Repeated, protein-encoding heterochromatic genes cause inactivation of a juxtaposed euchromatic gene

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Abstract Euchromatic genes are often silenced by rearrangements that place them within or near heterochromatin, a phenomenon known as position effect variegation (PEV). However, little is known about molecular structure of cis-acting heterochromatic fragments responsible for PEV. Here we report that heterochromatic cluster containing *Stellate* repeats, that encode putative regulatory subunit of protein kinase *CK*2 cause PEV of a reporter *white* 'mini-gene'. It is the first example of an euchromatic gene being silenced because of the proximity to the natural, well-defined heterochromatic repeat cluster.

Key words: Heterochromatic repeat; Position effect variegation; Mini-gene *white*

1. Introduction

The capability and specificity of cis-acting heterochromatic repeats to induce position effect variegation (PEV) is poorly understood [1]. It was shown in a model experiment that artificially generated repetitive sequence represented by several *white* 'mini-gene' transgenes produced phenotypes similar to heterochromatin induced PEV [2]. Silencing by artificial repeat arrays was observed for the *white* 'mini-gene' repeated within array. However, this construction does not simulate the conventional silencing by heterochromatin where repeated heterochromatic elements affect expression of the nearby situated euchromatic gene. On the other hand, transformants carrying artificial constructions containing natural heterochromatic repeats juxtaposed to euchromatic genes may better simulate the phenomenon of PEV produced through eu-heterochromatic chromosomal rearrangements. We used this approach to study peculiarities of heterochromatin induced silencing of the adjacent euchromatic genes. The heterochromatic *Stellate* repeats were used, which are located in the heterochromatin of the X-chromosome. The structure of the cluster of tandemly repeated *Stellate* units encoding putative regulatory subunit of protein kinase *CK*2 was recently reported [3].

2. Materials and methods

2.1. Construction of recombinant P-elements with Stellate tandem

A *Bam*HI/*Eco*RI fragment of heterochromatic *Stellate* cluster [3] containing the six *Stellate* repeats and 18S pseudogene was subcloned from the phage clone into vector *pCaSpeR-AUG-*β*gal* [4] (plasmid p52). The control plasmid pC with a single copy of *Stellate* and 18S pseudogene was generated by *Bgl*II digestion of the plasmid p52 (Fig. 1).

2.2. Drosophila transformation

Host flies for germline transformation were $Df(1)w^{67c23(2)},y$. The bipartite reporter gene constructs were co-injected with *pTurbo-*Δ*2-3* helper [5] essentially as described by Spradling [6] with modifications [7]. Transformants were identified by red eye color. Genome DNA was purified from adult flies [8].

2.3. Southern-blot analysis

Total genome DNA was digested by appropriate restriction endonucleases, fractionated by agarose electrophoresis in TBE buffer with ethidium bromide and transferred to nylon membrane HyBond-N (Amersham). The cloned in pUC19 1.15-kbp *Bgl*II fragment of *Stellate* gene and plasmid *pCaSpeR-AUG-*β*gal* were used as a probe for hybridization. Labeling probes were prepared by random priming using [α-^{32}P]ATP. Filter prehybridization and hybridization were performed as described [8].

2.4. In situ hybridization

Chromosome squashes were performed on glands fixed in 45% acetic acid. Transgene plasmid DNA was labeled by nick-translation [8] in the presence of biotin-11-dUTP. In situ hybridization and detection were done by the method of Engels et al. [9].

2.5. Histochemical localization of β-galactosidase

Staining was done according to Ashburner [10] with modifications from the protocol in http://info.pitt.edu/~carthew/manual/Head.html#GALACTOSIDASE.

3. Results and discussion

3.1. Stability of inserted construction carrying Stellate repeats

The fragment of *Stellate* cluster containing six *Stellate* units and a flanking region with 18S rDNA fragment was subcloned into *pCaSpeR-AUG-*β*gal* vector (plasmid p52) (Fig. 1) carrying the so-called *white* 'mini-gene' responsible for eye pigment accumulation. P-element mediated transformation of the $Df(1)w^{67c23(2)},y$ stock allowed us to obtain transformants with mosaic coloration of eyes. Southern-blot analysis of ten transformants (third generation) demonstrated stable inheritance of the 8-kbp *Bam*HI/*Eco*RI fragments encompassing six *Stellate* repeats (Fig. 2a). The single stock, 46.2, preserves only three *Stellate* copies judging by the appearance of approximately 3.6-kbp fragment instead of the 8-kbp one. Notice that unexpectedly high mobility in agarose gels of *Stellate* bearing fragments was previously shown (unpublished data of the authors).

