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Silencing at *Drosophila* telomeres: nuclear organization and chromatin structure play critical roles

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Transgenes inserted into the telomeric regions of *Drosophila melanogaster* chromosomes exhibit position effect variegation (PEV), a mosaic silencing characteristic of euchromatic genes brought into juxtaposition with heterochromatin. Telomeric transgenes on the second and third chromosomes are flanked by telomeric associated sequences (TAS), while fourth chromosome telomeric transgenes are most often associated with repetitious transposable elements. Telomeric PEV on the second and third chromosomes is suppressed by mutations in *Su(z)2*, but not by mutations in *Su(var)2-5* (encoding HP1), while the converse is true for telomeric PEV on the fourth chromosome. This genetic distinction allowed for a spatial and molecular analysis of telomeric PEV. Reciprocal translocations between the fourth chromosome telomeric region containing a transgene and a second chromosome telomeric region result in a change in nuclear location of the transgene. While the variegating phenotype of the *white* transgene is suppressed, sensitivity to a mutation in HP1 is retained. Corresponding changes in the chromatin structure and inducible activity of an associated *hsp26* transgene are observed. The data indicate that both nuclear organization and local chromatin structure play a role in this telomeric PEV.

Keywords: chromatin structure/*Drosophila*/nuclear organization/PEV/telomeres

Introduction

Telomeres were first identified as distinctive structures at the natural ends of chromosomes by cytological and genetic studies in *Drosophila* (Muller, 1938). Telomeres play important roles in maintaining chromosome stability, complete DNA replication, correct chromosome segregation and correct positioning of chromosomes within the nucleus (Kipling, 1995). Organisms must possess mechanisms to maintain telomere length. Without such mechanisms, telomeric sequences are lost during each round of replication (Lingner and Cech, 1998). In most metazoans, short G-rich tracks are laid down at the ends of chromosomes by telomerase, using an RNA primer as a template (Zakian, 1995). *Drosophila* telomeres lack G-rich tracks, but instead possess tandem repeats of the retrotransposons *HeT-A* and *TART* (Biessmann *et al*., 1992; Levis *et al*., 1993; Walter *et al*., 1995; Danilevskaya *et al*., 1997). Many parallels have been noted between telomerase-based telomere elongation and this retrotransposon-based system (Pardue *et al*., 1997).

Telomeric and pericentric regions of most eukaryotic genomes are packaged into heterochromatin, the relatively gene-poor, late replicating material that remains condensed throughout the cell cycle (Weiler and Wakimoto, 1995). In contrast, most regions between centromeres and telomeres are packaged into euchromatin, the gene-rich, early replicating material that decondenses during interphase. When euchromatic genes are brought into juxtaposition with heterochromatin by chromosomal rearrangement or transposition, they can exhibit position effect variegation (PEV), a silencing of the gene in a subset of the cells in which it is normally expressed (Weiler and Wakimoto, 1995). Such silencing is not gene specific, and can affect flanking DNA up to 100 kb from the breakpoint (Weiler and Wakimoto, 1995). Silencing is observed when genes are near telomeres in *Trypanosoma brucei* (Horn and Cross, 1995; Rudenko *et al*., 1995), *Saccharomyces cerevisiae* (Gottschling *et al*., 1990; Palladino and Gasser, 1994), *Schizosaccharomyces pombe* (Nimmo *et al*., 1994) and *Drosophila melanogaster* (Gehring *et al*., 1984; Hazelrigg *et al*., 1984; Karpen and Spradling, 1992; Levis *et al*., 1993; Wallrath and Elgin, 1995); at the silent mating type loci in *S.cerevisiae* (Stone and Pillus, 1998); and at pericentric regions of the chromosomes in *S.pombe* (Allshire *et al*., 1994), *D.melanogaster* (Wallrath and Elgin, 1995; Zhang and Spradling, 1995) and mammals (Dobie *et al*., 1997; Kioussis and Festenstein, 1997). Although telomeric and pericentric PEV share common properties, different responses to genetic modifiers have been noted (Nimmo *et al*., 1994; Wallrath and Elgin, 1995).

To understand the molecular mechanisms underlying PEV, a P-element mobilization screen was performed to recover *D.melanogaster* stocks in which well-studied euchromatic genes were placed within regions inducing PEV (Wallrath and Elgin, 1995). The P-element included a *white*$^+$ reporter gene expressed from an *hsp70* promoter, allowing detection of a variegating eye phenotype, and a tagged version of the *hsp26* gene. The *hsp26* gene, encoding one of the small heat shock proteins, has been extensively characterized; the heat shock genes can be induced in most cell types, facilitating molecular analysis of gene expression and chromatin structure. *In situ* hybridization of polytene chromosomes from stocks showing PEV of the *hsp70–white*$^+$ transgene revealed that the P-element had inserted within pericentric regions, telomeric sites, or the fourth chromosome (Wallrath and Elgin, 1995). [The small fourth chromosome contains blocks of repetitious DNA with characteristics of heterochromatin

(Carmena and Gonzalez, 1995; Pimpinelli *et al*., 1995).] The telomeric inserts from this collection have been used here to identify *cis*- and *trans*-acting components involved in this gene silencing.

A large number of dominant mutations that modify the expression of variegating genes juxtaposed to pericentric heterochromatin have been characterized (Weiler and Wakimoto, 1995). Such mutations are frequently found to be in genes that encode chromosomal proteins or modifiers of chromosomal proteins (Wallrath, 1998). Mutations that decrease or increase the extent of gene silencing are designated suppressors or enhancers of PEV, *Su(var)*s or *E(var)*s, respectively. In general, those mutations that suppress the PEV associated with chromosomal rearrangements also suppress variegation of *hsp70–white*$^+$ transgenes in pericentric regions or along the fourth chromosome, including those in fourth chromosome telomeric locations. In contrast, these *Su(var)*s have no effect on variegating *hsp70–white*$^+$ transgenes associated with second and third chromosome telomeres (Wallrath and Elgin, 1995). Two such examples are *Su(var)2-5*^{02}, a missense mutation in heterochromatin protein 1 (HP1) and *Su(var)2-1*^{01}, which causes an increase in the amount of acetylated histone H4 (Dorn *et al.*, 1986; Eissenberg *et al.*, 1992; Wallrath and Elgin, 1995). These data suggest that fourth chromosome telomeric PEV is mechanistically similar to that operating in pericentric regions, but that PEV at the second and third chromosome telomeres differs, perhaps involving a different set of chromosomal proteins.

Here we use transgenes as molecular entry points to examine the characteristics of silencing at different telomeres. We find that a particular class of DNA sequences surrounds the variegating transgenes in the second and third chromosome telomeres; a different class of DNA sequences is associated with the variegating transgenes at the fourth chromosome telomere. Genetic analysis implicates different sets of chromosomal proteins as the limiting factor(s) in silencing at these two types of telomere. Using X-rays to generate translocations between distal chromosome regions (telomere swapping), we assayed for effects on several chromosomal properties. Changes in nuclear organization, gene expression and chromatin structure are observed upon translocation. However, sensitivity to specific modifiers of PEV is unaltered. These data suggest that both nuclear organization and chromatin structure play a role in telomeric PEV.

Results

Mutations in Su(z)2 and Psc suppress telomeric PEV on the second and third chromosomes

*Su(var)*s identified as modifiers of *w*^{m4} rearrangements consistently modify PEV of transgenes at pericentric and fourth chromosome locations, but not those at telomeres of the second and third chromosome (Wallrath and Elgin, 1995). To identify proteins that might be involved in second and third chromosome telomeric gene silencing, a survey was performed using *Drosophila* stocks with mutations in known chromosomal proteins, exclusive of *Su(var)*s. Stocks carrying these mutations were crossed to stocks possessing a variegating *hsp70–white*$^+$ transgene at second or third chromosome telomeres and progeny were scored for dominant effects on telomeric PEV.

Table I. Effects of *Su(z)2* and *Psc* alleles on telomeric PEV

Gene	Mutant allele	Suppression of telomeric PEV
Supressor 2 of zeste	*Su(z)*^{1}	–
	Su(z)^4	–
	Su(z)2$^{1.a1}$	+
	Su(z)2$^{1.a5}$	–
	Su(z)2$^{1.b7}$	+
	Su(z)1$^{1.b8}$	–
	Su(z)2D^{eos}	–
	[a]*Su(z)2*^{5}	++
Posterior sex combs	*Psc*^{1}	–
	Psc$^{1.d19}$	+
	Psc$^{1.d20}$	+
	Psc^{14P4}	–
	Psc^{epb}	–
	Psc^{e22}	–
	Psc^{1433}	–
	Psc^{h27}	–
	Psc^{h28}	–
	Psc^{h30}	–
	Psc^{e23}	–
	Psc^{e24}	–
	Psc^{e25}	–

–, no effect; +, weak suppression; ++, strong suppression.
Su(z)2 and *Psc* alleles are described in Wu and Howe (1995).
[a]Also deletes *Psc*.

Mutations tested that showed no effect on telomeric PEV were *Pc*^{1}, *Pcl*^{1}, *Asx*^{1}, *Scm*^{01}, *z*^{1}, *z*^{e}, *brm*^{2}, *ISWI*^{1}, *ISW*^{2}, *k43*^{1}, *k43*$^{\gamma 4}$, *E(z)*^{1} and *E(z)*^{53} (mutations are defined in Flybase and Lindsley and Zimm, 1992). In contrast, particular mutations of the Polycomb group genes *Psc* and *Su(z)2* suppressed second and third chromosome telomeric PEV (Table I). Effective *Psc* alleles include *Psc*$^{1.d19}$ and *Psc*$^{1.d20}$; effective *Su(z)2* alleles include *Su(z)2*$^{1.b7}$ and *Su(z)2*$^{1.a1}$. The most pronounced effect was observed with a deletion of both *Psc* and *Su(z)2*, designated *Su(z)2*^{5} (Wu and Howe, 1995). This suppression of PEV was observed for all telomeric variegating *hsp70–white*$^+$ transgenes tested on the left arm of chromosome two, the right arm of chromosome two and the right arm of chromosome three (Table II). It is unlikely that the effects observed with these mutations are due to background differences, since several of these mutant stocks were derived from separate mutagenesis experiments using fly stocks with different genetic backgrounds. In addition, the *Su(z)2*^{5}-bearing chromosome was introduced into the *yw*^{67c23} host stock, the resulting stock was crossed to the telomeric insert stocks and suppression of telomeric PEV was still observed. However, it is a formal possibility that a second mutation on the *Su(z)2* chromosome is responsible for the effect. Interestingly, the *Psc*- and *Su(z)2*-bearing chromosomes have no effect on PEV associated with the pericentric and fourth chromosome regions, including the fourth chromosome telomere (Figure 1; data not shown). Thus, *Su(z)2*^{5} and *Su(var)2-5*^{02} were used as genetic 'tools' to monitor the effects of chromosomal rearrangements on telomeric PEV.

