Molecular Biology of the Cell
Vol. 17, 2158–2165, May 2006

The Mcp Element Mediates Stable Long-Range Chromosome–Chromosome Interactions in *Drosophila*

Julio Vazquez,*† Martin Müller,†‡ Vincenzo Pirrotta,§ and John W. Sedat‖

*Division of Shared Resources, Fred Hutchinson Cancer Research Center, Seattle, WA 98109; ‡Department of Zoology, University of Basel, CH-4056 Basel, Switzerland; §Department of Molecular Biology and Biochemistry, Rutgers University, Piscataway, NJ 08544; and ‖Department of Biophysics and Biochemistry, University of California, San Francisco, San Francisco, CA 94143-2240

Submitted January 17, 2006; Accepted February 10, 2006
Monitoring Editor: Yixian Zheng

Chromosome organization inside the nucleus is not random but rather is determined by a variety of factors, including interactions between chromosomes and nuclear components such as the nuclear envelope or nuclear matrix. Such interactions may be critical for proper nuclear organization, chromosome partitioning during cell division, and gene regulation. An important, but poorly documented subset, includes interactions between specific chromosomal regions. Interactions of this type are thought to be involved in long-range promoter regulation by distant enhancers or locus control regions and may underlie phenomena such as transvection. Here, we used an in vivo microscopy assay based on Lac Repressor/operator recognition to show that *Mcp*, a polycomb response element from the *Drosophila* bithorax complex, is able to mediate physical interaction between remote chromosomal regions. These interactions are tissue specific, can take place between multiple *Mcp* elements, and seem to be stable once established. We speculate that this ability to interact may be part of the mechanism through which *Mcp* mediates its regulatory function in the bithorax complex.

INTRODUCTION

The term transvection, originally described by Ed Lewis in *Drosophila* (Lewis 1954), referred to an unusual mechanism of genetic complementation at the bithorax complex, thought to require physical pairing between alleles. Since then, a variety of pairing-dependent phenomena have been documented in various species, including vertebrates. These include enhancer and silencer action in trans, the spreading of silenced states between homologous chromosomes, and pairing-dependent bypassing of insulator elements (reviewed in Pirrotta 1999; Duncan 2002; Kassis 2002). Pairing of alleles at specific *Drosophila* loci is also required to achieve wild-type levels of transcription (Goldsborough and Kornberg 1996), whereas the silencing effect of polycomb response elements (PREs) is often greatly enhanced by the pairing of two allelic copies of the PRE, the so-called pairing-sensitive silencing (reviewed in Pirrotta 1999). Although transvection phenomena are thought to rely on somatic pairing of homologous chromosomes, certain PRE regions, alone or aided by the presence of gypsy insulator elements, have been found to bypass this requirement (Hopmann *et al*., 1995; Sigrist and Pirrotta 1997; Müller *et al*., 1999). This finding suggested that *Drosophila* PREs might be able to pair autonomously, i.e., independently of the somatic pairing mechanism normally found in this organism. Cytological studies using conventional cytological methods have confirmed that copies of the *Fab-7* PRE are able to pair in *Drosophila* tissues and embryos (Bantignies *et al*., 2003). Here, we used an in vivo assay based on green fluorescent protein (GFP)-Lac repressor/operator recognition to show that multiple remote copies of *Mcp*, another PRE from the *Drosophila* bithorax complex, are able to establish stable interactions in imaginal disk nuclei. Interactions of the type described here may provide the physical basis for the observed sensitivity of the bithorax complex to transvection effects. More importantly, the ability of Mcp to interact over large distances may be part of the mechanism through which this element performs its regulatory function within the bithorax complex.

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E06–01–0049) on February 22, 2006.

The online version of this article contains supplemental material at *MBC Online* (http://www.molbiolcell.org).

† These authors contributed equally to this work.

Address correspondence to: Julio Vazquez (jvazquez@fhcrc.org).

Abbreviations used: PRE, polycomb response element.

MATERIALS AND METHODS

DNA Clones

psOMws′ is essentially identical to w#15 (described in Müller *et al*., 1999) except for the presence of a 2.5-kb fragment with 64 lacO sites. Therefore, the P-element w#15 and the LacO-fragment clone pAFS153 (a gift from A. F. Straight, Stanford University, Stanford, CA; Robinett *et al*., 1996; Straight *et al*., 1996) served as a starting point for its construction. In a first step, w#15 was digested with KpnI and XhoI. The resulting vector fragment and the scs fragment were gel purified. Then, the 2.5-kb LacO fragment was isolated from pAFS153 after SalI and KpnI restriction digests. The w#15 vector fragment was subsequently ligated with the LacO fragment leading to the intermediate pOMws′. Compared with w#15, in pOMws′, the scs fragment is replaced by the LacO fragment. In addition, the XhoI site separating the *white* enhancer from the LacO sites is abolished by the ligation of the SalI and XhoI compatible sticky ends. However, a new XhoI site located just next to the KpnI site in the LacO fragment was introduced. Hence, in the second cloning step, pOMws′ was cut with KpnI and XhoI. Then, it was ligated with the scs KpnI/XhoI-fragment, which was isolated in the first cloning step. The resulting P-element was named psOMws′. The construction of the GFP-Lac repressor protein fusion in GAL UAS vector pUASP has been described previously (Vazquez *et al*., 2001).

Table 1. Transgenic lines established for construct psOMws′

Insert	Isolation no.	Linkage	Location[a]	Eye color P/+[b]	Eye color P/P[b]	PS?[c]
OM1	67.68.1	X	ND	Orange-red, u	Orange, v	Yes
OM2	67.B	II	30–32	Dull red, u	Yellow, v	Yes
OM3	67.N	II	50–52	Dull red, u	Orange-red, v	Yes
OM4	67.12.1	III	64–66	Dull red, u	Yellow, v	Yes
OM5	67.90.1	III	82–84	Dull red, u	White, v	Yes
OM6	67.J	III	82–84	Dull red, u	Yellow, v	Yes
OM7	67.A	III	94–96	Dull red, u	Yellow, v	Yes
OM8	67.44.1	II	ND	Yellow, v	White	Yes
OM9	67.19.1	II	ND	Brown, u	Red, u	No
OM10	67.M	III	ND	Dull red, u	Red, u	No

ND, not determined; u, uniform eye color; v, variegated eye color.

[a] Approximate insertion sites of P-elements were deduced from recombination mapping relative to each other and relative to other genetic markers on the second or third chromosome. For details, see *Materials and Methods*.

[b] Eye colors from heterozygous (P/+) and homozygous (P/P) flies are indicated as scored from 3-d-old flies. For OM5, white, v means that there are a few scattered pigmented ommatidia in an otherwise white background.

[c] PS stands for pairing-dependent silencing of mini-*white*. Lines are scored as pairing sensitive when the eye color of homozygous flies is lighter than the eye color of heterozygous flies (for example, see Figure 1D). In contrast, generic P[mini-*white*] inserts show a clear dosage dependence where homozygous flies have darker eye color than heterozygous siblings (see Figure 1C).