The Southern analysis after 8–9 generations (Fig. 2b) revealed the 9.5-kbp *Hin*dIII fragment comprising *Stellate* repeats in all the stocks except 46.2, although several short fragments were detected in the stocks 44.4 and 50.3 revealing instability of insertion in some flies.

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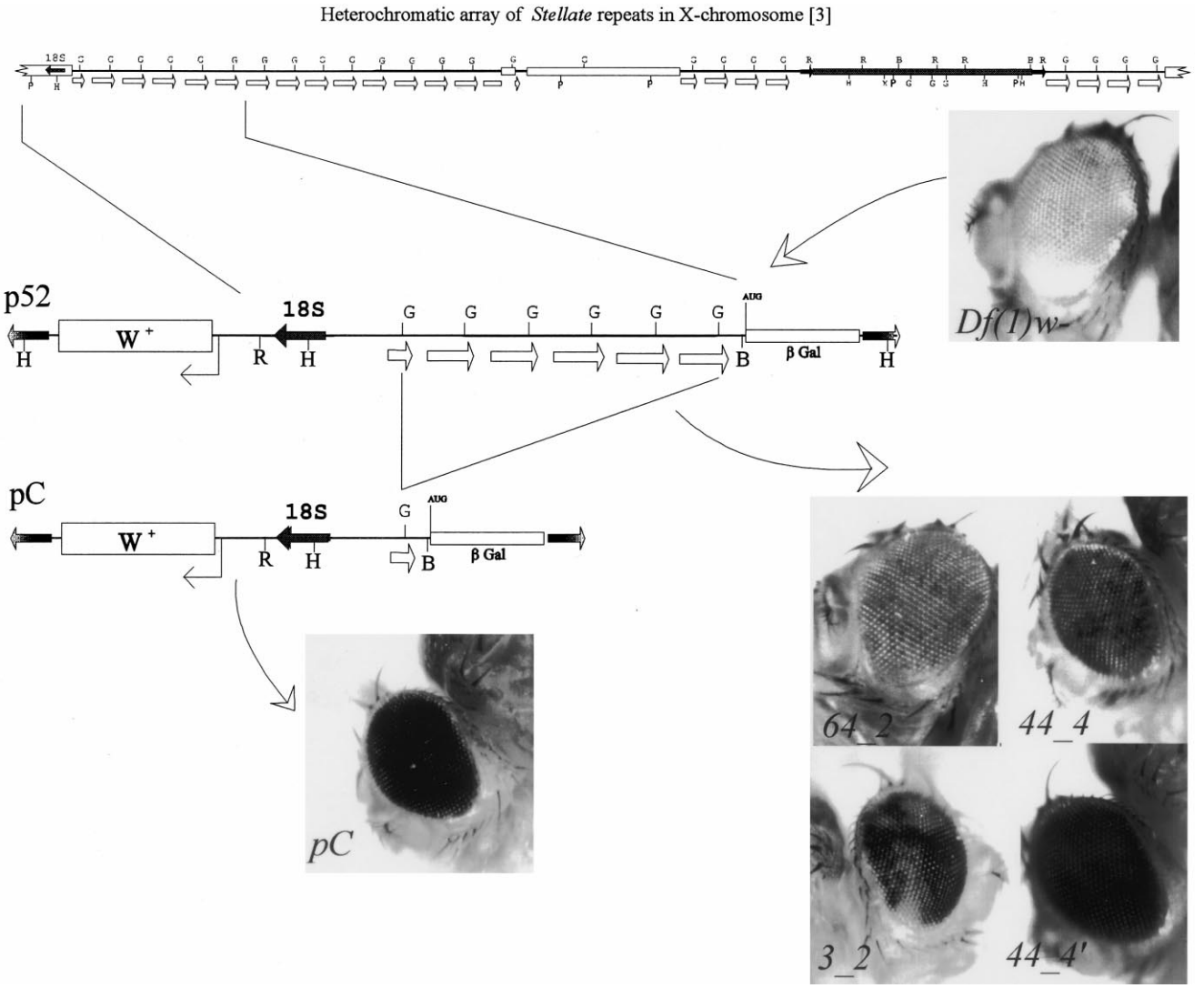

Fig. 1. *White* 'mini-gene' variegation caused by *Stellate* repeats cluster. Flanking region and six *Stellate* units (p52) or a single truncated *Stellate* unit with flanking sequences (pC) were cloned into *pCaSpeR-AUG-βgal* vector to recover transformants. Empty arrows indicate *Ste* repeats, black arrow indicates 185 rDNA fragment, P-element inverted repeats are partially blackened. G, *Bgl*II; R, *Eco*RI; X, *Xba*I; H, *Hin*dIII; B, *Bam*HI. The eyes of transformants carrying pC, p52 insertions (stocks 44.4, 64.2, 3.2) or lacking most *Stellate* repeats (44.4′) are presented.