DNA sequences associated with telomeric PEV

One possible explanation for the differences in response of the telomeric transgenes to modifier mutations is the nature of the adjacent DNA sequences. Using inverse PCR

Table II. Properties of telomeric insert stocks

Stock	Location	Eye phenotype	HP1	Su(z)2	Adjacent DNA
39C-5	2L	pale yellow/orange spots	–	+	0.4 kb repeat
39C-27	2R	orange w/ red spots	–	+	0.8 kb TAS
39C-50	2R	dark orange w/ red spots	–	+	0.8 kb TAS
39C-58	2R	dark orange w/ red spots	-	+	0.8 kb TAS
39C-31	3R	dark orange w/ red spots	–	+	1.0 kb TAS
39C-51	3R	dark orange w/ red spots	–	+	1.0 kb TAS
39C-62	3R	dark orange w/ red spots	–	+	1.0 kb TAS
118E-16	3R	dark orange w/ red spots	–	+	1.0 kb TAS
118E-26	3R	dark orange w/ red spots	–	+	1.0 kb TAS
118E-21	60E	dark orange w/ red spots	–	+	TAS
118E-15	4	red and white variegating	+	–	unique
39C-56	4	red and white variegating	+	–	*hoppel*
39C-68	4	red and white variegating	+	–	*hoppel*
39C-72	4	red and white variegating	+	–	*Fw*
HS-4	4	red and white variegating	+	–	*Fw*
HS-6	4	red and white variegating	+	–	*hoppel*

+ denotes suppression by mutations in the gene encoding HP1 or Su(z)2.
– denotes no effect.

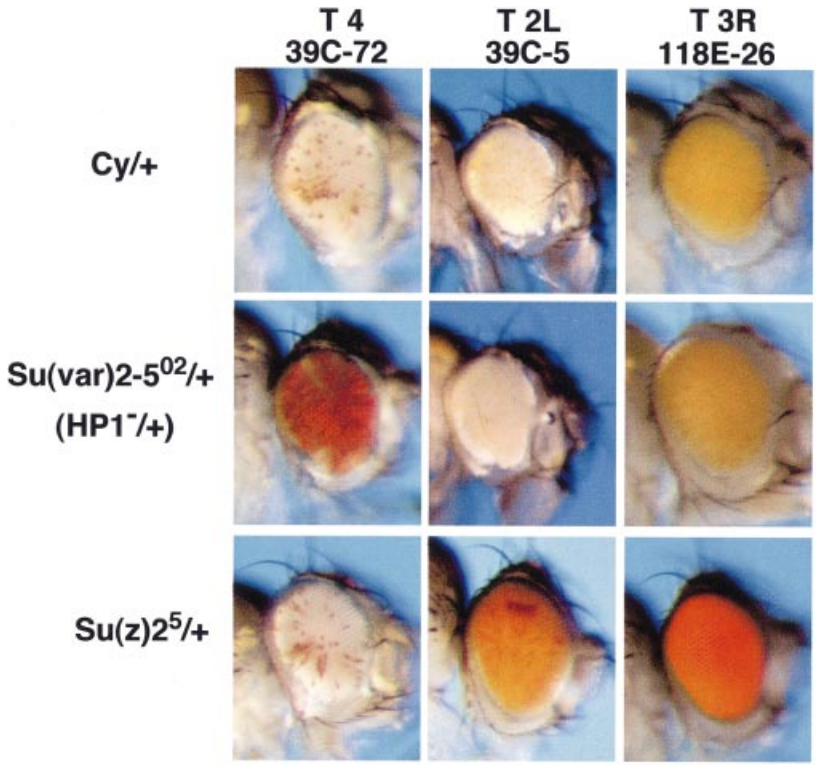

Fig. 1. Eye phenotypes of flies with a telomeric *hsp70–white*$^{+}$ transgene in different genetic backgrounds. Transgenes near the telomeric region of the fourth chromosome, such as in stock 39C-72, show suppression of PEV when heterozygous for a mutation in the gene encoding HP1, but not when heterozygous for a mutation in *Su(z)2*. Transgenes inserted near the telomeric regions of the second and third chromosomes, such as in stocks 39C-5 and 118E-26, show suppression of PEV when heterozygous for a mutation in *Su(z)2*, but not when heterozygous for a mutation in the gene encoding HP1. Top line: names of stocks and locations of P-element inserts. Left column: genotypes of the flies. Abbreviations: T 4, telomere of the fourth chromosome; T 2L, telomere of the left arm of chromosome two; T 3R, telomere of the right arm of the third chromosome; +, wild-type chromosome; Cy, inversion balancer chromosome carrying the *Curly* mutation.

and direct cloning, we have characterized the sequences adjacent to the telomeric transgenes (Figure 2A). Sequences on both sides of the 2L telomeric transgene, stock 39C-5, are identical to the 0.4 kb satellite sequences previously characterized in the subtelomeric region of 2L (DDBL/EMBL/GenBank accession No. U35404; Walter *et al.*, 1995). The transgene is flanked by 4 kb of the satellite sequence on the proximal side and 5.3 kb on the distal side; a *HeT-A* element is adjacent to the distal block of satellite sequence.

The sequences flanking the 2R and 3R telomeric transgenes have been determined in a collaborative study (Pavlova *et al.*, 1996); a detailed report will be published elsewhere (M.Pavlova and R.Levis, in preparation). Four transgenes at the 3R telomere are flanked by a subtelomeric repetitive element termed the 1.0 kb telomere associated

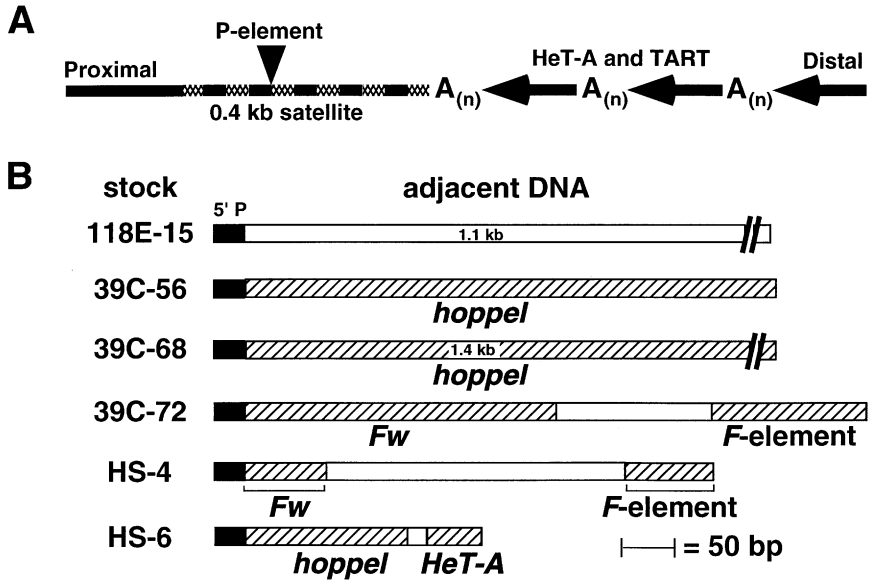

Fig. 2. Diagram of the sequences adjacent to telomeric *hsp70–white*$^+$ transgenes. (**A**) The second chromosome telomeric transgene in stock 39C-5 is within the 0.4 kb satellite sequences. The distal ends of the chromosome contain tandem arrays of the retrotransposons *HeT-A* and *TART*. (**B**) Fourth chromosome telomeric transgenes are adjacent to unique sequences (open box) and middle repetitive sequences (hatched box). Sequences that are >80% identical to a known sequence are labeled as such. Names of the stocks are listed on the left.

sequences (TAS) (Table II), while three transgenes at the 2R telomere are flanked by similar sequences designated the 0.8 kb TAS. Insertion within a TAS does not appear to occur at specific positions; the transgenes are inserted in both orientations relative to the end of the chromosome (Pavlova *et al.*, 1996). Such subterminal telomeric repeats, referred to here in general terms as TAS, were first identified in the telomeric region of the mini-X chromosome *Dp1187* as the site of frequent P-element insertions (Karpen and Spradling, 1992). Interestingly, stock 118E-21, which possesses a P-element at cytological region 60E (several bands proximal to the end of 2R) is also adjacent to sequences that are 93% identical to the X-chromosome TAS (DDBL/EMBL/GenBank accession No. L03284; Karpen and Spradling, 1992). This transgene responds to *Su(z)2*^{5}, but not to *Su(var)2-5*^{02}.

Sequences adjacent to the fourth chromosome telomeric transgenes are different from the 0.4 kb satellite and TAS; they are either unique or middle repetitive DNA sequences, frequently *hoppel* (Kurenova *et al.*, 1990) or *F*-element sequences (Di Nocera and Dawid, 1983) (Figure 2B). Adjacent to the *hsp70–white*$^+$ transgene of stock 118E-15 is at least 1.1 kb of unique DNA (DDBL/EMBL/GenBank accession No. AF103941). This sequence hybridizes to a single band on a Southern blot of genomic DNA digested with either *Hpa*II, *Sal*I or *Eco*RI, enzymes which do not cleave within the cloned fragment. In addition, one band at the most distal region of the fourth chromosome is recognized by *in situ* hybridization to polytene chromosomes using the cloned sequences as a probe (data not shown).