Fly Stocks

Transgenic lines for psOMws′ were established either according to standard procedures by injecting *Df(1)w*^{67c23},*y*$^{-}$ embryos (Rubin and Spradling 1982) or by mobilization (Robertson *et al*., 1998) of the X-linked transgene OM1 to chromosomes 2 or 3 (OM2, OM3, OM6, OM7, and OM10). Eight of 10 OM lines were pairing sensitive (80%). This is in good agreement with the value obtained for construct w#15, which lacks the lacO sites (71% pairing-sensitive lines; Müller *et al*., 1999). This indicates that the presence of the 2.5-kb DNA fragment containing 64 lacO sites does not significantly influence Mcp activity with respect to its pairing-dependent *mini-white* inactivation.

Recombinants between two transgenes were established by crossing P1,+/+, P2 virgin flies to *w*^{1} males. The progeny of these crosses was screened for recombinant males of the genotype P1,P2/+,+ by looking for the characteristic eye pigmentation indicative of long-distance pairing interaction. Such males were crossed individually with appropriate balancer lines and stocks were established. At the same time, recombination frequencies between pairs of interacting inserts were obtained and the inserts could be positioned relative to each other. To determine the actual position of each insert more precisely, inserts on the second and third chromosomes were also mapped relative to known markers. The combination of all the recombination data allowed an approximate mapping of OM2, OM3, OM4, OM5, OM6, and OM7 (Table 1). Details are available upon request.

Recombinants between *grappa*^{1A} (*gpp*^{1A}; Shanower *et al*., 2005) and OM4, OM6, or OM7 were established as follows. *gpp*^{1A} +/+ OM virgins were crossed to homozygous *gpp*^{1A} males. Among the male progeny, *gpp*^{1A} OM/*gpp*^{1A} + flies could be selected due to the eye pigmentation of the OM transgene and the strong loss of pigmentation in abdominal segments A5 and A6 characteristic of homozygous *gpp*^{1A} males. Such males were crossed individually with appropriate balancer lines, and stocks were established. These lines served as a basis to establish OM4 *gpp*^{1A} OM6, OM4 *gpp*^{1A} OM7, *gpp*^{1A} OM6 OM7 triple recombinants. Such recombinants have clearly darker eye color than flies heterozygous for a single OM transgene and homozygous for *gpp*^{1A}. To drive expression of the GFP-Lac repressor protein, we used line 1.3hs. This recombinant line carries 2 P-element insertions on chromosome 2: a GFP-lac repressor fusion gene, which is expressed under the control of the UAS enhancer in vector pUASP (line 1.3; for details, see Vazquez *et al*., 2001), and a heat shock-inducible hsp70::GAL4 insertion (Brand and Perrimon 1993; transgenic line was a gift from Markus Affolter, University of Basel, Basel, Switzerland). Control P[*lacO*, *white*] constructs M2.1 and M6.1 that do not carry Mcp were described in Vazquez *et al*. (2001).

Scoring Eye Colors

The pigmentation of the fly eye as a consequence of mini-*white* gene expression depends strongly on the age and sex of the fly (Qian and Pirrotta 1995). Therefore, care was taken to only compare and score eye color of flies of very similar age and sex, and also in the absence of balancer chromosomes. Flies were collected within a 4-h window after eclosion and subsequently aged for 3 d before scoring their eye color. Pictures were taken with a Nikon Coolpix 4500 digital camera mounted on a Leica MZ75 stereomicroscope, and processed with Adobe Photoshop (Adobe Systems, Mountain View, CA).

Microscopy

To induce expression of the GFP-Lac repressor protein, young third instars were heat shocked for 45–90 min in a 36°C incubator and were left to recover at room temperature for at least 16 h before imaging. Typically, the imaging was done 24–48 h after the initial heat shock. The relatively mild heat shock conditions and long recovery time were used to minimize potential heat-induced artifacts. Larvae were rinsed and dissected in saline (*Drosophila* testis isolation buffer; Casal *et al*., 1990). For short-term imaging, a small chamber was made by applying a ring of several layers of nail polish to a microscopy slide. After the nail polish had dried out, tissues were placed inside the ring in a drop of buffer and covered with a coverslip. For longer term imaging, tissues were imaged in *Drosophila* SL3 medium (Invitrogen, Carlsbad, CA) supplemented with 7% fetal calf serum, inside a sealed microscope chamber, as described previously (Vazquez *et al*., 2001, 2002). Similar imaging conditions have been shown to preserve spermatocyte viability for up to 12 h, including their progression through the meiotic cell cycle. Dead or damaged cells typically show a much greater degree of chromatin Brownian motion, possibly due to the destruction of chromatin–nuclear cytoskeleton interaction. Therefore, nuclei with unusual Brownian motion patterns were not included in the present study. To ascertain that the animals under study had the desired number of P[Mcp, lacO] inserts, polytene tissues and/or young spermatocytes dissected from the same animals were also analyzed. In those tissues, Mcp elements do not associate, therefore yielding one GFP spot per insertion site. With our lines, expression of GFP-Lac repressor protein was achieved primarily in the posterior end of the eye-antenna imaginal disk, behind the morphogenetic furrow. Therefore, the cells analyzed are photoreceptor cells in their differentiating phase and are most likely to be in G_2. Imaging was done on an inverted Olympus IX-70 microscope through a high numerical aperture 60×/1.4 PlanApo or 100×/1.35 UPlanApo objective. Time-lapse series and three-dimensional (3-D) stacks were deconvolved using Applied Precision (Issaquah, WA) SoftWoRx software. Pairing efficiency was determined on deconvolved 3-D stacks with sections typically collected at 0.2- to 0.3-μm spacing. Spots were counted through examination of the 3-D data sets. Because the intensity of the fluorescent spots was very low, counting of the spots was done only on the best data sets, i.e., those where the number of spots could be determined unambiguously for >90% of the nuclei in a given field. Nuclei that were not scored usually fell into two categories: those where no clear spots could be clearly identified, and those that showed a single blurred spot or two closely spaced spots. The former probably represent nuclei with unpaired spots that are too weak to clearly identify from the background GFP levels. The latter may represent either nuclei with unpaired spots or nuclei with a single spot that got blurred because of motion during imaging. Among nuclei that were suitable for scoring, although there was a great deal of variability in the intensity of GFP-Lac repressor spots, paired spots gave a signal that was on average twice as intense as that of unpaired spots (Figure 3), and the two types could therefore be easily distinguished. Because ambiguous nuclei most likely represent nuclei with fainter, unpaired spots, the proportion of unpaired nuclei may be slightly underestimated. Time-lapse movies were collected either as 3-D stacks or as optical sections of a single focal plane. Because of the lower optical resolution along the vertical

