: *attB*, sites for specific recombination into the genome; 5xUAS, GAL4-binding site; *Hsp70Bb*, *Hsp70* gene minimal promotor; *eGFP*, coding sequence of the *eGFP* reporter gene. Positions of primers *eGFPs2* and *eGFPas2* are shown. b) Activation of the *UAS-eGFP* reporter gene expression. Upper panel, phenotypes of *eGFP/+*; *pAct(GS)/+* flies after incubation in the medium with increasing RU486 concentration; the lower panel, phenotypes of *eGFP/+*; *tubGal4/tubGal80^{ts}* flies after incubation at 30°C for 1, 3, and 24 h. c) Profiles of *eGFP* expression in GAL4+GAL80^{ts} and GeneSwitch+RU486 systems. Axis *y*, the logarithm of the ratio of *eGFP* expression to that of *Rpl32* determined by quantitative PCR. Axis *x*, incubation time at 30°C for *eGFP/+*; *tubGal4/tubGal80^{ts}* flies (blue graph) or hours after feeding with RU486-containing yeast mix for *eGFP/+*; *pAct(GS)/+* flies (orange graph). Blue dashed horizontal line, the relative level of *eGFP* expression in *eGFP/+* flies without transactivator (basic expression). Red dashed horizontal line, *eGFP* expression level in *eGFP/+*; *tubGal4/+* flies (maximal activation of *eGFP* expression). Orange area, the period of feeding with the RU486-containing mix. d) *eGFP* expression levels without activation. From left to right: *eGFP/+*; *tubGal80^{ts}/+* flies (without a source of *Gal4* in the genome); *eGFP/+*; *tubGal80^{ts}/tubGal4* flies at 18°C (maximum repression by *Gal80^{ts}*); *eGFP/+*; *pAct(GS)/+* flies (in the absence of RU486). e) Dependence of *eGFP* activation in *eGFP/+*; *tubGal80^{ts}/tubGal4* flies on temperature. The relative amount of *eGFP* mRNA was measured 24 h after incubation at 18, 25 and 29°C (for description, see Fig. 2c).

acterized by high and stable expression levels in the majority of tissues and at all developmental stages (gene profile in the FlyBase database (http://flybase.org); our data). The relative amounts of *eGFP* mRNA were measured using the RealTime_PCR software from the amplifier manufacturer.

The sequences of used primers are below (8.25 pmol of each primer per standard 30-μl PCR reaction):

for *Rpl32* gene:
Rp49up: ATGACCATCCGCCCAGCATAC
Rp49rev2: GCTTAGCATATCGATCCGACTGG;

for *eGFP* gene:
eGFPs2: CCTGGGGCACAAGCTGGAGT
eGFPas2: GGGTAGCGGCTGAAGCACTGC.

Amplification protocol included denaturation for 5 min at 95°C; 45 cycles of amplification (15 s at 94°C, 10 s at 64°C, 10 s at 72°C); final elongation of PCR products for 5 min at 72°C before reading of the melting curves. Amplification was carried out using Hot Start Taq DNA polymerase and PCR buffer (SibEnzyme, Russia) in the presence of intercalating fluorescent dye SYTO13 (Thermo Fisher Scientific). The presented values are the result of at least three independent experiments.

Microscopy. The images of eGFP fluorescence were obtained on Leica MZ6 binocular microscope (Leica Microsystems, Germany) equipped with a Leica DC300 camera. Fluorescence was excited at 450 nm (blue LED Luxeon Royal Blue 3W); the filter cut-off was <500 nm.

RESULTS

***Trans*-inactivation of the reporter constructions by the *In(2)A4* inversion.** As it was shown earlier [11], chromosomal rearrangement *In(2)A4* causes *trans*-inactivation of the reporter genes located on the homologous non-rearranged chromosome (Fig. 1b). Taking into account the *trans*-inactivation distribution data, we chose the *Ret* gene genomic region as the place where the inserted reporter gene would be repressed with a high probability. The *Ret* gene is the closest to the breakpoint in *In(2)A4*; the fly stock 43099 (Bloomington) contains the MiMIC insertion within the first exon of the *Ret* at a distance of 14 kb from the breakpoint. The *Ret* encodes cadherin superfamily protein and is expressed at a low level and mainly in the nervous system. Insertion of a reporter gene in the *Ret* is not lethal, the resulting homozygous flies are viable.