Sequences adjacent to the *hsp70–white*$^+$ transgene in fourth chromosome insert stocks 39C-56, 39C-68 and HS-6 are 95, 94 and 92% identical, respectively, to portions of the transposable element *hoppel* (DDBL/EMBL/GenBank accession No. X78388). The junction fragments between the P-element and *hoppel* are different in each case; thus there does not appear to be a specific 'hot spot' for P-element insertion within *hoppel*. In stock HS-6, the *hoppel* sequences are followed by sequences 100% identical to sequences of the *HeT-A* retrotransposon (DDBL/EMBL/GenBank accession No. M84199), which is primarily located at the distal ends of telomeres (Danilevskaya *et al.*, 1997). The DNA sequences immediately adjacent to the *hsp70–white*$^+$ transgenes of stocks 39C-72 and HS-4 are 91 and 84% identical, respectively, to *Fw*, an *F*-element family member (DDBL/EMBL/GenBank accession Nos. S82273 and M17214). The *F*-element is similar to the LINE transposable elements found in mammals (Di Nocera and Casari, 1987). In stocks 39C-72 and HS-4, the sequences with identity to *Fw* are followed by a short stretch of DNA that gives no alignment with sequences in GenBank, and then by sequences with identity to the *F*-element. Thus, one of the fourth chromosome telomeric P-element inserts is immediately adjacent to a unique sequence, while five inserts are adjacent to middle repetitive DNA elements previously known to be associated with heterochromatin. Despite the differences in DNA sequence, the variegating phenotype of all of these fourth chromosome transgenes is suppressed by mutations in HP1 (Figure 1; Wallrath and Elgin, 1995; data not shown).

Telomere swapping results in a loss of gene silencing for HP1-sensitive transgenes

Transgenes that mapped to the fourth chromosome telomeric region show similar responses to genetic modifiers as the pericentric and other fourth chromosome transgenes, possibly due to the close proximity to pericentric heterochromatin. To address whether the length of the chromosome arm and hence nuclear position influences PEV, we performed X-ray mutagenesis to generate telomeric translocations. Males of stock 118E-15, possessing a variegating *hsp70–white*$^+$ transgene near the telomere of the fourth chromosome, were mutagenized and used in a mating scheme that allowed for the detection of transloca-

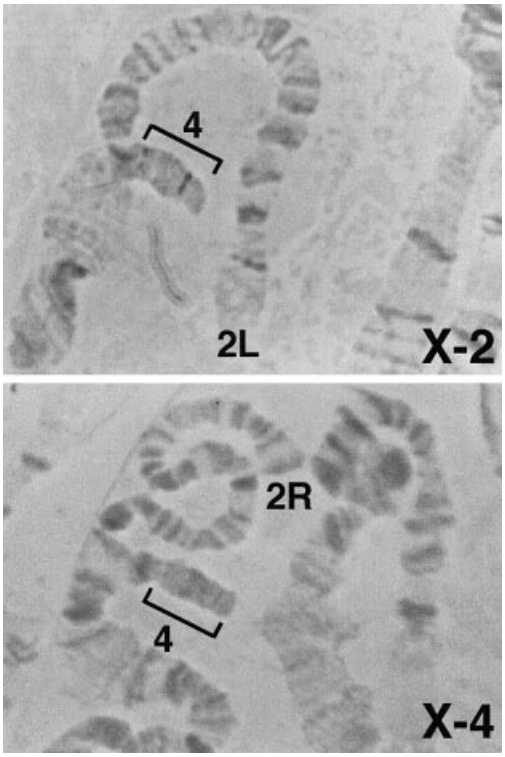

Fig. 3. Cytology of the fourth chromosome translocations. Cytological regions 102A–F of the fourth chromosome are translocated onto 2L (at cytological region 25A) in stock X-2. Cytological regions 102A–F of the fourth chromosome are translocated onto 2R (at cytological region 56D–F) in stock X-4.

tions. Eleven stocks were recovered in which the P-element no longer segregated as a free fourth chromosome. Cytology of polytene chromosomes from these possible translocation stocks revealed that two stocks, X-2 and X-4, have the desired genotype (Figure 3). Stock X-2 carries a translocation of the fourth chromosome onto the distal region of 2L, with the second chromosome breakpoint at cytological position 25A, and the fourth chromosome breakpoint within the proximal banded region 102A. Similarly, stock X-4 carries a translocation of the fourth chromosome to the distal region of 2R at cytological position 56D–F; the breakpoint on the fourth chromosome is again near 102A. In both stocks the proximal–distal orientation of the fourth chromosome is maintained, leaving the P-element near the tip of the chromosome arm (Figure 3 and shown diagrammatically in Figure 4). The fourth chromosome region involved in the translocation (cytological region 102A–102F) is ~1 Mb in size (J.Locke, personal communication).

The most apparent phenotype of stocks X-2 and X-4 is the dramatic loss of gene silencing (Figure 4). These stocks exhibit a weak PEV phenotype. However, that phenotype is still suppressed by mutations in the gene encoding HP1 (Figure 4), and is not affected by the *Su(z)2*^{5} mutation (data not shown). Not all chromosomal rearrangements have similar phenotypic consequences. Stock X-10 resulted from a transposition event in which the fourth chromosome (cytological region 102A–F) was inserted into the proximal region of 3L at cytological position 80. The proximal–distal orientation of the fourth chromosome is inverted (illustrated in Figure 4). The eye phenotype of this stock is virtually indistinguishable from that of the starting stock 118E-15 (Figure 4). PEV in this stock is strongly suppressed by mutations in HP1. The other stocks from the screen possess complex rearrangements that are not easily defined by cytological observation.

To obtain translocations of a second chromosome telomere carrying a variegating *hsp70–white*$^+$ transgene to the fourth chromosome distal region, a genetic screen similar to that described above was performed using males of stock 39C-27 possessing a P-element insert near the telomere of 2R. One stock, X-12, was recovered in which the second chromosome telomere was translocated to the fourth chromosome. *In situ* hybridization to the polytene chromosomes revealed that the breakpoint on the fourth chromosome was again in the proximal 102A region (data not shown). The breakpoint on the second chromosome was within region 60. However, the translocated portion appeared to include at least several bands from the 2R telomeric region, maintained in the original orientation.

Flies of translocation stock X-12 had an eye phenotype nearly identical to flies of the starting stock 39C-27 (Figure 4). The variegating phenotype of the translocated *hsp70–white*$^+$ transgene was not suppressed by mutations in the gene encoding HP1, but was suppressed by the *Su(z)2*^{5} mutation, an identical phenotypic profile to that of the transgene in stock 39C-27. Thus proximity to a large block of heterochromatin, such as the centromere, does not alter the degree of PEV for a second chromosome telomeric transgene, nor does it induce sensitivity to mutations in the gene encoding HP1. The results from both types of translocation indicate that local DNA sequences dictate which modifiers have an effect, but that the extent of gene silencing at the chromosome four telomere is sensitive to a major change in chromosome position relative to the pericentric heterochromatin.

The translocated fourth chromosome has an altered nuclear location

The *hsp70–white*$^+$ transgene on the translocated fourth chromosome retained ~1 Mb of adjacent sequences, and yet gene silencing was dramatically reduced. This suggests that elements other than the local chromatin structure, such as nuclear position, might play a role in gene silencing. Using salivary gland polytene nuclei, we determined the nuclear location of the translocated fourth chromosome. Spatial relationships between different chromosomal segments in polytene chromosomes are similar to those observed in diploid cells, as seen for the variegating *brown* gene. In that case, an insertion of ~1 Mb of heterochromatin within *brown* (Platero *et al*., 1998) causes that locus, and a paired wild-type *brown*$^+$ homolog, to associate with centric heterochromatin in polytene and diploid cells (Talbert *et al.*, 1994; Csink and Henikoff, 1996; Dernburg *et al.*, 1996).

We asked whether the 1 Mb of fourth chromosome material translocated to a distal location on the second chromosome would associate with the chromocenter, or would maintain the 'Rabl' orientation typical of the second chromosome in wild-type nuclei (the chromocenter near the nuclear periphery at one pole, and the telomeres associated with the nuclear periphery near the opposite pole of the nucleus) (Mathog *et al.*, 1984; Hochstrasser

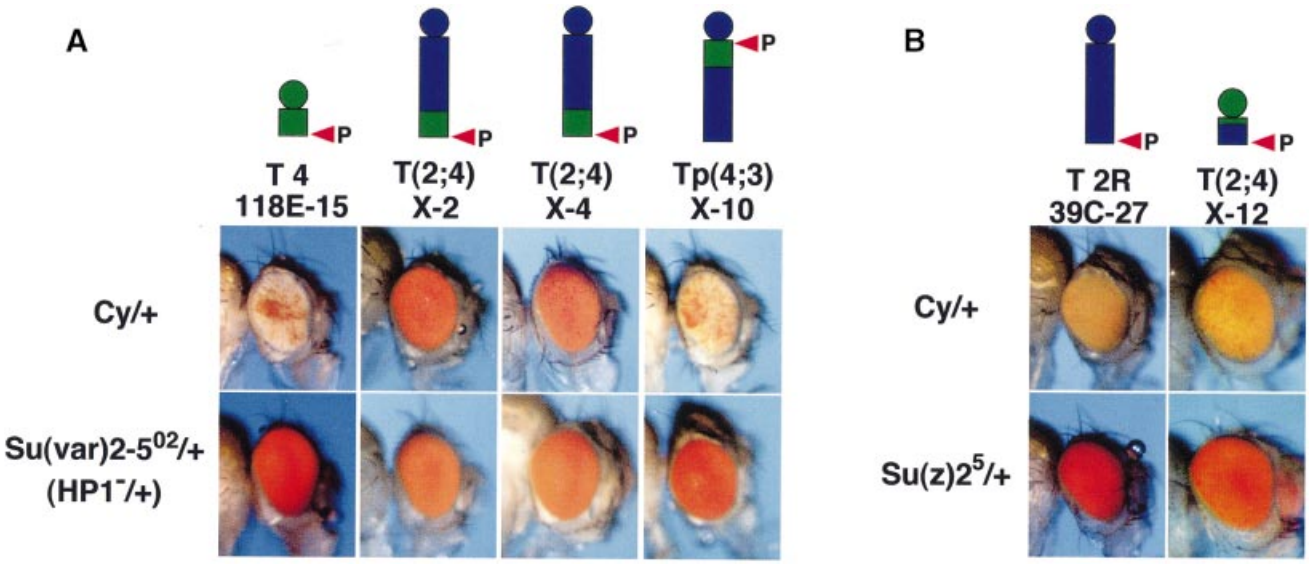

Fig. 4. Eye phenotypes of fourth chromosome rearrangements in different genetic backgrounds. (**A**) Diagrams of the relevant chromosomes are shown at the top with a circle representing the centromere and the red arrowhead labeled with a 'P' representing the P-element. The fourth chromosome is represented in green, the second and third chromosomes in blue. Other designations are as for Figure 1. Translocation of ~1 Mb of the distal portion of a fourth chromosome carrying the P-element at its telomere to the distal end of chromosome two [stocks labeled T(2;4)] causes a reduction in the degree of silencing of the *white*$^{+}$ transgene. Transposition of approximately the same fourth chromosome fragment to a proximal region of the third chromosome [stock Tp(4;3)] does not significantly alter the phenotype. The PEV observed in all of these cases is suppressed when the flies are heterozygous for a mutation in the gene encoding HP1, *Su(var)2-5*^{02}. (**B**) Diagram of the relevant chromosomes at the top. Translocation of a distal region of chromosome two, carrying the *white*$^{+}$ transgene at the telomere, onto the distal region of the fourth chromosome [stock T(2;4)] does not alter the degree of PEV. In both cases shown, the PEV is suppressed when the flies are heterozygous for mutations in *Su(z)2*, but is not affected by mutations in the gene encoding HP1 (latter not shown).