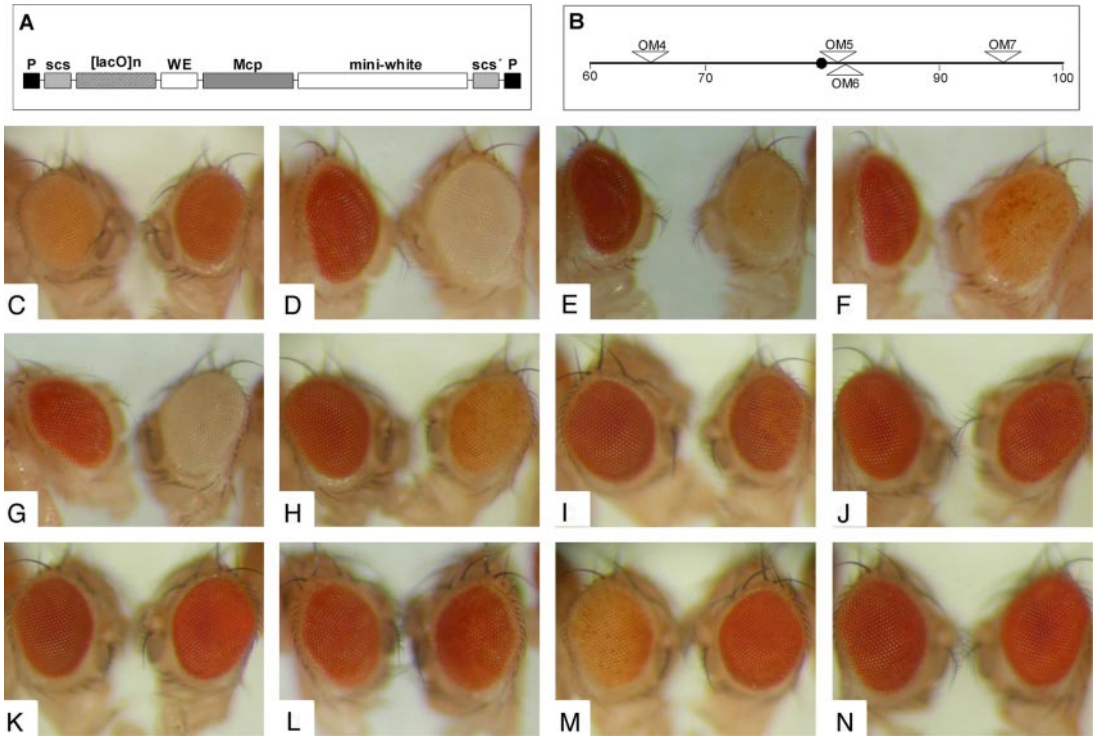

Figure 1. Mcp mediates pairing-dependent silencing of *white*. (A) Map of the P-element construct psOMws′ used to test long-range interactions in live flies. The construct contains an Mcp element (Mcp) between the *white* enhancer region (WE) and the *white* minigene (mini-*white*), and an array of 64 lac operator sequences ([lacO]n). The construct is flanked by the scs and scs′ insulator elements to minimize position effects. (B) Diagram showing the approximate location of four psOMws′ inserts on the third chromosome used in this study. Numbers below the map refer to cytological landmarks on chromosome 3. (C–N) Pairing-dependent silencing of *white* in flies carrying psOMws′ inserts. (C) Generic mini-*white* construct, without *white* enhancer (to show dosage dependent expression). P/+ versus P/P. In this, as in the following photographs, flies with the lowest number of constructs are on the left. Note the increase in eye pigmentation in the homozygous fly. (D–F) Typical examples of pairing-dependent silencing of mini-*white* mediated by Mcp. (D) OM5/+ versus OM5/OM5. (E) OM6/+ versus OM6/OM6. (F) OM4/+ versus OM4/OM4. (G–L) Examples of Mcp-mediated long-distance silencing. (G) OM5/+ versus OM5 OM6/+ +. (H) OM7/+ versus OM6 OM7/+ +. (I) OM4/+ versus OM4 OM6/+ +. (J) +OM7/+ + versus OM4 OM7/+ +. (K) OM3/+ versus OM3/+; OM7/+. (L) OM7/+ versus OM4 OM6+/+ OM6 OM7. (M and N) Suppression effect of a homozygous gpp^{1A} background on Mcp-mediated long-distance silencing. (M) OM6 OM7/+ + versus gpp^{1A} OM6 OM7/gpp^{1A} + +. (N) OM4 OM7/+ + versus OM4 gpp^{1A} OM7/+ gpp^{1A} +.

axis, and the small size of the eye disk nuclei, all spots could generally been seen in single sections focused roughly at the central plane of the nucleus.

RESULTS

As an attempt to identify novel euchromatic sequences capable of mediating chromosome–chromosome interactions, we turned our attention to the *Mcp* element from the *Drosophila* bithorax complex (Vazquez *et al*., 1993; Mihaly *et al*., 1998; Müller *et al*., 1999). *Mcp* is located between the *iab-4* regulatory region that directs expression of *abd-A* in parasegment 9, and *iab-5*, that directs expression of *Abd-B* in parasegment 10 (Lewis 1978; Karch *et al*., 1985). Deletions of *Mcp* cause ectopic activation of *Abd-B* in PS9 (Crosby *et al*., 1993), which led to the hypothesis that this element could function either as a silencer (Busturia and Bienz 1993; Busturia *et al*., 1997) or as a boundary element located between and functionally separating *iab-4* and *iab-5* (Gyurkovics *et al*., 1990; Karch *et al*., 1994). When present on a transgene, a 2.8-kb fragment containing *Mcp* was shown to mediate pairing-dependent silencing of a linked mini-*white* gene (Müller *et al*., 1999). The term pairing-dependent silencing describes a phenomenon in which the eye color of homozygous flies (containing two copies of the transgene) is lighter than the eye color observed in heterozygous flies (containing 1 copy of the transgene; reviewed in Kassis 2002). In general, the activity of the mini-*white* reporter gene is dosage dependent. Hence, eye pigmentation normally increases with the number of mini-*white* transgenes in the genome (Figure 1C). Pairing-dependent silencing is position dependent and its strength is variable (compare Figure 1, D–F). Similar silencing effects could also be observed when the two P[*Mcp*, mini-*white*] copies were inserted at different locations in the genome or when they were present on rearranged chromosomes. These genetic interactions suggested that two copies of the *Mcp* element could physically interact, independently of homologous chromosome pairing (Müller *et al*., 1999).