The reporter genes *eneye-mini-white* (contains the *mini-white* gene under the control of the eye-specific enhancer) and *mini-white* (no additional regulatory elements) were inserted into the *Ret* gene by specific integration with the MiMIC element in the fly stock 43099. Analysis of the *trans*-effect of the *In(2)A4* chromosomal rearrangement on the resulting constructions showed that the *mini-white* reporter gene is strongly inactivated, while the presence of the eye-specific enhancer prevents repression (Fig. 1c). Hence, the *In(2)A4* causes the *trans*- acting position effect of the reporter genes inserted into the *Ret* region in the non-rearranged chromosome. At the same time, active expression of the *mini-white* gene triggered by the eye-specific enhancer prevents *trans*-inactivation.

To study the influence of heterochromatin on transcription activation, we've constructed flies where the *eGFP* reporter gene with *Hsp70* promoter and UAS regulatory element was inserted into the *Ret* gene (Fig. 2a). In the absence of the GAL4 transactivator, the level of *eGFP* expression in these flies was low in both larvae and adults. In larvae, a specific pattern of expression associated with peripheral ganglia and, probably, corresponding to the expression pattern of the *Ret* gene was observed. In adult flies, no *eGFP* expression was visually detected (Fig. 1c). Insertion of the GAL4 source under the control of the α-tubulin promotor (*tubGal4*) into the genome of flies bearing the *UAS-eGFP* construction results in a high level of *eGFP* expression; at the same time, no *trans*-inactivation of the reporter gene was observed in *eGFP/A4*; *tubGal4* flies comparing to *eGFP/+*; *tubGal4* flies (Fig. 1c). Therefore, expression of the reporter gene at a high level prevents *trans*-inactivation (as in the case of the *mini-white* gene under the control of the eye-specific enhancer).

Regulation of the *eGFP* reporter gene expression in the GAL4+GAL80^{ts} and GeneSwitch systems. The level of the *UAS-eGFP* expression can be regulated using the systems based on the modulation of the GAL4 transactivator activity. These systems allow to identify the threshold level of *eGFP* expression above which no *trans*-inactivation of the reporter gene is observed. To choose the optimal tool for the regulation of the reporter gene expression, we analyzed the GAL4+GAL80^{ts} and GeneSwitch systems.

The results of our experiments are presented in the Fig. 2, c-e. Panels (d) and (e) (Fig. 2) show the levels of *eGFP* expression in control flies with blue (*eGFP*; *Gal4(–)*, negative control) and red (*eGFP*; *Gal4(+)*, positive control) dashed lines. The presence of *Gal4* gene under the control of the tubulin promotor (*tubGal4*) in the genome leads to the 600-fold increase in the *eGFP* expression. The level of *eGFP* expression almost reaches the level of expression of the ribosomal protein *Rpl32* gene used for normalization.

Inactivation of the GAL80^{ts} protein occurs gradually from 18 to 30°C; when the temperature rises from 20 to 25°C, the reporter gene expression level increases 15 times (Fig. 2e). At the same time, the level of *eGFP* expression at 30°C in *eGFP/+*; *tubGal4/tubGal80^{ts}* flies is two times lower than in *eGFP/+*; *tubGal4/+* flies, i.e.,

GAL80^{ts} partially retained its repressor activity. Higher temperatures have a negative effect on the viability of flies and were not tested. During the first hour of incubation of the flies at 30°C, the amount of *eGFP* mRNA increases 2.5 times and then continues to increase; after 3 h of incubation, the rate of mRNA accumulation drops down (Fig. 2c).

