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Homologous association of the Bithorax-Complex during embryogenesis: consequences for transvection in *Drosophila melanogaster*

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SUMMARY

Transvection is the phenomenon by which the expression of a gene can be controlled by its homologous counterpart in *trans*, presumably due to pairing of alleles in diploid interphase cells. Transvection or *trans*-sensing phenomena have been reported for several loci in *Drosophila*, the most thoroughly studied of which is the Bithorax-Complex (BX-C). It is not known how early *trans*-sensing occurs nor the extent or duration of the underlying physical interactions. We have investigated the physical proximity of homologous genes of the BX-C during *Drosophila melanogaster* embryogenesis by applying fluorescent in situ hybridization techniques together with high-resolution confocal light microscopy and digital image processing. The association of homologous alleles of the BX-C starts in nuclear division cycle 13, reaches a plateau of 70% in postgastrulating embryos, and is not perturbed by the transcriptional state of the genes throughout embryogenesis. Pairing frequencies never reach 100%, indicating that the homologous associations are in equilibrium with a dissociated state. We determined the effects of translocations and a *zeste* protein null mutation, both of which strongly diminish transvection phenotypes, on the extent of diploid homologue pairing. Although translocating one allele of the BX-C from the right arm of chromosome 3 to the left arm of chromosome 3 or to the X chromosome abolished *trans*-regulation of the *Ultrabithorax* gene, pairing of homologous alleles surprisingly was reduced only to 20-30%. A *zeste* protein null mutation neither delayed the onset of pairing nor led to unpairing of the homologous alleles. These data are discussed in the light of different models for *trans*-regulation. We examined the onset of pairing of the chromosome 4 as well as of loci near the centromere of chromosome 3 and near the telomere of 3R in order to test models for the mechanism of homologue pairing.

Key words: Ultrabithorax, abdominal-A, Abdominal-B, *Trans*-sensing, Chromosome painting, *Homologous* pairing, CLSM, FISH

INTRODUCTION

Pairing of homologous chromosomes in Diptera like *Drosophila* has been inferred from physical observations made in the early part of this century on the close apposition of homologues in prophase, metaphase and anaphase (Metz, 1916) as well as from a large body of genetic data. The genetic experiments showed that homologous chromosomes can influence each other, phenomena that are collectively called *trans*-sensing effects [reviewed in Henikoff, 1997]. The most intensively studied effect is transvection, the control of genes by regulatory sequences in *trans*, first described by Lewis (Lewis, 1954) and investigated in detail for the Bithorax Complex (BX-C) (Babu et al., 1987; Castelli-Gair et al., 1990; Martinez-Laborda et al., 1992; Mathog, 1990; Micol and García-Bellido, 1988). These papers postulate two models of how transvection might occur: (a) direct interaction of the homologous chromosome elements, or (b) transmission of a soluble macromolecule such as a short-lived RNA transcript from one homologue to the other. Common to both models is the assumption that transvection depends on the physical proximity of the two homologous alleles. Cytological examination of interphase cells, other than polytene salivary glands, has not been undertaken to investigate *trans*-sensing and has only been performed on the heterochromatic $brown^{Dominant}$ (bw^{D}) mutation (Csink and Henikoff, 1996; Dernburg et al., 1996) and the multicopy histone locus (Hiraoka et al., 1993), although very recently a number of loci outside the histone locus on chromosome 2 have also been examined (Fung et al., 1998).

Only a limited number of genes described so far, such as *eyes absent*, *brown*, *cubitus interruptus*, *white*, *light*, *compensatory response*, *decapentaplegic*, *yellow* and *Sex combs reduced*, show transvection, and a large number of mutants that carry heterozygous chromosome rearrangements appear normal (Leiserson et al., 1994; reviewed in Henikoff, 1997; Tartof and Henikoff, 1991). These observations suggest that *trans*-sensing phenomena may be the exception rather than the rule for most genetic loci.