To obtain direct evidence for the physical interaction of *Mcp* elements, we used the GFP-Lac repressor/operator chromosome tagging technique (Robinett *et al*., 1996; Vazquez *et al*., 2001). This live approach has the advantage of minimizing possible artifacts due to fixation and hybridization procedures. Furthermore, it offers high spatial resolution, because a cluster of GFP-lac repressor molecules bound to integrated lacO arrays occurs as a diffraction-limited spot in *Drosophila* nuclei (Vazquez *et al*., 2001). The P-element construct psOMws′ carrying the *Mcp* element, the mini-*white* gene, the *white* enhancer, and a ~2.5-kb array of *lac* operator (lacO) sequences, was generated and used to transform a *white* mutant *Drosophila* strain (Figure 1A). Out of 10 lines recovered, eight showed

Table 2. Interactions between psOMws' transgenes

Insert 1	Insert 2						
	OM1	OM2	OM3	OM4	OM5	OM6	OM7
OM1	L (v)						
OM2	D (v)	L (v)					
OM3	D (u)	L (v)	L (v)				
OM4	D (u)	D (v)	D (v)	L (v)			
OM5	D (v)	D (v)	D (v)	L (v)	L (v)		
OM6	D (v)	D (v)	D (v)	L (v)	L (v)	L (v)	
OM7	D (v)	D (v)	D (v)	I (v)	L (v)	L (v)	L (v)

This table indicates the silencing interactions observed in trans-combinations between seven OM transgenes. The data for the remaining three transgenes are not indicated because no silencing interactions were observed. The eye color of insert 1/insert 2 progeny from such a pairwise cross was compared to the eye colors of insert 1/+ and insert 2/+ control flies. The results are represented as follows. D, insert 1/insert 2 flies have darker eye color than both controls. I, insert 1/insert 2 flies have eye color very similar to both controls. L, insert 1/insert 2 flies have lighter eye color than both controls. u, eye pigmentation seems uniform; v, eye pigmentation seems variegated. In experiments that are scored as D(v), the variegation suggests that there probably is a weak degree of pairing dependent silencing of the mini-*white* gene. Very similar results were obtained in reciprocal crosses.

pairing-sensitive expression of mini-*white*, as demonstrated by the reduced and often variegated eye color of flies homozygous for the insert (Figure 1, D–F, and Table 1). Construct psOMws', therefore, behaved in a manner similar to that observed for a previously tested construct lacking the lacO array (construct w#15 in Müller *et al*., 1999). This result indicates that the *lacO* sequences did not interfere with the ability of *Mcp* to induce pairing-sensitivity silencing of *white*. Lines carrying a similar construct lacking the *Mcp* sequences did not show pairing sensitivity (Müller *et al*., 1999; Müller, Hogga and Pirrotta, unpublished data; and Vazquez, data unpublished), which indicates that Mcp is required for the pairing dependent reduction of mini-*white* gene expression levels in transgenic flies.

In a second step, the long-distance interaction potential between OM transgenes located on the X, the second or the third chromosome was assessed by 21 pairwise crosses (Table 2). Typically, transheterozygous combinations on the same chromosome showed a significant reduction in eye pigmentation levels and variable degrees of variegation, indicating pairing-dependent silencing of *mini-white*. The strength of the genetic interaction can be estimated by the difference in eye pigmentation between transheterozygous and heterozygous control flies. Silencing seems to decrease as the distance between inserts increases (compare Figure 1, G–K), with the lowest degree of silencing achieved between inserts located on different chromosomes. In the latter case, even though expression of *white* seemed to be largely dosage dependent, a weak degree of variegation in a number of lines still alluded to the possibility of a small level of long-distance interaction between the transgenes involved (Figure 1 and Table 2).

To visualize the inserts in live *Drosophila*, we used a heat shock-inducible system to express GFP-Lac repressor protein in the eye imaginal disk and other *Drosophila* tissues (see *Materials and Methods*). *lacO*-bound GFP-Lac repressor protein was detected by fluorescence deconvolution microscopy. Flies with a single P [*Mcp*, mini-*white*] insert in heterozygous condition showed a single GFP spot in ≥98% of the nuclei (Table 3). In rare cases, two spots could be observed. Such cases probably represented cells undergoing chromosome replication or segregation. When the same P-element insert was present in two copies (homozygous condition), the same frequency of nuclei with single spots was observed. This result indicates that allelic copies of the P [*Mcp*, mini-*white*] insert are associated. This association does not require the presence of *Mcp*, because constructs containing only the *lacO* array and *white* showed similar levels of association (Table 3), and it is due to the normal pairing of homologous chromosomes in somatic nuclei. When the two copies were not allelic, but rather were present at two different locations on the same chromosome, two types of nuclei were observed. Nuclei with a single GFP spot were most abundant (>90%), indicating widespread physical association of the two elements (Figure 2, D–G, and Table 3). A small fraction of nuclei (<10%) showed two distinct GFP spots of approximately half the fluorescence intensity (Figure 3) and represented nuclei in which the remote P-elements were not associated. The association of remote elements was dependent on the presence of *Mcp*, because

Table 3. Pairing frequencies between psOMws' inserts

	Insert	Paired	Unpaired	Total	Paired (%)	Comments
1	M2.1 M6.1	9	237	246	4	No Mcp
2	M6.1/M6.1	89	1	90	99	No Mcp, homozygous
3	OM4/OM4	124	4	128	97	Homozygous
4	OM4 OM6	96	3	99	97	Same chromosome
5	OM6 OM7	82	4	86	95	Same chromosome
6	OM5 OM7	183	11	194	94	Same chromosome
7	OM4 OM7	149	6	155	96	Same chromosome
8	OM3; OM7	157	6	163	96	Different chromosome
9	OM6 OM7/OM6 OM4	104	7	111	94	Same chromosome
10	OM4 OM7 1A/1A	91	8	99	92	Grappa background
11	OM4 OM6	1	65	66	2	Polytene nuclei
12	OM4 OM6	3	143	146	2	Spermatocyte nuclei

The pairing frequencies for different combinations of P element inserts was determined. Detailed genotypes are as follows: 1, M2.1 M6.1/+ +. 2, M6.1/M6.1. 3, OM4/OM4. 4, OM4 OM6/+ +. 5, OM6 OM7/+ +. 6, OM5 OM7/+ +. 7, OM4 OM7/+ +. 8, OM3/+; OM7/+. 9, OM4 OM6 +/+ OM6 OM7. 10, OM4 gpp^{1A} OM7/+ gpp^{1A} + 0. 11, OM4 OM6/+ + (in polytene nuclei). 12, OM4 OM6/+ + (in early spermatocytes). Control insertions M2.1 and M6.1 have been described (Vazquez *et al*., 2001).