Activation of the GeneSwitch by the hormone RU486 in *eGFP/+*; *pAct(GS)/+* flies increases the level of *eGFP* mRNA 3-fold during the first hour after feeding; then the mRNA accumulation continues. After 8 h, the mRNA level reaches its maximum which is close to the level of *eGFP* mRNA amount in the positive control flies (*eGFP/+*; *tubGal4/+*). The *eGFP* mRNA amount remains high 24 h after termination of hormone treatment (Fig. 2c). At the same time, the presence of *GeneSwitch* in the genome, even in the absence of RU486, results in a 47-fold increase in the reporter gene expression comparing to the background expression of *eGFP* in the flies without *GeneSwitch*. The level of *eGFP* expression in the presence of both *tubGal4* and *tubGal80^{ts}* in the genome at 18°C was only 3 times higher than the background expression (Fig. 2d). Therefore, *eGFP* expression can be regulated by both the GAL4+GAL80^{ts} and GeneSwitch systems; however, GAL80^{ts} is much more efficient in the suppression of the reporter gene than the GeneSwitch in the absence of RU486.

The level of reporter gene expression affects the degree of heterochromatin-mediated repression. The transgenic *eGFP* construction locates in the area affected by *In(2)A4*-caused *trans*-inactivation. The influence of heterochromatin on a euchromatin gene could be modulated by the level of expression of this gene at different developmental stages. Using the GAL4+GAL80^{ts} system, we found the temperature range (and consequently, the level of *eGFP* expression) at which the significant *trans*-inactivation of the reporter gene is observed (Fig. 3). Comparison of the *eGFP* expression in *eGFP/+*; *tubGal4/+* (control) and *eGFP/In(2)A4*; *tubGal4/+* flies (*trans*-inactivation condition) shows that the high expression levels block *trans*-inactivation of *UAS-eGFP* like in the case of the *eneye-mini-white* reporter gene (Fig. 1c). In the flies without GAL4 (*eGFP/+* and *eGFP/In(2)A4*), the eGFP fluorescence is not visually detectable; however, its expression level could be determined by quantitative PCR. We found that the background level (without GAL4 transactivator) of *eGFP* expression in *eGFP/In(2)A4* flies was ~3 times lower than in *eGFP/+* flies. Therefore, in absence of specific activation of the expression, *eGFP* is *trans*-inactivated by the *In(2)A4* rearrangement (Fig. 3a). Insertion of the sources of GAL4 transactivator and GAL80^{ts} repressor into the genome allows to regulate the level of *eGFP* expression by changing the incubation temperature. Comparison of the