Table 1
Stocks carrying p52 insertion (six *Stellate* repeats) causing *white* variegation; localization in polytene chromosomes is indicated according to Bridges map

Stocks	Localization	Phenotype (eye color)
3.2, 3.3	98B	patches of variegation
22.2	84F1	dispersed patches of variegation
64.2	32C	
13.5	95CD	varying extents of iridescent variegation and mottling
13.12	29E	
13.13	18C	
23.2	18D, 19E	
50.3	X-chromosome	
55.1	39C	
55.2	4D	
44.4	50E	
44.4′	derivative of 44.4, loss of all *Stellate* repeats except one	red eyes
16.1	99F1	posterior-anterior gradient of coloration, iridescent variegation, mottling
46.2	remained three *Stellate* repeats	orange eyes

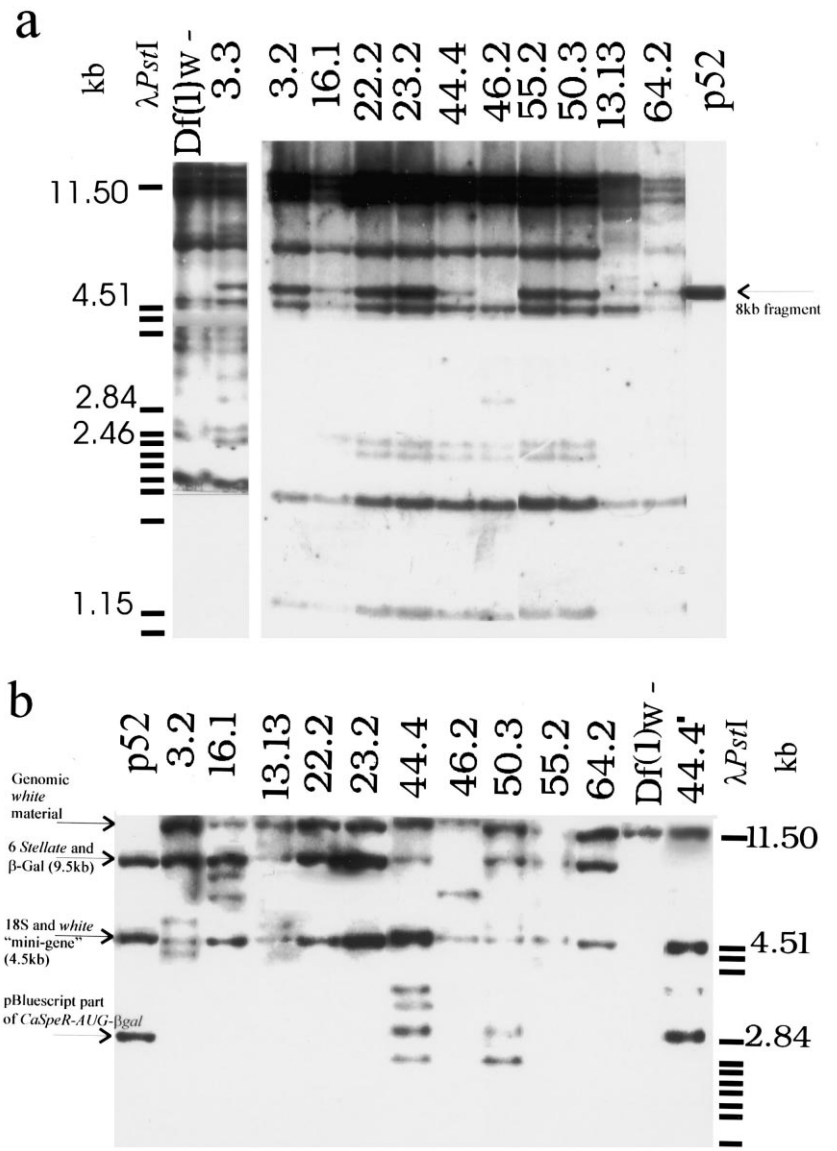

Fig. 2. Southern-blot analysis of transformants carrying p52 insertion. *Df(1)w-*, stock used for transformation. p52, plasmid containing six *Stellate* repeats, flanked by P-element termini. a: *Bam*HI/*Eco*RI digestion, hybridization to *Ste* probe. b: *Hin*dIII digestion, hybridization to *pCaSpeR-AUG-βgal* probe. The right-most lane (44.4′) demonstrates complete elimination of 9.5-kbp fragment (carrying *Ste* tandem) in the substock selected for enhanced eye color (see Fig. 1).