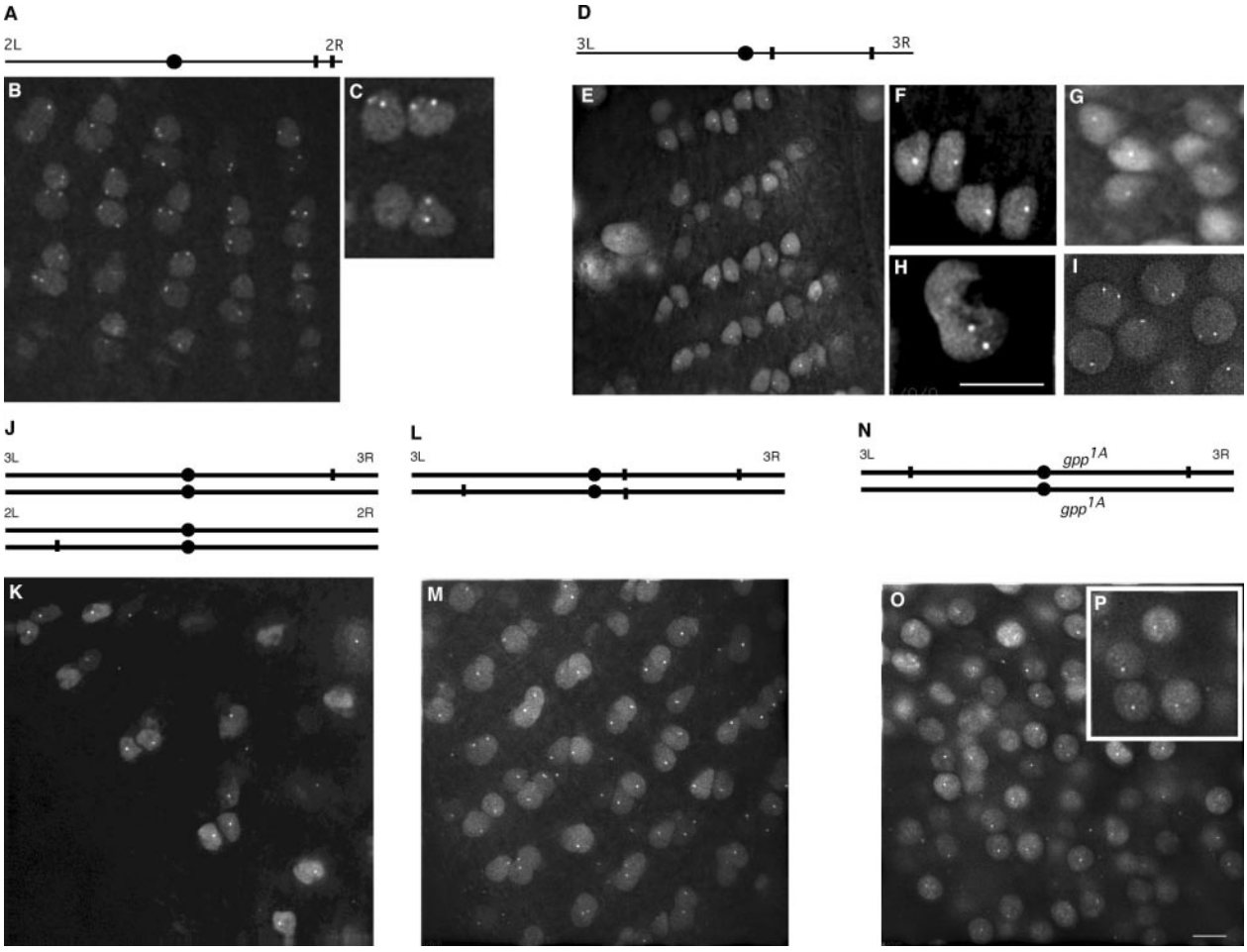

Figure 2. Long-distance interaction of Mcp elements in live eye disk nuclei. P-elements were detected in live, intact third instar imaginal eye discs, except where indicated otherwise. Diagrams show the approximate location of the inserts. (A–C) Control line with two inserts lacking Mcp (M2.1, M6.1/+ +). (D–G) Two different inserts containing Mcp on the same chromosome (OM6, OM7/+ +). (H) Polytene nucleus from the same animal shows the presence of two unpaired inserts. (I) Early spermatocyte nuclei showing unpaired loci (only a fraction of nuclei have both spots in focus). (J and K) Line with one insert on chromosome 2 and one insert on chromosome 3 (OM3/+; OM7/+). (L and M) Four different inserts at three different loci on chromosome 3 (OM4 OM6 +/+ OM6 OM7). (N–P) Two inserts on chromosome 3 (OM4 OM7/+ +) in a gpp^{1A} homozygous background. The inset shows a higher magnification view. Bars, 10 μm (H); 5 μm (O).

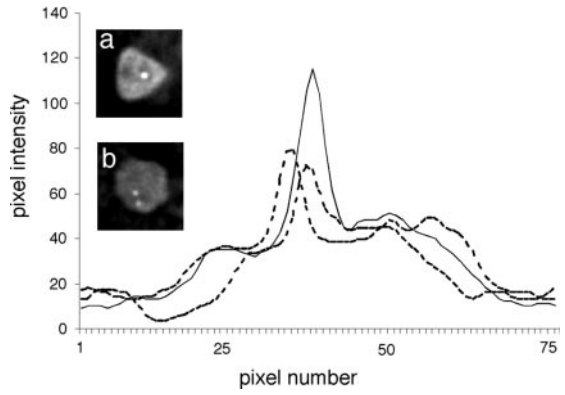

Figure 3. Intensity profile of paired versus unpaired spots. The insets show two representative nuclei from a single field, showing paired (a) and unpaired (b) GFP-Lac repressor spots. The graph shows an intensity profile through the paired (solid line) and unpaired spots (dashed lines). The transgenic line illustrated here has two inserts at locations 82–84 and 94–96, on chromosome 3.

virtually all nuclei from eye imaginal discs of control flies carrying similar P-element insertions that lacked *Mcp* sequences had two spots and hence no significant degree of association (Figure 2, A–C). Various combinations of same chromosome inserts were tested and showed similar levels of pairing (Table 3). These results provide direct evidence for an efficient physical interaction between remote chromosomal sequences, mediated by the *Mcp* element.

Previous studies have demonstrated a strong preference for intrachromosomal interactions between P[*Mcp*, mini-*white*] constructs in genetic tests (Table 2; Müller *et al*., 1999). Such preference might reflect an inability of nonhomologous chromosomes to interact, perhaps due to their confinement to distinct chromosome territories. To test this idea, we examined one line carrying one insertion of the psOMws′ element on the second chromosome (OM3) and a second insertion of the same element on the third chromosome (OM7; Figure 2, J and K). Surprisingly, we found a high degree of physical association comparable with that observed for inserts located on the same chromosome, even