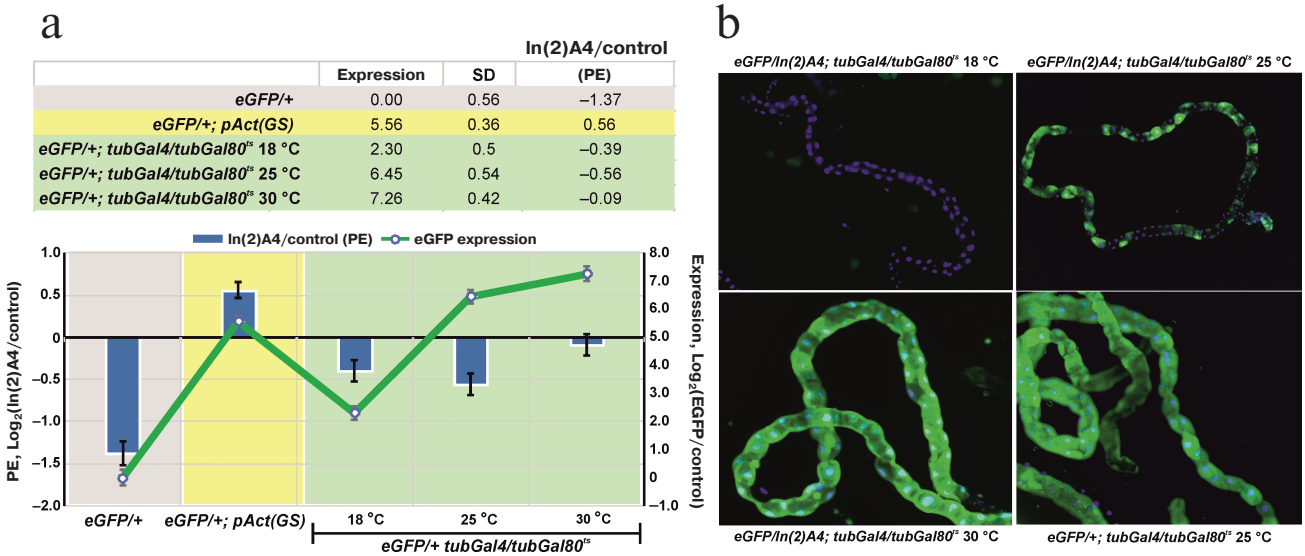

Fig. 3. The influence of the *eGFP* reporter gene expression level on the degree of its *trans*-inactivation by the *In(2)A4* inversion. a) *eGFP* expression level in the GeneSwitch and GAL4+GAL80^{ts} systems (graph, right scale) and the repression degree under the influence of *In(2)A4* (bars, left scale). Expression is presented as a logarithm of the ratio of the *eGFP* expression level in a given genotype to that in *eGFP/+* flies (with the reporter gene, but without the transactivator GAL4). The degree of *trans*-inactivation (*In(2)A4*/control) is presented as a logarithm of the ratio of the *eGFP* reporter expression in *eGFP/In(2)A4* flies to that in *eGFP/+* flies (control); negative values indicate *trans*-inactivation. *eGFP/+*; *pAct(GS)* – flies with the reporter gene and GeneSwitch in the absence of activation with RU486; *eGFP/+*; *tubGal4/tubGal80^{ts}* – flies with *eGFP* reporter gene in the presence of GAL4 and GAL80^{ts}; flies were grown at 18, 25, and 30°C. b) Expression of the *eGFP* reporter gene in Malpighian tubules of *eGFP/In(2)A4*; *tubGal4/tubGal80^{ts}* and *eGFP/+*; *tubGal4/tubGal80^{ts}* (control) flies at different temperatures; no *eGFP* expression is observed at 18°C; mosaic inactivation of the *eGFP* expression is observed at 25°C; at 30°C the *eGFP* expression level is high, and no significant position effect (PE) is observed. Green fluorescence, *eGFP*; blue fluorescence, DAPI-stained nuclei.

eGFP expression levels in *eGFP*/+; *tubGal4/tubGal80^{ts}* and *eGFP/In(2)A4*; *tubGal4/tubGal80^{ts}* flies at 18, 25, and 30°C shows that at 18 and 25°C, when the levels of *eGFP* expression are 3 and 87 times higher than the background, the ~1.5- to 2.0-fold repression of reporter by the *In(2)A4* is observed. No inactivation is detected at 30°C (where the level of *eGFP* expression is 153 times higher than the background level) (Fig. 3a). Hence, the ~100-time increase in the gene expression prevents heterochromatin repression. In some types of tissues (malpighian tubes), heterochromatin repression of the *eGFP* reporter gene leads to the classical mosaic PEV phenotype (Fig. 3b). The high background level of the reporter gene transcription in the flies with *GeneSwitch* source (*eGFP/In(2)A4*; *pAct(GS)*/+) prevents *trans*-inactivation (Fig. 3a).

DISCUSSION

Chromosomal rearrangement *In(2)A4* affects both the expression of euchromatin genes translocated near the heterochromatin (*cis*-position effect) and reporter genes in the corresponding regions of homologous non-rearranged chromosome (*trans*-inactivation), providing an opportunity to study and to compare the mechanisms of *cis*- and *trans*-inactivation. Earlier, we have found a number of unexpected features of the *cis*-position effect in the case of *In(2)A4* rearrangement. The effect of heterochromatin on the juxtaposed euchromatin (*cis*-effect) is not limited to the repression of genes located there. Only a small part of genes translocated to heterochromatin changed their level of expression, and both repression and activation of genes were observed. Moreover, in some cases, the character of heterochromatin influence on a gene depends on the developmental stage; the *cis*-inactivation of a gene at the late larval stage could be switched to the activation of its transcription in adults [9-11].