3.2. *Tandemly repeated Stellate genes cause position effect variegation (PEV) of the white 'mini-gene' as a reporter gene*

All transformants demonstrate mosaic distribution of eye pigmentation (variegation *white* 'mini-gene' expression), except the 46.2 stock were three preserved *Stellate* copies ensure orange-colored eyes, which is rather common for the expression of P-element mediated *white* 'mini-gene' transformants. Consequently, these three copies of *Stellate* repeats are apparently insufficient to cause a pronounced PEV. The stocks carrying undamaged insertions (3.2, 22.2, 64.2, 13.13, 23.2, 55.2) demonstrate different patterns of variegation (Table 1, Fig. 1). Several other stocks which were not checked by Southern analysis show mosaic distribution of eye pigmentation (*white* expression). In situ hybridization with *Stellate* probe to polytene chromosomes revealed localization of recombinant P-element insertions in euchromatic regions (ac-

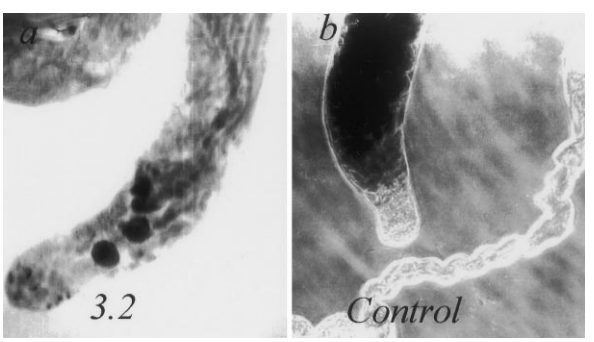

Fig. 3. Position effect silencing in testis. β-galactosidase activity throughout adult testis. a: Stock 3.2, mosaic expression. b: Control, non-variegated βgal activity. Terminal part of testis contain stem cells where expression of transgenes is often restricted.

cording to the polytene map), on the X-chromosome and chromosomes 2 and 3 (Table 1). The site of insertion may influence the phenote (peculiarities) of variegation.

The instability of insertion in the 44.4 stock led to the appearance of flies with non-variegated red eyes among the flies demonstrating pronounced levels of variegation (Fig. 1). We selected the 44.4′ stock with red eyes which is a derivative of the 44.4 stock which carries no 9.5-kbp *Hin*dIII fragments but displays the presence of a 2.9-kbp fragment (Fig. 2b). This 2.9-kbp fragment exhibits no hybridization with *Stellate* derived probe (not shown) and is represented by a region flanking *Stellate* cluster. Four transformed lines carrying single *Ste* repeat and flanking sequences (plasmid pC) (Fig. 1) expressed more intensive eye color but no *white* variegation. Thus, all these observations indicate an inherent ability of *Stellate* repeats to cause *white* variegation.

The degree of *white* variegation in the stocks was enhanced at low temperatures and also as a result of their maintenance using sterilized food. These environmental modifiers are known to be typical for heterochromatic PEV in *D. melanogaster*. In addition, the elimination of a Y-chromosome, the strong suppressor of PEV [1], enhanced PEV of the *white* 'mini-gene' in the transformants.

The high level of *Stellate* expression in testis of individuals lacking Y-chromosome is a well-known phenomenon [11]. Transgenic fly contains β-galactosidase gene adjacent to putative *Stellate* promoter. It is surprising that we have detected β-galactosidase expression by staining for β-galactosidase activity in testis of the transformants carrying Y-chromosome. Possibly, this expression is mediated by the *Stellate* promoter, since it is known that no β-galactosidase activity was detected in all five transformants carrying inserted vector *pCaSpeR-AUG-βgal*. β-galactosidase expression is variegated at least in the testis of three stocks, 3.2 (Fig. 3a), 13.13 and 50.3, yet no variegation was observed in control transformants where β-galactosidase gene was fused to the fragments of *Stellate* unit encompassing intergenic region and a start of coding region (Fig. 3b, control).

To our knowledge, these results demonstrate for the first time the ability of cloned genomic fragments of constitutive heterochromatin to cause PEV. Thus, it was shown that the proximity of an approximately 9.5-kbp fragment containing tandemly repeated heterochromatic genes, causes PEV of a reporter *white* 'mini-gene'.

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