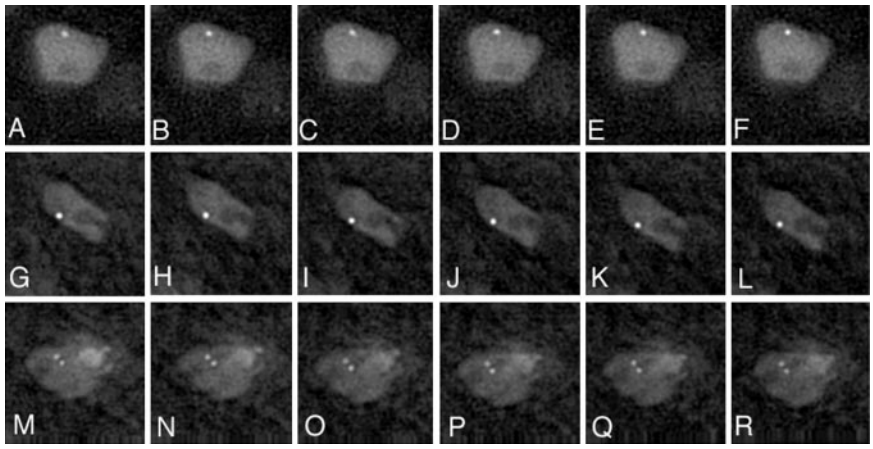

Figure 4. Mcp interactions are stable. Time-lapse shows a single nucleus with inserts OM5 OM7/+ +. (A–F) Images of a single focal plane were taken at 1-s intervals. Manual focusing was used to help track the spots. (G–L) Single two-dimensional (2-D) optical sections extracted from 3-D stacks taken at 1-min intervals. (M–R) Nucleus with unpaired inserts. Images are single 2-D optical sections extracted from 3-D stacks taken at 1-min intervals. See supplemental material for additional time-lapse movies.

though this combination of inserts showed little silencing of mini-*white* in the adult eye (Figure 1K; Tables 2 and 3). These results are consistent with early studies that showed that translocated copies of the bithorax complex with little or no ability to genetically interact still paired with high frequency in *Drosophila* embryos (Gemkow *et al*., 1998).

We next addressed whether the interaction was limited to two P-elements or whether it could involve more than two chromosomal regions. By crossing lines containing recombinant chromosomes, larvae containing four psOMws′ inserts (OM4, OM6 +/+OM6, and OM7) were obtained. Hence, two of the inserts were in heterozygous (OM4 and OM7) and one (OM6) was in homozygous condition. Inspection of the eye color of adult flies revealed that the four transgenes gave rise to eye pigmentation very similar to heterozygous OM7/+ control flies (Figure 1L). This observation is in agreement with a clear deviation from dosage dependence and suggests that the 4 P-elements are interacting. In fact, the analysis of eye imaginal tissue from such larvae revealed a single fluorescence spot in the majority of nuclei, indicating that all four inserts were physically associated, thereby bringing three distinct chromosomal loci into close contact (Figure 2, L–M, and Table 3).

To address the dynamics and stability of *Mcp* interaction, live eye disk nuclei carrying P[*Mcp*, mini-*white*] insertions tagged with the *lacO* array were tracked by time-lapse fluorescence microscopy. The difficulty of imaging live imaginal discs, combined with the need for high-resolution observation of relatively faint signals, imposes severe constraints on the length of time during which the tissues can be reliably observed. When tracked over periods of up to 10 min at frame rates of one image per second, paired loci were never seen to dissociate, despite substantial Brownian motion (Figure 4, A–L, and supplemental movies of the corresponding data sets). Similarly, de novo associations between the rare unpaired loci were never observed during similar time-intervals (Figure 4, M–R). Attempts at tracking the behavior of paired loci over extended periods (1–2 h) also failed to reveal any separation of the loci (our unpublished data). These results suggest that the interactions, once established, are stable for periods of minutes, and possibly hours. Similarly, the rare unpaired loci do not seem to be able to associate in nuclei of third instar larval eye discs.

The isolation of mutants that suppress *Mcp*-dependent silencing of *mini-white* could potentially uncover chromosomal proteins that play a role in chromosome–chromosome interaction. One such mutation, *grappa* (*gpp*), has been described in detail previously (Shanower *et al*., 2005). *gpp* encodes the *Drosophila* homologue of the yeast Dot1p, a Histone H3 methyltransferase that modulates chromatin structure and gene silencing in yeast (Singer *et al*., 1998; San-Segundo and Roeder 2000; Lacoste *et al*., 2002; van Leeuwen *et al*., 2002; van Leeuwen and Gottschling 2002; Ng *et al*., 2003). In *Drosophila*, the dominant *grappa* allele gpp^{1A} is homozygous viable. When tested on various double recombinant P[*Mcp*, *mini-white*] chromosomes, long-distance *Mcp*-mediated *mini-white* silencing is often (but not always) suppressed in heterozygous gpp^{1A} flies. If occurring, suppression is always enhanced in a homozygous gpp^{1A} background (Shanower *et al*., 2005; Figure 1, M and N). Therefore, we wanted to examine the colocalization of inserts OM4 and OM7 in gpp^{1A}/gpp^{1A} flies. As shown in Figure 2, N–P, and Table 3, *Mcp* elements were paired in >90% of the nuclei, a frequency similar to that observed in flies wild-type for *grappa*. Although limited, these results suggest that gpp^{1A} does not prevent the establishment or maintenance of chromosome–chromosome interactions.

DISCUSSION

A variety of genetic phenomena are thought to rely on the physical interaction or communication between distant chromosomal elements. Examples include modulation of promoter activity by remote regulatory elements, homology search during DNA recombination and repair, and pairing-dependent phenomena such as transvection. For example, it has been proposed that developmentally regulated transcription at the human betaglobin locus relies on dynamic, short-lived interactions between promoter elements at the globin locus and a distant locus control region (Wijgerde *et al*., 1995, 1996). More recently, long-range associations, both intra- and interchromosomal, were demonstrated in human T-helper cells (Spilianakis and Flavell, 2004; Spilianakis *et al*., 2005).

This study identifies a short chromosomal region from the bithorax complex, *Mcp*, that is able to interact with other copies of the same element present at remote locations in the genome. After the direct demonstration of pairing of the *Fab-7* PRE (Bantignies *et al*., 2003), this is the second example of a discrete chromosomal region able to mediate sequence-specific, long-range chromosomal interactions in the *Drosophila* nucleus.

The frequency of association of our *Mpc* construct in the eye disk was very high; it was observed in ≥90% of nuclei. The frequency of association was substantially higher than that observed by in situ hybridization for *Fab-7*. It could be

argued that the conditions used for in situ hybridization might disrupt potentially fragile interactions. Limited experiments in our laboratory, however, showed that the fixation procedures generally used for in situ hybridization did not significantly affect the frequency of paired sites in the eye disk, compared with the in vivo method (our unpublished data). The differences, therefore, may reflect variable strengths of different pairing-sensitive elements, or stage or tissue-specific effects. Indeed, although we observed pairing of the psOMws′ element in other larval tissues such as brain and wing discs, the frequency of pairing in such tissues was often much lower (20–60% of that observed in the eye disk; our unpublished data). Other tissues, such as polytene nuclei, showed virtually no pairing (Figure 2). One possible explanation is that tissue-specific factors present in the eye disk might contribute to the pairing. Because the *white* gene present on our constructs is expressed in the eye, it is possible that *white* sequences might act in conjunction with *Mcp* to increase the level of association of the constructs in the eye disk. It is also possible that the embryonic stages analyzed in the *Fab-7* studies may represent the early stages in the establishment of this type of long-range interactions (Bantignies *et al*., 2003). In agreement with the work of these authors, however, we found no evidence of pairing of the *Mcp* element in the male or female germ line (Figure 2I; our unpublished data).