et al., 1986). Stocks X-2 and X-4 were examined for nuclear location of the translocated fourth chromosome using immunofluorescence and confocal microscopy. Larvae heterozygous for the translocated chromosome, or sibling larvae carrying non-rearranged chromosomes as controls, were used for the analysis. Salivary glands were isolated and immunostained with a monoclonal antibody against HP1 (James *et al.*, 1989). Nuclei with wild-type chromosomes typically show intense staining of HP1 at the chromocenter, including staining of chromosome four (Figure 5A). In 52% (488/937) of the nuclei from the translocation heterozygotes of stock X-4, both a large chromocenter region and a second smaller HP1-staining region are apparent (Figure 5B); for stock X-2, 69% (360/524) of the nuclei showed a similar smaller second region of HP1 staining in addition to the chromocenter (data not shown). In contrast, a second small region of HP1 immunofluorescence, distinct from the large chromocenter, was observed in <8% (81/905) of the cells of wild-type sibling larvae. To verify that the second region recognized by HP1 in stocks X-2 and X-4 was in fact the translocated fourth chromosome, combined immunofluorescence and *in situ* hybridization was performed using antibodies against HP1 and a series of overlapping cosmids that span 412 kb of the medial region of the fourth chromosome (cytological region 102B–102D); all of these cosmid clones hybridize to both the translocated fourth chromosome fragment and the non-rearranged fourth chromosome in a translocation heterozygote (Figure 5C–E). In 100% (205/205) of the nuclei examined, there was colocalization of the DNA probes and the anti-HP1 antibody (example shown in Figure 5E).

In the above experiments, the translocated fourth chromosome appeared to be associated with the nuclear periphery. Double immunofluorescence staining with antibodies to HP1 and to nuclear lamin confirmed that in 100% (33/33) of the cases studied, the translocated fourth chromosome (seen as a distinct second HP1 spot) associated with the nuclear lamin (Figure 5F). The fourth chromosome signal was frequently observed towards the opposite pole from the chromocenter. The results suggest that the reduction in PEV observed in the translocation stocks (X-2 and X-4) reflects the change in nuclear location of the fourth chromosome.

Translocation alters heat shock induced expression and chromatin structure of the hsp26 transgene

In order to correlate nuclear location with gene expression within one cell type, we analyzed the *hsp26* gene of the P-element in salivary gland nuclei. To obtain a quantitative assessment of the relative expression of the *hsp26* transgene in the salivary glands of the various stocks, we established the DNA copy number of the transgene for each stock. In general, heterochromatin is underrepresented in polytene nuclei (Weiler and Wakimoto, 1995). Representation was assessed by quantitative Southern analysis using *Eco*RI, which generates different sized DNA fragments from the endogenous *hsp26* gene and the *hsp26* transgene (see Materials and methods). The stocks used were homozygous for the endogenous *hsp26* gene and heterozygous for the fourth chromosome rearrangement. For stock 39C-X, heterozygous for a euchromatic P-element insert, the ratio of transgene to endogenous gene DNA is 0.42, reasonably close to the expected value of 0.50 (data not shown). Ratio values for the telomeric transgenes were as follows: stock 118E-15, 0.29; X-2, 0.45; X-4, 0.40; X-10, 0.32 (data not shown). While these changes are relatively

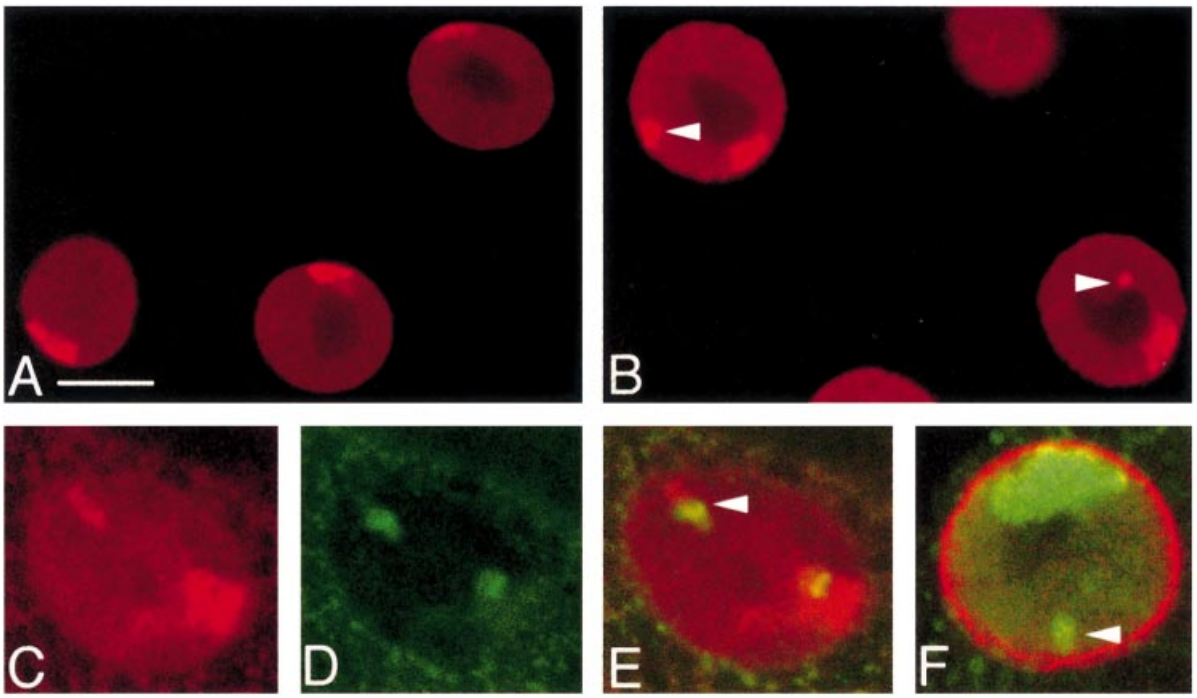

Fig. 5. Localization of HP1 and the fourth chromosome in salivary gland nuclei. (**A**) Immunolocalization of HP1 in salivary gland nuclei with unrearranged chromosomes. (**B**) Immunolocalization of HP1 in salivary gland nuclei from stock X-4. Fluorescence is observed at the chromocenter and at the translocated fourth chromosome (arrow). (**C**) Immunolocalization of HP1 (red) and (**D**) fourth chromosome cosmids (green) in a salivary gland nucleus carrying the X-4 translocation. (**E**) The merged image of C and D showing colocalization of protein and DNA to the translocated fourth chromosome (arrow). (**F**) Immunolocalization of HP1 (green) and lamin (red) showing both the chromocenter and translocated fourth chromosome (arrow) associated with the nuclear periphery. Scale bar is 10 μm for all panels.

small, the relative copy number values are essential for quantifying the relative expression from the *hsp26* genes.

To assay heat shock induced expression of *hsp26*, larvae hemizygous for the P-element or heterozygous for a translocation carrying the P-element were given a heat shock treatment; salivary glands were dissected and total RNA prepared. RT–PCR was performed to make cDNAs from the endogenous *hsp26* gene and the *hsp26* transgene transcripts. The values for the amount of cDNA produced were quantitated by radioactive hybridization and then corrected for DNA copy number and amount of RNA in the reaction. Finally, the values were normalized to that observed for stock 39C-X, set at 100% expression (Figure 6A). The telomeric fourth chromosome transgene of stock 118E-15 is inducible to 13% of the level observed for the euchromatic control, a level very similar to that observed using whole larvae as a source of RNA (15%) (Wallrath and Elgin, 1995). In the translocation stocks X-2 and X-4, the *hsp26* transgenes are heat shock inducible to 150 and 210% of the level observed for the euchromatic control, respectively. In contrast, the transposition stock X-10 [Tp(4;3)] shows only 21% heat shock inducible expression relative to the control. There is an obvious correlation between the nuclear location and inducible gene expression, here observed within the same cell type.

Previously, we have shown that heterochromatic transgenes exhibiting reduced heat shock inducible *hsp26* expression have alterations in chromatin structure (Wallrath and Elgin, 1995). In these cases, the *hsp26* promoter region is less accessible to restriction enzyme digestion and the transgene is packaged in a very regular nucleosome array. We wondered whether the *hsp26* transgene on the translocated fourth chromosome would show a chromatin configuration more like that in euchromatin, given the high level of inducible expression, or more like that in heterochromatin, given the HP1 association. In euchromatin, the *hsp26* regulatory region is characterized by two nucleosome-free regions over the heat shock elements (HSEs) (Thomas and Elgin, 1988; see Figure 6B). Accessibility of the *Xba*I sites within the HSEs can be used to monitor the chromatin configuration (Lu *et al.*, 1993; Cryderman *et al.*, 1998).

We determined the accessibility of the *hsp26* transgene promoter using restriction enzyme treatment of nuclei isolated from salivary glands from non-heat shocked third instar larvae. In stock 39C-X, the proximal *Xba*I site of the euchromatic *hsp26* transgene is 85% accessible. In contrast, the *Xba*I site of the fourth chromosome telomeric transgene is only 9% accessible. The proximal *Xba*I sites in the two translocation stocks, X-2 and X-4, are 67 and 65% accessible, respectively, while that of the transposition stock X-10 is 7% accessible (Figure 6B). Thus, there is a direct correlation between the level of *hsp26* heat shock inducible expression and the accessibility of the proximal *Xba*I site within the same cell type.