The different sensitivity of euchromatin genes to the heterochromatin position effect was reported for the position effect caused by *In(1)w^{m4h}* inversion. In this case, detectable repression was observed only for the *white* gene, and the authors [23] suggested that this gene is specifically sensitive to heterochromatin. This hypothesis, however, contradicts a large number of PEV cases described in the literature for different genes [3].

The data presented above do not correspond to traditional expectations on the influence of heterochromatin on euchromatin, according to which the position effect is a result of nonspecific repression of genes caused by the chromatin compaction. Heterochromatin interacts in a complex manner with the transcriptional machinery, and the result of such interactions varies for different genes. Heterochromatin environment can prevent chromatin remodeling (changes in the histone code and nucleosome positioning under the influence of promoter-associated chromatin-remodeling protein complexes) during transcription induction. This model suggests that the effect of heterochromatin environment is manifested at the moment of expression activation, whereas the established level of expression does not change.

Expression of some genes is induced during metamorphosis, and comparison of gene expression profiles at the pupal stage in flies with PEV and in control wild-type flies could reveal the influence of heterochromatin on the expression activation. We verified this suggestion in experiments with the genes potentially exposed to the position effects of inversions *In(2)A4* and *In(1)w^{m4h}*. It was shown that the genes, whose expression does not change at the pupal stage, are not subjected to the heterochromatin position effect in the *In(2)A4* and *In(1)w^{m4h}* inversions, while the genes whose expression was activated at the pupal stage experienced the position effect, and their activation was perturbed (preliminary data).

To study the link between *trans*-inactivation and expression, a genetic system containing the reporter gene with adjustable expression located in the *trans*-inactivated region of a normal chromosome was developed. Constructions carrying the *mini-white* or *mini-white* under the control of the eye-specific enhancer (*eneye*) were inserted into the same position in the region affected by *trans*-inactivation. It was shown that the *mini-white* reporter gene underwent strong *trans*-inactivation by *In(2)A4*, while the presence of an enhancer in the transgene construction significantly increases expression of the *mini-white* gene and suppresses *trans*-inactivation (Fig. 1c). Two possible explanations of the observed effect of the enhancer can be proposed: either the high level of gene expression prevents position effect or the enhancer itself blocks heterochromatinization of a neighbor gene. To verify these hypotheses, the transgene construction containing the *eGFP* gene under the control of the regulatory UAS element was inserted into the same position as *mini-white*-containing transgenes, and the fly stocks with combination of *UAS-eGFP*, *In(2)A4* inversion, and a system of the *UAS-eGFP* expression control were generated.

We controlled the reporter gene expression using the GAL4 activator and its thermosensitive repressor GAL80^{ts} or the artificial protein GeneSwitch, which is able to bind UAS and activate the expression in the presence of RU486. Both systems can burst the expression of the reporter gene hundreds of times, however, the GeneSwitch protein significantly activates transcription even in the absence of RU486 (Fig. 2). Analysis of the changes in *eGFP* expression in the presence of *In(2)A4* shows that the *trans*-inactivation is suppressed when the reporter gene expression is adjusted to a high level by GAL4+GAL80^{ts} system. At lower expression levels, the position effect is observed; the *eGFP* expression level in

eGFP/In(2)A4 flies is 1.5-2.0 times lower than in *eGFP/+* flies (Fig. 3). Therefore, strong activation of the reporter gene expression is enough to prevent heterochromatin repression. The position effect is observable in a wide range of expression levels (from 3 to 87 times over the background; Fig. 3) but disappears when the expression is ~150 times higher than the background. This indicates the existence of some threshold level of transcription above which the gene is resistant to heterochromatinization.