The eye color assay for long-distance interactions showed that insertions located on the same chromosome are much more likely to show genetic interaction (as evidenced by the stronger silencing of *white*). Our assay, however, revealed similar (and high) levels of association between sequences located on different chromosomes. These results are consistent with previous studies, where a substantial amount of residual pairing between alleles of the bithorax complex was still observed for translocations that abolished transvection (Gemkow *et al*., 1998). Our assay also revealed that the long-range association may involve at least up to four elements located at three different chromosomal loci. Although not tested in this study, such interactions are also likely to involve the endogenous *Mcp* elements (Bantignies *et al*., 2003). This raises the possibility that *Mcp* and similar elements may be involved in the formation of higher order chromatin complexes comprising multiple genes or regulatory regions.

Previous studies have identified a mutation in *grappa*, gpp^{1A}, that substantially suppresses the pairing-dependent silencing of *white* mediated by *Mcp* (Müller *et al*., 1999). *grappa* encodes the *Drosophila* homologue of the yeast Histone H3 methyltransferase Dot1p. Our results clearly show that although gpp^{1A} drastically reduces the level of pairing-dependent silencing mediated by *Mcp*, it has little or no effect on the observed pairing of *Mcp* elements in the eye imaginal disk. This suggests that pairing may be an initial necessary step in the regulatory process mediated by *Mcp* and that *grappa* acts subsequently to induce chromatin changes required for silencing. In the absence of additional data, however, other possibilities cannot be excluded. For example, the timing of pairing could be critical to allow developmentally regulated factors to associate to, and repress transcription around the *Mcp* element. In such a model, gpp^{1A} could be delaying the onset of pairing, resulting in reduced levels of silencing. Additional studies will be necessary to establish the series of events that lead to pairing-dependent silencing of *Mcp*-associated genes.

The use of a live system has also allowed us, for the first time, to also address the dynamics of long-distance chromosome–chromosome interaction. Once established, the interactions seem to be stable, because we saw no evidence of separation of initially paired loci. Due to the finite resolution of the light microscope, this does not exclude local transient separation of short DNA regions. However, given that chromatin is naturally subject to diffusive motion, a complete separation of the paired regions, even for a brief moment, would be expected to lead to a drifting away of the tagged regions and the appearance of two separate GFP spots (Vazquez *et al*., 2001). Rare, unpaired loci were also never seen to associate. The presence of a small fraction of nuclei with unpaired loci at any given time therefore does not seem to be the result of an equilibrium state between a population of rapidly associating and dissociating loci. Therefore, *Mcp* elements, possibly by the action of specific chromosome-associated proteins, are able to lock remote chromosomal regions in the paired state for extended periods, even in the presence of substantial chromatin movement. The stable contacts we describe are in contrast with the short-lived dynamic interactions that have been postulated to occur between remote regulatory elements, such as between the human betaglobin LCR and promoter regions (Wijgerde *et al*., 1995). Our studies suggest that the rate-limiting step in the pairing process could be the establishment of the initial contact between remote *Mcp* elements early during development and possibly renewed early at the beginning of each new cell cycle. This situation is reminiscent of the rapid and stable pairing of homologous chromosomes observed in somatic cells (Fung *et al*., 1998) and of meiotic pairing in *Drosophila* spermatocytes (Vazquez *et al*., 2002). This interpretation is consistent with the hypothesis that interactions between Polycomb-group response elements might be involved in the transmission of chromatin states during *Drosophila* development (Bantignies *et al*., 2003).

We have presented a live system for the direct analysis of long-distance chromosome interactions in *Drosophila*. This system allowed us to identify a discrete DNA sequence from the bithorax complex, *Mcp*, that is able to promote stable physical interactions between distant chromosomal regions. The presence of pairing elements at the bithorax complex had long been suspected, due to the susceptibility of this locus to transvection effects. Although the pairing properties of *Mcp* (and *Fab-7*) were originally inferred from the ability of this element to silence a linked *white* gene in a pairing-dependent manner, it is not clear at the moment what function pairing serves in the context of the bithorax complex. It has been proposed that association between these elements might play a role in the transmission of regulatory chromatin states (Bantignies *et al*., 2003). It is also possible that pairing elements might play a role in bringing together remote regulatory regions or stabilize regulatory interactions within the complex. The ability to track such associations both in live and fixed tissues should help clarify the relationship between chromosome organization and gene regulation.

ACKNOWLEDGMENTS

We thank Markus Affolter for the heat shock-inducible hsp70::GAL4 fly line. Pam Geyer, R. Scott Hawley, Ivan Dellino, Henrik Gyurkovics, François Karch, Daniel Pauli, Christian Sigrist, and members of the Sedat laboratory contributed valuable discussions, suggestions, and critical comments on the manuscript. This work was funded by National Institutes of Health Grant GM-58460 (to J.W.S.) and by a grant from the Swiss National Science Foundation (to V. P.).

REFERENCES

Bantignies, F., Grimaud, C., Lavrov, S., Gabut, M., and Cavalli, G. (2003). Inheritance of Polycomb-dependent chromosomal interactions in *Drosophila*. Genes Dev. *17*, 2406–2420.

Brand, A. H., and Perrimon, N. (1993). Targeted gene expression as a means of altering cell fates and generating dominant phenotypes. Development *118*, 401–415.

Busturia, A., and Bienz, M. (1993). Silencers in Abdominal-B, a homeotic *Drosophila* gene. EMBO J. *12*, 1415–1425.

Busturia, A., Wightman, C. D., and Sakonju, S. (1997). A silencer is required for maintenance of transcriptional repression during *Drosophila* development. Development *124*, 4343–4350.

Casal, J., Gonzalez, C., and Ripoll, P. (1990). Spindles and centrosomes during male meiosis in *Drosophila melanogaster*. Eur. J. Cell Biol. *51*, 38–44.

Crosby, M. A., Lundquist, E. A., Tautvydas, R. M., and Johnson, J. J. (1993). The 3′ regulatory region of the Abdominal-B gene: genetic analysis supports a model of reiterated and interchangeable regulatory elements. Genetics *134*, 809–824.

Duncan, I. W. (2002). Transvection effects in *Drosophila*. Annu. Rev. Genet. *36*, 521–556.

Fung, J. C., Marshall, W. F., Dernburg, A., Agard, D. A., and Sedat, J. W. (1998). Homologous chromosome pairing in *Drosophila melanogaster* proceeds through multiple independent initiations. J. Cell Biol. *141*, 5–20.