Discussion

Subtelomeric sequences have an altered chromatin structure

The variegating second and third chromosome telomeric transgenes recovered here are within TAS, indicating that these sequence elements form a silencing environment. It has recently been reported that multimerization of the

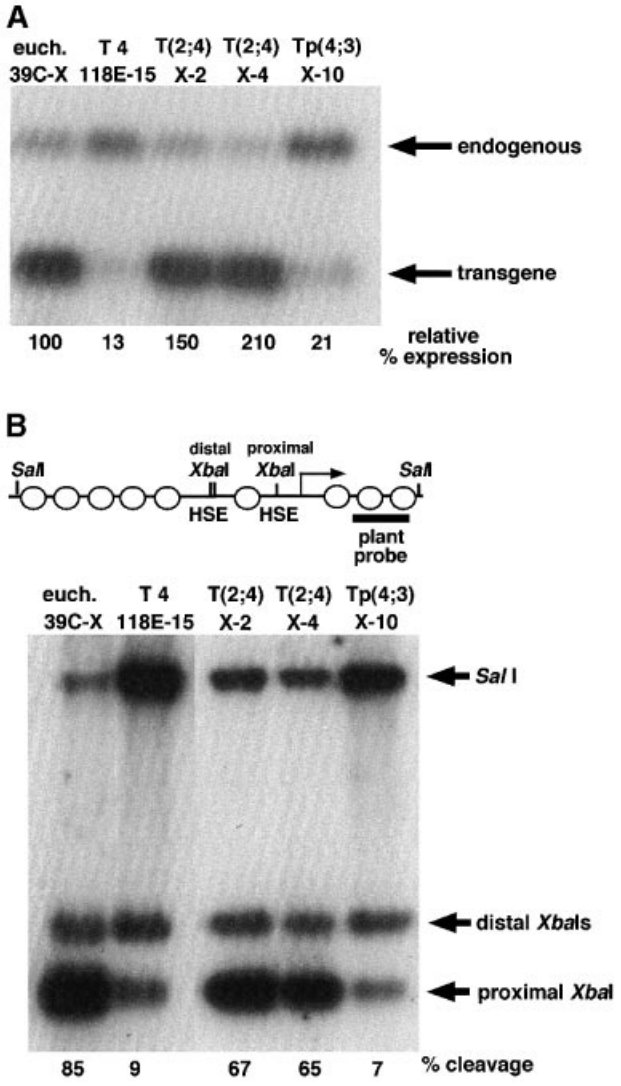

Fig. 6. Inducible expression and chromatin structure of *hsp26* on the translocated fourth chromosome. (**A**) RT–PCR results show an increase in expression from the *hsp26* transgene on the translocated fourth chromosome. Expression values are corrected for DNA copy number (see text) and shown relative to that of 39C-X, set at 100%. (**B**) Schematic representation of the *hsp26* promoter region of the euchromatic gene (top), with circles representing the approximate location of nucleosomes, and the two nucleosome-free regions upstream of the transcription start (arrow) indicated: the plant sequence used as a probe for the Southern blot is shown. Nuclei were isolated from the transgenic stocks and treated with an excess amount of *Xba*I; the DNA was purified, cleaved to completion with *Sal*I, and analyzed by Southern blot using the plant DNA fragment as the probe. The percentage cleavage at the proximal *Xba*I site is shown.

0.4 kb 2L satellite causes direction-dependent repression of an adjacent *white*$^{+}$ reporter gene (Kurenova *et al.*, 1998). Most of the fourth chromosome telomeric transgenes are adjacent to the known repetitious elements *hoppel* or *Fw*. Interestingly, no variegating transgenes were identified within *HeT-A* or *TART* elements. Such flies would not be recovered in this screen if insertion into these transposable elements completely silenced the *hsp70–white*$^{+}$ reporter gene, or allowed for its full expression. Alternatively, the most distal regions of telomeres may be packaged in a manner that does not allow for P-element insertion. A variety of species have telomeric repeats associated with non-histone chromosomal proteins that form specialized chromatin structures (Muniyappa and Kironmai, 1998).

The role of the middle repetitive DNA sequences in the subtelomeric regions of chromosomes is unclear. It is hypothesized that subtelomeric regions might serve as buffer zones between the atypical chromatin structure of the distal ends of chromosomes, and the euchromatic gene-containing regions proximal to the telomere (Kipling, 1995). Recent data suggest that these regions have a distinctive heterochromatic structure. In *S.cerevisiae*, two classes of middle repetitive elements, Y′ and X, reside in subtelomeric locations (Louis, 1995). Y′ elements are packaged with hypoacetylated histones, a feature of repressive chromatin (Braunstein *et al.*, 1993). The subtelomeric X elements exhibit a general resistance to nuclease digestion, suggesting a tightly packaged chromatin structure (Vega-Palas *et al.*, 1998). Here we observe that transgenes showing telomeric PEV in *Drosophila*, associated with repetitious elements, are less accessible to digestion by restriction enzymes (Figure 6B; see also Wallrath and Elgin, 1995). Transgenes located within TAS also show a reduction in accessibility to *Escherichia coli dam* methylase when the enzyme is expressed in *Drosophila* (D.Cryderman, S.Smith and L.Wallrath, unpublished data). Therefore, it appears that subterminal telomeric sequences are packaged in a heterochromatin structure that is not conducive to gene expression.

It appears that repetitive elements found in telomeric locations are associated with chromosomal proteins that play a role in gene silencing at other genomic locations. The results reported above implicate HP1 in silencing at the fourth chromosome telomere; similar genetic analysis indicates a role in pericentric heterochromatin. The extent of cleavage of a pericentric *hsp26* transgene is a function of the amount of HP1 protein; accessibility is 5% in a wild-type background, but increases to 28% when heterozygous for a mutation in the gene encoding HP1 (Cryderman *et al.*, 1998). Psc and Su(z)2 proteins are part of the Polycomb group, structural chromosomal proteins involved in maintaining the silent state of homeotic genes (Pirrotta, 1997). Su(z)2 and Psc localize to telomeric locations (as well as many euchromatic sites) (Rastelli *et al.*, 1993); this, in conjunction with the genetic results, suggests a direct effect on telomere structure. Although HP1 is associated primarily with pericentric and fourth chromosome heterochromatin, it is also seen at specific euchromatic sites and at all telomeres (James *et al.*, 1989; Fanti *et al.*, 1998). While mutations in HP1 do not suppress PEV at second and third chromosome telomeres (Figure 1), they do lead to an increase in telomere fusions in general (Fanti *et al.*, 1998).

Nuclear organization as a mechanism of gene regulation

Many lines of evidence suggest that nuclear organization plays a role in regulating gene expression (Lamond and Earnshaw, 1998). Several studies in *S.cerevisiae* demonstrate a link between gene silencing and nuclear location (Maillet *et al.*, 1996; Marcand *et al.*, 1996; Andrulis *et al.*, 1998). Nuclear organization has also been suggested to contribute to PEV in *Drosophila* (Wakimoto and Hearn, 1990; Henikoff, 1997). An allele of the *brown* gene, *bw*^{D}, generated by an insertion of ~1 Mb of centric satellite

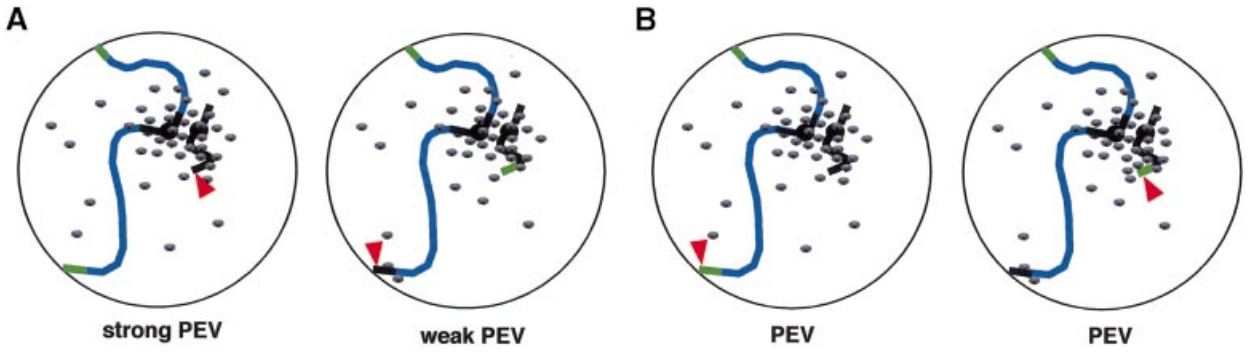

Fig. 7. Model for nuclear compartments of silencing components. (**A**) HP1-sensitive chromatin domains are affected by nuclear position. The location of a telomeric transgene within an HP1-sensitive domain (black) is marked by a red arrowhead. This transgene shows strong PEV due to its proximity to centric heterochromatin, which contains many binding sites for silencing components such as HP1 (small shaded ovals; left cartoon). Numerous binding sites generate an increased effective concentration of silencing factors within this region of the nucleus. Upon translocation to the telomeric region of a longer chromosome, the translocated material is mislocalized to a different compartment within the nucleus (right cartoon). An association of the translocated region with silencing factors is maintained due to the cotranslocation of *cis*-acting sequences; however, the total number of binding sites occupied is presumed to decrease due to a lower effective concentration of these silencing factors in this region of the nucleus. This results in increased gene expression. (**B**) HP1-insensitive telomeric chromatin domains are unaffected by nuclear position. The location of a telomeric transgene within an HP1-insensitive domain (green) is marked by an arrowhead (left cartoon). Upon translocation to the telomeric region of a shorter chromosome, the translocated material is mislocalized within the nucleus into a compartment that has a high concentration of HP1 and associated silencing factors (right cartoon). The translocated telomeric region retains its local chromatin structure, which does not depend on HP1; therefore, gene expression remains unchanged.

sequences (Platero *et al.*, 1998), causes silencing of *brown in trans*. Cytological studies show that the mutant *brown* allele pairs with its wild-type homolog during interphase in diploid and polytene nuclei (Talbert *et al.*, 1994; Csink and Henikoff, 1996; Dernburg *et al.*, 1996). The satellite DNA sequences present on the mutant allele can associate with centric heterochromatin, causing the mutant allele to 'drag' the wild-type homolog into close proximity with heterochromatin (Henikoff, 1997).