Interestingly, when using the GeneSwitch-based system (*eGFP/In(2)A4*, *pAct(GS)/+* flies), no position effect was observed despite rather low levels of the reporter gene expression (Fig. 3). This observation can be possibly explained by the fact that *GeneSwitch* is under the *Actin5C* gene promoter, whereas the *Gal4* of the GAL4-GAL80^{ts} system is under the α-tubulin promotor. Although both promotors control housekeeping genes and provide high levels of transcription, α-tubulin promotor shows lower activity (compared to the *Actin5C* promotor) at the late embryonic stages and in the middle of metamorphosis at the pupal stage (FlyBase data). A higher level of expression of the *GeneSwitch* and, as a result, of the reporter *eGFP* gene at these stages can lead to the complete suppression of *trans*-inactivation.

Authors of [24] studied *trans*-inactivation of the *UAS-eGFP* reporter gene in the *brown^{Dominant}* system. In this case, the expression of the reporter gene was driven by the sources of GAL4 under the control of tissue-specific enhancers [24]. Unlike the results of our study, the degree of the *UAS-eGFP trans*-inactivation in [24] was not affected by the level of its expression. We believe that tissue-specificity and complex expression profiles of the GAL4 sources during development (see [24]) complicated the identification of correlations between the level of the reporter gene expression and the degree of its inactivation under the influence of heterochromatin.

The experimental system including the *UAS-eGFP* reporter gene and the system to control its expression using a combination of GAL4+GAL80^{ts} can be used in the studies of interconnections between the chromatin structure and gene expression, e.g., for identification of critical steps in the formation of transcription-inactive chromatin structure in ontogenesis and understanding of how the changes in the gene expression influence this process. It would be interesting to study the competition between heterochromatin components and transcription factors for binding to the promoter and track the possible changes in the intranuclear reporter gene localization in the case of *trans*-inactivation.

In this work, the system of the regulated reporter gene expression was used for the first time to study heterochromatin repression. In the future, this system might be used to investigate the dynamics of interactions between the gene transcriptional machinery and its heterochromatin environment.

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REFERENCES

1. Elgin, S. C., and Reuter, G. (2013) Position-effect variegation, heterochromatin formation, and gene silencing in *Drosophila*, *Cold Spring Harb. Perspect. Biol.*, **5**, a017780.
2. Muller, H. J. (1930) Types of visible variations induced by X-rays in *Drosophila*, *J. Genet.*, **22**, 299.
3. Spofford, J. B. (1976) Position-effect variegation in *Drosophila*, in *The Genetics and Biology of Drosophila*, Academic Press.
4. Weiler, K. S., and Wakimoto, B. T. (1995) Heterochromatin and gene expression in *Drosophila*, *Annu. Rev. Genet.*, **29**, 577-605.
5. Zhimulev, I. F., Belyaeva, E. S., Fomina, O. V., Protopopov, M. O., and Bolshakov, V. N. (1986) Cytogenetic and molecular aspects of position effect variegation in *Drosophila melanogaster*, *Chromosoma*, **94**, 492-504.
6. Prokofyeva-Belgovskaya, A. A. (1945) Heterochromatinization as a change of chromosome cycle, *Zh. Obshch. Biol.*, **4**, 93-124 (Russian version); Prokofyeva-Belgovskaya, A. A. (1947) Heterochromatinization as a change of chromosome cycle, *J. Genet.*, **48**, 80-98 (English version).
7. Reuter, G., and Spierer, P. (1992) Position effect variegation and chromatin proteins, *Bioessays*, **14**, 605-612.
8. Singh, J., Freeling, M., and Lisch, D. (2008) A position effect on the heritability of epigenetic silencing, *PLoS Genet.*, **4**, e1000216.
9. Abramov, Y. A., Shatskikh, A. S., Maksimenko, O. G., Bonaccorsi, S., Gvozdev, V. A., and Lavrov, S. A. (2016) The differences between *cis*- and *trans*-gene inactivation caused by heterochromatin in *Drosophila*, *Genetics*, **202**, 93-106.
10. Lavrov, S. A., Shatskikh, A. S., Kibanov, M. V., and Gvozdev, V. A. (2013) Correlation on a cellular level of gene transcriptional silencing and heterochromatin compartment dragging in case of PEV-producing eu-heterochromatin rearrangement in *Drosophila melanogaster*, *Mol. Biol. (Moscow)*, **47**, 286-291.
11. Abramov, Y. A., Kibanov, M. V., Gvozdev, V. A., and Lavrov, S. A. (2011) Genetic and molecular analysis of gene trans-inactivation caused by homologous eu-heterochromatic chromosome rearrangement in *Drosophila melanogaster*, *Dokl. Biochem. Biophys.*, **437**, 72-76.
12. Csink, A. K., Bounoutas, A., Griffith, M. L., Sabl, J. F., and Sage, B. T. (2002) Differential gene silencing by *trans*-heterochromatin in *Drosophila melanogaster*, *Genetics*, **160**, 257-269.
13. Csink, A. K., and Henikoff, S. (1996) Genetic modification of heterochromatic association and nuclear organization in *Drosophila*, *Nature*, **381**, 529-531.