Gemkow, M. J., Verveer, P. J., and Arndt-Jovin, D. J. (1998). Homologous association of the bithorax-complex during embryogenesis: consequences for transvection in *Drosophila melanogaster*. Development *125*, 4541–4552.

Goldsborough, A. S., and Kornberg, T. B. (1996). Reduction of transcription by homologue asynapsis in *Drosophila* imaginal discs. Nature *381*, 807–810.

Gyurkovics, H., Gausz, J., Kummer, J., and Karch, F. (1990). A new homeotic mutation in the *Drosophila* bithorax complex removes a boundary separating two domains of regulation. EMBO J. *9*, 2579–2585.

Hopmann, R., Duncan, D., and Duncan, I. (1995). Transvection in the iab-5,6,7 region of the bithorax complex of *Drosophila*: homology independent interactions in trans. Genetics *139*, 815–833.

Karch, F., Galloni, M., Sipos, L., Gausz, J., Gyurkovics, H., and Schedl, P. (1994). Mcp and Fab-7, Molecular analysis of putative boundaries of cis-regulatory domains n the bithorax complex of *Drosophila melanogaster*. Nucleic Acids Res. *22*, 3138–3146.

Karch, F., Weiffenbach, B., Peifer, M., Bender, W., Duncan, I., Celniker, S., Crosby, M., and Lewis, E. B. (1985). The abdominal region of the bithorax complex. Cell *43*, 81–96.

Kassis, J. A. (2002). Pairing-sensitive silencing, polycomb group response elements, and transposon homing in *Drosophila*. Adv. Genet. *46*, 421–438.

Lacoste, N., Utley, R. T., Hunter, J. M., Poirier, G. G., and Cote, J. (2002). Disruptor of telomeric silencing-1 is a chromatin-specific histone H3 methyltransferase. J. Biol. Chem. *277*, 30421–30424.

Lewis, E. B. (1954). The theory and application of a new method of detecting chromosomal rearrangements in *Drosophila melanogaster*. Am. Nat. *88*, 225–239.

Lewis, E. B. (1978). A gene complex controlling segmentation in *Drosophila*. Nature *276*, 565–570.

Mihaly, J., *et al*. (1998). Chromatin domain boundaries in the Bithorax complex. Cell Mol. Life Sci. *54*, 60–70.

Müller, M., Hagstrom, K., Gyurkovics, H., Pirrotta, V., and Schedl, P. (1999). The Mcp element from the *Drosophila melanogaster* bithorax complex mediates long-distance regulatory interactions. Genetics *153*, 1333–1356.

Ng, H. H., Ciccone, D. N., Morshead, K. B., Oettinger, M. A., and Struhl, K. (2003). Lysine-79 of histone H3 is hypomethylated at silenced loci in yeast and mammalian cells: a potential mechanism for position-effect variegation. Proc. Natl. Acad. Sci. USA *100*, 1820–1825.

Pirrotta, V. (1999). Transvection and chromosomal *trans*-interaction effects. Biochim. Biophys. Acta *1424*, M1–M8.

Qian, S., and Pirrotta, V. (1995). Dosage compensation of the *Drosophila white* gene requires both the X-chromosome environment and multiple intragenic elements. Genetics *139*, 733–744.

Robertson, H. M., Preston, C. R., Phillis, R. W., Johnsonschlitz, D. M., Benz, W. K., and Engels, W. R. (1998). A stable genomic source of P-element transposase in *Drosophila melanogaster*. Genetics *118*, 461–470.

Robinett, C. C., Straight, A., Willhelm, C., Sudlow, G., Murray, A. W., and Belmont, A. S. (1996). In vivo localization of DNA sequences and visualization of large-scale chromatin organization using lac operator/repressor recognition. J. Cell Biol. *135*, 1685–1700.

Rubin, G. M., and Spradling, A. C. (1982). Genetic transformation of *Drosophila* with transposable element vectors. Science *218*, 348–353.

San-Segundo, P. A., and Roeder, G. S. (2000). Role for the silencing protein Dot1 in meiotic checkpoint control. Mol. Biol. Cell. *11*, 3601–3615.

Shanower, G. A., Müller, M., Blanton, J. L., Honti, V., Gyurkovics, H., and Schedl, P. (2005). Characterization of the grappa gene, the *Drosophila* histone H3 lysine 79 methyltransferase. Genetics *169*, 173–184

Sigrist, C. J., and Pirrotta, V. (1997). Chromatin insulator elements block the silencing of a target gene by the *Drosophila* polycomb response element (PRE), but allow trans interactions between PREs on different chromosomes. Genetics *147*, 209–221.

Singer, M. S., Kahana, A., Wolf, A. J., Meisinger, L. L., Peterson, S. E., Goggin, C., Mahowald, M., and Gottschling, D. E. (1998). Identification of high-copy disrupters of telomeric silencing in *Saccharomyces cerevisiae*. Genetics *150*, 613–632.

Spilianakis, C. G., and Flavell, R. A. (2004). Long-range intrachromosomal interactions in the T helper type 2 cytokine locus. Nat. Immunol. *5*, 1017–1027.

Spilianakis, C. G., Lalioti, M. D., Town, T., Lee, G. R., and Flavell, R. A. (2005). Interchromosomal associations between alternatively expressed loci. Nature *435*, 637–645.

Straight, A. F., Belmont, A. S., Robinett, C. C., and Murray, A. W. (1996). GFP tagging of yeast chromosomes reveals that protein protein interactions can mediate sister chromatid cohesion. Curr. Biol. *12*, 1599–1608.

van Leeuwen, F., Gafken, P. R., and Gottschling, D. E. (2002). Dot1p modulates silencing in yeast by methylation of the nucleosome core. Cell *109*, 745–756.

van Leeuwen, F., and Gottschling, D. E. (2002). Genome-wide histone modifications: gaining specificity by preventing promiscuity. Curr. Opin. Cell Biol. *14*, 756–762.

Vazquez, J., Belmont, A. S., and Sedat, J. W. (2001). Multiple regimes of constrained chromosome motion are regulated in the interphase *Drosophila* nucleus. Curr. Biol. *11*, 1227–1239.

Vazquez, J., Belmont, A. S., and Sedat, J. W. (2002). The dynamics of homologous chromosome pairing during male *Drosophila* meiosis. Curr. Biol. *12*, 1473–1483.

Vazquez, J., *et al*. (1993). Genetic and molecular analysis of chromatin domains. Cold Spring Harb. Symp. Quant. Biol. *58*, 45–54.

Wijgerde, M., Gribnau, J., Trimborn, T., Nuez, B., Philipsen, S., Grosveld, F., and Fraser, P. (1996). The role of EKLF in human beta-globin gene competition. Genes Dev. *10*, 2894–2902.

Wijgerde, M., Grosveld, F., and Fraser, P. (1995). Transcription complex stability and chromatin dynamics in vivo. Nature *377*, 209–213.