The results presented here using *hsp26* and those described above emphasize the importance of nuclear organization in gene silencing, suggesting that the nucleus is made up of subregions that differ in their capacity to support gene expression. Our results indicate that changes in the local chromatin structure occur with changes in nuclear organization. Such changes in local chromatin structure (loss of *Xba*I accessibility) have previously been shown to be associated with a loss of inducible gene expression (Lu *et al.*, 1993, 1995). Furthermore, changes in the accessibility of an *hsp26* transgene at a pericentric site have been correlated with alterations in the levels of HP1 (Cryderman *et al.*, 1998). HP1 is unequally distributed throughout the genome (James *et al.*, 1989; Figure 4); its concentration in the pericentric heterochromatin may serve to elevate its concentration in the region of the nucleus where the centromere resides (Figure 7). This, and the contributions of other proteins found in association with pericentric heterochromatin [e.g. Arp4 (Frankel *et al.*, 1997), Su(var)3-7 (Cleard *et al.*, 1997)], may generate a local nuclear environment that induces silencing of euchromatic genes such as *white* and *brown*, while supporting expression of heterochromatic genes such as *light* (Wakimoto and Hearn, 1990; Weiler and Wakimoto, 1998). Translocation of the fourth chromosome telomeric region to the distal second chromosome telomeric region places it in a region of the nucleus that has a lower concentration of HP1, and presumably of other heterochromatic factors. This could result in a more 'open' chromatin structure, resulting in a loss of gene silencing.

In contrast to the changes in chromosomal properties for the fourth chromosome telomeric transgenes, the second chromosome telomeric transgenes are unaffected by nuclear position. The second chromosome P-element is within TAS; levels of expression are not sensitive to levels of HP1, implying a local chromatin structure that is not determined by levels of HP1. A change in location to an HP1-enriched nuclear compartment has no effect on gene expression (Figure 7). The fact that the same gene, *hsp70–white*$^{+}$, may or may not respond to changes in nuclear position depending on the adjacent sequences, demonstrates the importance of local DNA sequences, presumably dictating a specific local chromatin structure. Furthermore, these findings indicate that HP1 has an impact on structural organization specifically, and does not simply 'coat' the DNA, or serve to drive all repetitive DNA sequences into a repressive structure.

While several studies have shown that either chromatin structure or nuclear organization can have an impact on PEV, this study demonstrates that both are critical. The placement of a gene on a chromosome relative to the centromere and telomere will have an impact on its nuclear position (Marshall *et al.*, 1997), and can have an impact on its expression pattern. Consequently, chromosomal translocations that alter the linear relationship of sequences along a chromosome could affect three dimensional relationships within the nucleus that ultimately dictate chromosomal processes, including gene expression. These findings also suggest that alterations in chromosomal properties may be the molecular mechanism responsible for certain cases of human genetic diseases in which a translocation breakpoint maps kilobases from the candidate disease gene (Kleinjan and van Heyningen, 1998).

Materials and methods

Drosophila genetics

All *Drosophila* stocks were raised on standard corn meal sucrose media (Shaffer *et al.*, 1994) at 25°C unless otherwise stated. All mutations are

as previously described (Lindsley and Zimm, 1992; Wu and Howe, 1995). To assay for dominant modification of PEV, males from stocks heterozygous for a modifier of PEV, a mutation in a Polycomb group protein or other mutations were crossed with females from stocks with a P-element at a telomeric location. The eye phenotype of resulting progeny with the mutation were compared with their siblings lacking the mutation.

X-ray mutagenesis. To recover cases in which the fourth chromosome telomere was translocated to the second or third chromosome telomeric region, 3-day-old males of stock 118E-15, carrying the P-element at a telomeric location on the fourth chromosome, were subjected to 4500 R gamma rays (Torrex 150D, Scanray Corporation). These males were mated to *w; net; sbd; spa^{pol}* virgin females for 5 days in culture bottles. Resulting *white^{+}* male progeny were mated individually to *w; net; sbd; spa^{pol}* females in vials. Of 820 males tested, six lacked progeny possessing both eye pigmentation and net wings, the phenotype expected from a translocation of the fourth telomere (possessing the *hsp70–white^{+}* transgene) onto the second chromosome of the male parent, and five lacked progeny possessing both eye pigmentation and stubbloid bristles, the phenotype expected from a translocation of the fourth chromosome onto the third chromosome. Male and female progeny carrying the P-element were used to establish a stock. *In situ* hybridization to larval salivary gland polytene chromosomes (Cryderman *et al.*, 1998) was performed to determine the rearrangement and the location of the P-element in each stock.

To recover cases in which the second chromosome telomeric region was translocated onto the fourth chromosome, 3-day-old males of stock 39C-27, possessing a 2R telomeric *hsp70–white^{+}* transgene, were treated with X-rays as above. Of 1266 male offspring tested, one yielded the desired phenotype (eye pigmentation, net wings and stubbloid bristles). *In situ* hybridization to larval salivary gland polytene chromosomes revealed that a fragment including several of the most distal bands from 2R had been translocated to the fourth chromosome. This stock, X-12, showed a sparkling eye phenotype when heterozygous with *spa^{pol}* indicating a deficiency for some fourth chromosome material. This stock thrived for several months after the analysis but has subsequently been lost.

Larvae for salivary glands. For procedures involving salivary gland isolation (see below), males containing a translocation were mated to *w; net; sbd; spa^{pol}* virgin females at 18°C. Translocation-bearing third instar larval progeny were selected based on expression of the *white^{+}* transgene, which gives yellow coloration to the Malpighian tubules. Sibling larvae with non-rearranged chromosomes having white Malpighian tubules were used as controls.

Cloning the DNA adjacent to the P-element insert

Direct cloning. The DNA surrounding the telomeric 2L P-element insert of stock 39C-5 was directly cloned. High molecular weight DNA was prepared from 50 adults as described elsewhere (Walter *et al.*, 1995). A genomic library was generated by partially digesting the DNA with *Sau*3A, and partially filling the *Sau*3A sites with Klenow enzyme, dGTP, dATP; 1.2 μg of DNA was used with 1.2 μg of λBlueSTAR phage arms with partially filled *Xho*I sites (Novagen). Recombinant phage DNA was packaged with Gigapack Gold (Stratagene) and infected into *E.coli* DB1316. Approximately 4×10^5 recombinant phage from this unamplified library were screened with a 1.9 kb *Sst*I fragment of the mini-*white* gene. Fifteen positive phage were plaque-purified and grown in 30 ml liquid cultures of *E.coli* DB1316 for DNA preparation. The desired restriction fragments were subcloned into pBluescript I, KS (Stratagene) for mapping and sequencing. Sequencing was done from double-stranded Bluescript subclones using Sequenase 2.0 according to the manufacturer's instructions (USB).

Inverse PCR. The DNA adjacent to the P-element inserts on the fourth chromosome was cloned by inverse PCR using primers specific for the 5′ inverted P-element repeat and the 3′ region of the *white* gene as described (Cryderman *et al.*, 1998). Clones were sequenced manually using dideoxy nucleotides and Sequenase (USB), and were also sequenced using an automated sequencing system (ABI, University of Iowa Sequencing Core Facility). A BLAST search was performed to identify matching sequences from DDBJ/EMBL/GenBank database.

Chromosome cytology. Salivary glands were dissected from third instar larvae and squashed as previously described (Wallrath *et al.*, 1996). The chromosomes were stained with 5% Giesma (Sigma) in 10 mM phosphate buffer (pH 6.8) to accentuate the banding patterns.

Immunostaining

Immunofluorescence. Immunostaining of salivary glands was performed as described (Frankel *et al.*, 1997) with minor modification. Salivary glands were dissected at 4°C in PBS from larvae cultured at 18°C and placed on ice. Glands were transferred to 2% paraformaldehyde in PBS at room temperature for 20 min, and washed three times for 5 min each in PBS^{2+} (130 mM NaCl, 7 mM Na_2HPO_4, 3 mM $Na_2H_2PO_4$, 10 mM EGTA, 0.1% Triton X-100). Glands were blocked in PBS^{2+} with 1% BSA for 60 min prior to incubation for 60 min in a 1:250 dilution of mouse monoclonal anti-HP1 antibody C1A9 (James *et al.*, 1989) in PBS^{2+} with 1% BSA. Glands were again washed three times for 5 min each in PBS^{2+} and blocked in PBS^{2+} with 1% BSA for 60 min. The primary antibody was detected using a Rhodamine RED™ anti-IgG conjugated antibody (Molecular Probes) in a 1:5000 dilution in PBS^{2+} with 1% BSA, incubating for 60 min. Glands were washed three times, 10 min each in PBS^{2+}, prior to mounting on a slide in PPD mounting medium (1 mg/ml *p*-phenylenediamine in 50% glycerol, pH 8.0).

Double label experiments utilized a polyclonal anti-HP1 antibody #365 detected with anti-rabbit fluorescein-conjugated antibody (Cappel), and anti-lamin monoclonal antibody t40 (gift of B.Harmon and J.Sedat) detected with anti-IgG Rhodamine RED™ conjugate. Samples were analyzed and images collected on a Bio-Rad MRC 1024 scanning laser confocal microscope equipped with a krypton/argon laser.

Combined immunofluorescence and in situ hybridization. DNA *in situ* hybridization was performed using modifications of two published protocols (Dernburg *et al.*, 1996; Frankel *et al.*, 1997). All washes and incubation volumes were 500 μl; washes were performed at room temperature unless noted otherwise. Salivary glands were dissected at 4°C in PBS from larvae cultured at 18°C, and placed on ice. Glands were transferred into 4% paraformaldehyde in PBS at 4°C, allowed to warm to room temperature for 30 min and rinsed in three changes of 2× SSCT (0.3 M NaCl, 0.3 M sodium citrate pH 7.0, 0.1% Triton X-100) for a total of 30 min. Glands were incubated in 50% formamide in 2× SSCT for a minimum of 60 min, and then transferred to a 0.5 ml tube containing 160 μg of cosmid DNA labeled by nick translation (Sambrook *et al.*, 1989) with biotin-16-dUTP (Boehringer Mannheim) in 20 μl of hybridization solution (50% formamide, 3× SSCT, 10% dextran sulfate). The DNA used was a pool of 16 cosmids (10•03, 14b14, 56p02, 54a21, 92E5, 37107, 5b10, 48f16, 16n07, 25•01, 58I20, 19m21, ey7, 19f04, 32d22, 7n10) which comprise a 412 kb cosmid contig spanning the medial region of the fourth chromosome (gift of J.Locke, Canadian Fourth Chromosome Genome Project). The DNA was denatured by heating the tube with the hybridization mix and the glands at 95°C for 4 min; the tube was then cooled for 5 min on ice. Hybridization was carried out overnight in a 37°C waterbath.