14. Talbert, P. B., Leciel, C. D., and Henikoff, S. (1994) Modification of the *Drosophila* heterochromatic mutation brown^{Dominant} by linkage alterations, *Genetics*, **136**, 559-571.
15. Henikoff, S., and Dreesen, T. D. (1989) *Trans*-inactivation of the *Drosophila* brown gene: evidence for transcriptional repression and somatic pairing dependence, *Proc. Natl. Acad. Sci. USA*, **86**, 6704-6708.
16. Mcguire, S. E., Le, P. T., Osborn, A. J., Matsumoto, K., and Davis, R. L. (2003) Spatiotemporal rescue of memory dysfunction in *Drosophila*, *Science*, **302**, 1765-1768.
17. Fujimoto, E., Gaynes, B., Brimley, C. J., Chien, C. B., and Bonkowsky, J. L. (2011) Gal80 intersectional regulation of cell-type specific expression in vertebrates, *Dev. Dyn.*, **240**, 2324-2334.
18. Osterwalder, T., Yoon, K. S., White, B. H., and Keshishian, H. (2001) A conditional tissue-specific transgene expression system using inducible GAL4, *Proc. Natl. Acad. Sci. USA*, **98**, 12596-12601.
19. Roman, G., Endo, K., Zong, L., and Davis, R. L. (2001) P[Switch], a system for spatial and temporal control of gene expression in *Drosophila melanogaster*, *Proc. Natl. Acad. Sci. USA*, **98**, 12602-12607.
20. Bischof, J., Maeda, R. K., Hediger, M., Karch, F., and Basler, K. (2007) An optimized transgenesis system for *Drosophila* using germ-line-specific phiC31 integrases, *Proc. Natl. Acad. Sci. USA*, **104**, 3312-3317.
21. Groth, A. C., Fish, M., Nusse, R., and Calos, M. P. (2004) Construction of transgenic *Drosophila* by using the site-specific integrase from phage phiC31, *Genetics*, **166**, 1775-1782.
22. Venken, K. J., Schulze, K. L., Haelterman, N. A., Pan, H., He, Y., Evans-Holm, M., Carlson, J. W., Levis, R. W., Spradling, A. C., Hoskins, R. A., and Bellen, H. J. (2011) MiMIC: a highly versatile transposon insertion resource for engineering *Drosophila melanogaster* genes, *Nat. Methods*, **8**, 737-743.
23. Vogel, M. J., Pagie, L., Talhout, W., Nieuwland, M., Kerkhoven, R. M., and Van Steensel, B. (2009) High-resolution mapping of heterochromatin redistribution in a *Drosophila* position-effect variegation model, *Epigenetics Chromatin*, **2**, 1.
24. Sage, B. T., Wu, M. D., and Csink, A. K. (2008) Interplay of developmentally regulated gene expression and heterochromatic silencing *in trans* in *Drosophila*, *Genetics*, **178**, 749-759.