After hybridization, glands were washed at 37°C for 45 min with three changes of 50% formamide in 2× SSCT followed by three 5-min washes in 2× SSCT and a rinse for 5 min in PBS^{2+}. Glands were then treated with anti-HP1 antibody C1A9 and blocked as described above. Fluorescent detection of the biotinylated probe was carried out using a fluorescein isothiocyanate Ultra-Neutral Lite Avidin™ conjugate (Molecular Probes). Simultaneous detection of the HP1 antibody was accomplished using a 1:5000 dilution of Rhodamine RED™ anti-IgG conjugated antibody (Molecular Probes) in PBS^{2+} with 1% BSA for 60 min. Glands were washed three times for 10 min each in PBS^{2+} prior to mounting on a slide in PPD mounting medium. Samples were analyzed and images collected on a Bio-Rad MRC 1024 scanning laser confocal microscope equipped with a krypton/argon laser.

DNA copy number analysis

Fifty pairs of salivary glands from larvae heterozygous for the P-element were dissected in 0.8% NaCl and collected on ice. DNA was extracted from the glands according to published procedures (Wallrath *et al.*, 1996). The DNA was cleaved with *Eco*RI, separated by size on a 1% agarose TAE gel, transferred to nylon membrane (Hybond, Amersham) and hybridized to α-^{32}P- labeled *Eco*RI fragment corresponding to the 5′ region of the *hsp26* gene. After washing, the radioactivity remaining on the membrane was measured using an Instant Imager (Packard Instruments). Independent trials using different samples from the same stock show average variations of ±0.05 for the ratio of transgene to endogenous gene.

Quantitative assessment of *hsp26* expression

RNA isolation. Larvae were heat shocked for 45 min at 37°C. Sixteen pairs of third instar larval salivary glands were collected in dissection buffer (130 mM Tris, 5 mM KCl and 1.5 mM $CaCl_2$) on ice. The glands

were transferred to a microfuge tube containing 200 µl of dissection buffer, centrifuged for 30 s in a microfuge at 4°C, and the supernatant discarded. Total RNA was extracted according to a published procedure (Andres and Thummel, 1994). The final pellet was resuspended in 200 µl of DEPC-treated water; 20 µl of 8 M LiCl was added and the mixture incubated on ice for 2 h. This step was repeated and the pellet vacuum dried. The pellet was resuspended in 15 µl of DEPC-water and the concentration of RNA determined by spectrophotometry.

PCR. One microgram of total RNA was used to make cDNA according to the Superscript Preamplification System for First Stand cDNA Synthesis kit (BRL). To eliminate PCR products from contaminating genomic DNA, the samples were digested with 10 U of *Sac*I (Promega) and 0.5 µl of RNase H (2 U/µl). *Sac*I cleaves at position +490 in the *hsp26* gene, a location that is between the primers used for PCR. Two microliters of the digest was used in a PCR containing 1× PCR buffer (BRL), 1.5 mM MgCl, 100 µM of each dNTP, 2 U of Amplitaq (Perkin-Elmer), 0.1 mM of each gene-specific primer and 0.2 mM of the primer common to both transgene and endogenous gene. The primer specific for the endogenous *hsp26* gene is 5′-CTGGTGTTT-ACGAATGGGTCTTCACC-3′. The primer specific for the *hsp26* transgene is 5′-CTCAAGATATGGAACATGAACAAGTGC-3′ which corresponds to barley sequences. The primer common to both corresponds to *hsp26* sequences 5′-CCTTTGCTTACAAGTCAAACAAGTTC-3′. The reaction mixture was incubated at 95°C for 3 min to denature the RNA:cDNA hybrids. Twenty amplification cycles, 1 min at 95°C, 2 min at 60°C and 3 min at 72°C, were performed. Ten microliters of each PCR mixture were electrophoresed on a 1% agarose TAE gel and the DNA transferred to nylon membrane (Hybond, Amersham). The membrane was hybridized with the α-^{32}P-labeled *Eco*RI–*Sac*I fragment (+7 to +490) of *hsp26* according to standard procedures (Sambrook *et al.*, 1989). The radioactivity on the membrane was counted using an Instant Imager (Packard Instruments). The values for the transgene were corrected for template copy number according to the results obtained from the salivary gland copy number studies (see above). The values for the *hsp26* transgene were normalized relative to the amount of PCR product from the endogenous *hsp26* gene. The expression values were then made relative to that of 39C-X, set at 100%. Several samples were each analyzed three times using the same and different preparations of cDNA; standard deviations were <10%.

Chromatin structure analysis

Salivary glands from 100 larvae hemizygous for the P-element were dissected in dissection buffer (see above) and collected on ice. Nuclei were isolated (Cartwright *et al.*, 1999) and treated with 400 U of *Xba*I (NEB). The DNA was purified (Wallrath *et al.*, 1998) and cleaved with 60 U of *Sal*I (NEB) in the presence of 10 µg of RNase A. The DNA was separated by size on a 1% agarose TAE gel, transferred to nylon membrane (Hybond, Amersham) and hybridized with the α-^{32}P-labeled barley cDNA fragment (plant probe, Figure 5). The radioactivity on the membrane was measured by an Instant Imager (Packard Instruments). The percentage cleavage at the proximal *Xba*I site was determined as the ratio of the amount of the proximal *Xba*I fragment to the sum of the proximal *Xba*I, distal *Xba*I and *Sal*I fragments, as described previously (Wallrath and Elgin, 1995).

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References

Allshire,R.C., Javerzat,J.-P., Redhead,N.J. and Cranston,G. (1994) Position effect variegation at fission yeast centromeres. *Cell*, **76**, 157–169.

Andres,A.J. and Thummel,C.S. (1994) Methods for quantitative analysis of transcription in larvae and prepupae. *Methods Cell Biol.*, **44**, 565–573.

Andrulis,E.D., Neiman,A.M., Zappulla,D.C. and Sternglanz,R. (1998) Perinuclear localization of chromatin facilitates transcriptional silencing. *Nature*, **394**, 592–595.

Biessmann,H., Valgeirsdottir,K., Lofsky,A., Chin,C., Ginther,B., Levis,R.W. and Pardue,M.L. (1992) HeT-A, a transposable element specifically involved in 'healing' broken chromosome ends in *Drosophila melanogaster*. *Mol. Cell. Biol.*, **12**, 3910–3918.

Braunstein,M., Rose,A.B., Holmes,S.G., Allis,C.D. and Broach,J.R. (1993) Transcriptional silencing in yeast is associated with reduced nucleosome acetylation. *Genes Dev.*, **7**, 592–604.

Carmena,M. and Gonzalez,C. (1995) Transposable elements map in a conserved pattern of distribution extending from β-heterochromatin to centromeres in *Drosophila melanogaster*. *Chromosoma*, **103**, 676–684.

Cartwright,I.L., Cryderman,D.E., Gilmour,D.S., Pile,L., Tang,H., Wallrath,L.L., Weber,J. and Elgin,S.C.R. (1999) Analysis of *Drosophila* chromatin structure *in vivo*. *Methods Enzymol.*, **304**, 462–496.

Cleard,F., Delattre,M. and Spierer,P. (1997) SU (VAR)3-7, a *Drosophila* heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation. *EMBO J.*, **16**, 5280–5288.

Cryderman,D.E., Cuaycong,M.H., Elgin,S.C.R. and Wallrath,L.L. (1998) Characterization of sequences associated with position effect variegation at pericentric sites in *Drosophila* heterochromatin. *Chromosoma*, **107**, 277–285.

Csink,A.K. and Henikoff,S. (1996) Genetic modification of heterochromatic association and nuclear organization in *Drosophila*. *Nature*, **381**, 529–531.

Danilevskaya,O.N., Arkhipova,I.R., Traverse,K.L. and Pardue,M.L. (1997) Promoting in tandem: the promoter for telomere transposon HeT-A and implications for the evolution of retroviral LTRs. *Cell*, **88**, 647–655.

Dernburg,A.F., Broman,K.W., Fung,J.C., Marshall,W.F., Philips,J., Agard,D.A. and Sedat,J.W. (1996) Perturbation of nuclear architecture by long-distance chromosome interactions. *Cell*, **85**, 745–759.

Di Nocera,P.P. and Dawid,I.B. (1983) Interdigitated arrangement of two oligo(A)-terminated DNA sequences in *Drosophila*. *Nucleic Acids Res.*, **11**, 5475–5482.

Di Nocera,P.P. and Casari,G. (1987) Related polypeptides are encoded by *Drosophila* F elements, I factors and mammalian L1 sequences. *Proc. Natl Acad. Sci. USA*, **84**, 5843–5847.

Dobie,K., Mehtali,M., McClenaghan,M. and Lathe,R. (1997) Variegated gene expression in mice. *Trends Genet.*, **13**, 127–130.

Dorn,R., Heymann,S., Lindigkeit,R. and Reuter,G. (1986) Suppressor mutation of position-effect variegation in *Drosophila melanogaster* affecting chromatin properties. *Chromosoma*, **93**, 398–403.

Eissenberg,J.C., Morris,G.D., Reuter,G. and Hartnett,T. (1992) The heterochromatin-associated protein HP-1 is an essential protein in *Drosophila* with dosage-dependent effects on position-effect variegation. *Genetics*, **131**, 345–352.

Fanti,L., Giovinazzo,G., Berloco,M. and Pimpinelli,S. (1998) The heterochromatin protein 1 (HP1) prevents telomere fusions in *Drosophila melanogaster*. *Mol. Cell*, **2**, 527–538.

Frankel,S., Sigel,E.A., Craig,C., Elgin,S.C.R., Mooseker,M.S. and Artavanis-Tsakonas,S. (1997) An actin-related protein in *Drosophila* colocalizes with heterochromatin protein 1 in pericentric heterochromatin. *J. Cell Sci.*, **110**, 1999–2012.

Gehring,W.J., Klemenz,R., Weber,U. and Kloter,U. (1984) Functional analysis of the *white*$^{+}$ gene of *Drosophila* by P-factor-mediated transformation. *EMBO J.*, **3**, 2077–2085.

Gottschling,D.E., Aparicio,O.M., Billington,B.L. and Zakian,V.A. (1990) Position effect at *S.cerevisiae* telomeres: reversible repression of Pol II transcription. *Cell*, **63**, 751–762.

Hazelrigg,T., Levis,R. and Rubin,G.M. (1984) Transformation of *white* locus DNA in *Drosophila*: dosage compensation, *zeste* interaction and position effects. *Cell*, **36**, 469–481.

Henikoff,S. (1997) Nuclear organization and gene expression: homologous pairing and long-range interactions. *Curr. Opin. Cell Biol.*, **9**, 388–395.

Hochstrasser,M., Mathog,D., Gruenbaum,Y., Saumweber,H. and Sedat,J.W. (1986) Spatial organization of chromosomes in the salivary gland nuclei of *Drosophila melanogaster*. *J. Cell Biol.*, **102**, 112–123.

Horn,D. and Cross,G.A. (1995) A developmentally regulated position effect at a telomeric locus in *Trypanosoma brucei*. *Cell*, **83**, 555–561.

James,T.C., Eissenberg,J.C., Craig,C., Dietrich,V., Hobson,A. and Elgin,S.C.R. (1989) Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of *Drosophila*. *Eur. J. Cell Biol.*, **50**, 170–180.

Karpen,G.H. and Spradling,A.C. (1992) Analysis of subtelomeric heterochromatin in the *Drosophila* minichromosome Dp1187 by single P element insertional mutagenesis. *Genetics*, **132**, 737–753.

Kioussis,D. and Festenstein,R. (1997) Locus control regions: overcoming heterochromatin-induced gene inactivation in mammals. *Curr. Opin. Genet. Dev.*, **7**, 614–619.

Kipling,D. (1995) *The Telomere*. Oxford University Press, New York, NY.

Kleinjan,D.J. and van Heyningen,V. (1998) Position effect in human genetic disease. *Hum. Mol. Genet*. **7**, 1611–1618.

Kurenova,E.V., Leibovich,B.A., Bass,I.A., Bebikhov,D.V., Pavlova,M.N. and Danilevskaya,O.N. (1990) Hoppel-family of mobile elements of *Drosophila melanogaster*, flanked by short inverted repeats and having preferential localization in the heterochromatin regions of the genome. *Genetika*, **26**, 1701–1712.

Kurenova,E., Champion,L., Biessmann,H. and Mason,J.M. (1998) Directional gene silencing induced by a complex subtelomeric satellite from *Drosophila*. *Chromosoma*, **107**, 311–320.

Lamond,A.I. and Earnshaw,W.C. (1998) Structure and function in the nucleus. *Science*, **280**, 547–553.

Levis,R.W., Ganesan,R., Houtchens,K., Tolar,L.A. and Sheen,F.M. (1993) Transposons in place of telomeric repeats at a *Drosophila* telomere. *Cell*, **75**, 1083–1093.

Lindsley,D.L. and Zimm,G.G. (1992) *The Genome of Drosophila melanogaster*. Academic Press, San Diego, CA.

Lingner,J. and Cech,T.R. (1998) Telomerase and chromosome end maintenance. *Curr. Opin. Genet. Dev.*, **8**, 226–232.

Louis,E.J. (1995) The chromosome ends of *Saccharomyces cerevisiae*. *Yeast*, **11**, 1553–1573.

Lu,Q., Wallrath,L.L., Granok,H. and Elgin,S.C.R. (1993) $(CT)_n \cdot (GA)_n$ repeats and heat shock elements have distinct roles in chromatin structure and transcriptional activation of the *Drosophila hsp26* gene. *Mol. Cell. Biol.*, **13**, 2802–2814.

Lu,Q., Wallrath,L.L. and Elgin,S.C.R. (1995) The role of a positioned nucleosome at the *Drosophila melanogaster hsp26* promoter. *EMBO J.*, **14**, 4738–4746.

Maillet,L., Boscheron,C., Gotta,M., Marcand,S., Gilson,E. and Gasser,S.M. (1996) Evidence for silencing compartments within the yeast nucleus: a role for telomere proximity and Sir protein concentration in silencer-mediated repression. *Genes Dev.*, **10**, 1796–1811.

Marcand,S., Gasser,S.M. and Gilson,E. (1996) Chromatin: a sticky silence. *Curr. Biol.*, **6**, 1222–1225.

Marshall,W.F., Fung,J.C. and Sedat,J.W. (1997) Deconstructing the nucleus: global architecture from local interactions. *Curr. Opin. Genet. Dev.*, **7**, 259–263.

Mathog,D., Hochstrasser,M., Gruenbaum,Y., Saumweber,H. and Sedat,J. (1984) Characteristic folding pattern of polytene chromosomes in *Drosophila* salivary gland nuclei. *Nature*, **308**, 414–421.

Muller,H.J. (1938) The remaking of chromosomes. *Collecting Net*, **XIII**, 181–195, 198.

Muniyappa,K. and Kironmai,K.M. (1998) Telomere structure, replication and length maintenance. *Crit. Rev. Biochem. Mol. Biol.*, **33**, 297–336.

Nimmo,E.R., Cranston,G. and Allshire,R.C. (1994) Telomere-associated chromosome breakage in fission yeast results in variegated expression of adjacent genes. *EMBO J.*, **13**, 3801–3811.

Palladino,F. and Gasser,S.M. (1994) Telomere maintenance and gene repression: a common end? *Curr. Opin. Cell Biol.*, **6**, 373–379.

Pardue,M.L., Danilevskaya,O.N., Traverse,K.L. and Lowenhaupt,K. (1997) Evolutionary links between telomeres and transposable elements. *Genetica*, **100**, 73–84.

Pavlova,M., Wallrath,L., Elgin,S. and Levis,R. (1996) Telomeric position effects: variegating transgenes are flanked by related sequences in the 2R and 3R telomeres and are inserted in both orientations. 37th Annual *Drosophila* Research Conference Program and Abstracts Volume, The Genetics Society of America, 159.

Pimpinelli,S., Berloco,M., Fanti,L., Dimitri,P., Bonaccorsi,S., Marchetti,E., Caizzi,R., Caggese,C. and Gatti,M. (1995) Transposable elements are stable structural components of *Drosophila melanogaster* heterochromatin. *Proc. Natl Acad. Sci. USA*, **92**, 3804–3808.

Pirrotta,V. (1997) PcG complexes and chromatin silencing. *Curr. Opin. Genet. Dev.*, **7**, 249–258.

Platero,J.S., Csink,A.K., Quintanilla,A. and Henikoff,S. (1998) Changes in chromosomal localization of heterochromatin-binding proteins during the cell cycle in *Drosophila*. *J. Cell Biol.*, **140**, 1297–1306.

Rastelli,L., Chan,C.S. and Pirrotta,V. (1993) Related chromosome binding sites for zeste, suppressors of zeste and Polycomb group proteins in *Drosophila* and their dependence on Enhancer of zeste function. *EMBO J.*, **12**, 1513–1522.

Rudenko,G., Blundell,P.A., Dirks-Mulder,A., Kieft,R. and Borst,P. (1995) A ribosomal DNA promoter replacing the promoter of a telomeric VSG gene expression site can be efficiently switched on and off in *T. brucei*. *Cell*, **83**, 547–553.

Sambrook,J., Fritsch,E.F. and Maniatis,T. (1989) Preparation of radiolabeled DNA and RNA probes. In Nolan,C. (ed.), *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, Vol. 2, pp.10.16–10.20.

Shaffer,C.D., Wuller,J.M. and Elgin,S.C.R. (1994) Raising large quantities of *Drosophila* for biochemical experiments. *Methods Cell Biol.*, **44**, 99–108.

Stone,E.M. and Pillus,L. (1998) Silent chromatin in yeast: an orchestrated medley featuring Sir3p. *Bioessays*, **20**, 30–40.

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.

Thomas,G.H. and Elgin,S.C.R. (1988) Protein/DNA architecture of the DNase I hypersensitive region of the *Drosophila hsp26* promoter. *EMBO J.*, **7**, 2191–2201.

Vega-Palas,M.A., Venditti,S. and Di Mauro,E. (1998) Heterochromatin organization of a natural yeast telomere: changes of nucleosome distribution driven by the absence of Sir3p. *J. Biol. Chem.*, **273**, 9388–9392.

Wakimoto,B.T. and Hearn,M.G. (1990) The effects of chromosome rearrangements on the expression of heterochromatic genes in chromosome 2L of *Drosophila melanogaster*. *Genetics*, **125**, 141–154.

Wallrath,L.L. (1998) Unfolding the mysteries of heterochromatin. *Curr. Opin. Genet. Dev.*, **8**, 147–153.

Wallrath,L.L. and Elgin,S.C.R. (1995) Position effect variegation in *Drosophila* is associated with an altered chromatin structure. *Genes Dev.*, **9**, 1263–1277.

Wallrath,L.L., Guntur,V.P., Rosman,L.E. and Elgin,S.C.R. (1996) DNA representation of variegating heterochromatic P-element inserts in diploid and polytene tissues of *Drosophila melanogaster*. *Chromosoma*, **104**, 519–527.

Wallrath,L.L., Swede,M.J. and Elgin,S.C.R. (1998) Mapping chromatin structure in *Drosophila*. In Gould,H. (ed.), *Chromatin: A Practical Approach*. Oxford University Press, New York, NY, pp. 59–77.

Walter,M.F., Jang,C., Kasravi,B., Donath,J., Mechler,B.M., Mason,J.M. and Biessmann,H. (1995) DNA organization and polymorphism of a wild-type *Drosophila* telomere region. *Chromosoma*, **104**, 229–241.

Weiler,K.S. and Wakimoto,B.T. (1995) Heterochromatin and gene expression in *Drosophila*. *Annu. Rev. Genet.*, **29**, 577–605.

Weiler,K.S. and Wakimoto,B.T. (1998) Chromosome rearrangements induce both variegated and reduced, uniform expression of heterochromatic genes in a development-specific manner. *Genetics*, **149**, 1451–1464.

Wu,C.T. and Howe,M. (1995) A genetic analysis of the *Suppressor 2 of zeste* complex of *Drosophila melanogaster*. *Genetics*, **140**, 139–181.

Zakian,V.A. (1995) Telomeres: beginning to understand the end. *Science*, **270**, 1601–1607.

Zhang,P. and Spradling,A.C. (1995) The *Drosophila* salivary gland chromocenter contains highly polytenized subdomains of mitotic heterochromatin. *Genetics*, **139**, 659–670.

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