Transvection is scored in genetic studies by investigating alterations of phenotypes resulting from gene expression in translocation mutants and heterozygotic deletions. Although

such experiments have been used to support the homologous pairing model, there has been no direct demonstration that the rearrangements disrupt pairing. In addition, rearrangements in the BX-C produce conflicting data concerning homologous pairing for the three genes in the complex. Whereas a translocation of one allele of the BX-C to the X chromosome, namely *Tp(3;1)P115*, leads to a complete loss of transvection at the *Ultrabithorax* (*Ubx*) locus (Micol and García-Bellido, 1988), transvection phenotypes at the *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) loci are strongly reduced but not eliminated (Jijakli and Ghysen, 1992). Also, there is no strict rule for the proximity of breakpoints to disrupt transvection at different genes suggesting that this property is intrinsic to the gene (see Gelbart, 1982; Smolik-Utlaut and Gelbart, 1987; Gubb et al., 1990, 1997). There is a clear need for assays of homologous chromosome pairing that do not rely solely on phenotypes of adult flies.

Goldsborough and Kornberg (1996) undertook a molecular analysis of transvection at the *Ubx* locus by measuring transcription from both homologues in wild-type as well as flies carrying a *Contrabithorax* (*Cbx*^{1}) mutation in one chromosome combined with a *zeste*^{a} (*z*^{a}) background or translocations of the wild-type homologue. Their data suggest that disruption of the transvection phenotype can occur by different mechanisms since the *z*^{a} decouples the transcription rates of the alleles whereas translocations reduce transcription from both *Ubx* and *Cbx*^{1}. They conclude that normal homologous pairing generally enhances transcription.

One of the most interesting results Lewis found was that breakpoints proximal to the BX-C were more effective in disrupting transvection, suggesting that pairing of the homologous chromosomes initiates at the centromere and proceeds towards the telomere (Lewis, 1954). The same results were obtained by an indirect physical study of chromosome pairing performed recently by measuring frequencies of site-specific recombination (Golic and Golic, 1996). These authors used the FLP/FRT system under control of a heat-shock promoter to induce recombination in male flies, which normally do not show recombination. Their data again showed that recombination is inhibited in sites distal to a rearrangement breakpoint. A complementary observation is the partial restoration of transvection in flies that carry an inversion heterozygosity and are also mutant for a prolonged cell cycle.

In this study, we investigated the physical proximity of the genes in the BX-C in embryos of *Drosophila melanogaster* by fluorescent in situ hybridization (FISH) techniques together with high-resolution confocal light microscopy (CLSM) and image processing. We measured the extent of diploid homologue pairing of translocations and a *zeste* ^{a} (protein null) mutation, both of which strongly diminish transvection phenotypes. These data were compared with the results from wild-type embryos for which we determined the time of onset, the stability and the effect of transcription on pairing at the BX-C. In our experiments, we define paired alleles as a single resolvable hybridization signal, the minimal distance between two resolvable signals was 400 nm. Finally, we addressed the mechanism of homologue pairing by comparing the results at this locus with those for probes near the centromere and telomere of chromosome 3 as well as whole chromosome painting of chromosome 4.

MATERIALS AND METHODS

Fly stocks and egg collection

The following fly stocks were used: *D. melanogaster* wild-type strain Oregon R-P2 (Allis et al., 1977), *zeste* alleles: *y*^{1} *z*^{a} and *z*$^{a6\,94}$ *ct*^{6}, and transpositions: *Tp(3;3)P47, Ubx*$^{bx\text{-}34e}$*/TM1, Me*^{1} *ri*^{1} *Sb*$^{sbd\text{-}1}$ and *Tp(3;1)P115, e*^{11}*/TM1, Me*^{1} *ri*^{1} *Sb*$^{sbd\text{-}1}$. All of these stocks are described in Lindsley and Zimm (1992) and additional information can be found in FlyBase (gopher://www.ebi.ac.uk). As marker, we used *UAS·Ubx 62.1* (Castelli-Gair et al., 1994) combined with a *Gal4* line (*Mz798hII:gal4*) from the laboratory collection of G. Technau that expresses in the salivary glands after germband retraction and is inserted into the second chromosome (M. A. González-Gaitán, personal communication).

Larvae were raised in plastic bottles on a medium of cornmeal, agar, soy bean meal, malt extract, molasses, yeast, 0.5% (v/v) propionic acid and the mold inhibitor methyl p-hydroxybenzoate (Nipagin^{R}, Caesar and Lorentz, Hilden, FRG). Flies were held in bottles at 18, 22 or 25˚C. Eggs were collected on apple-juice agar plates containing fresh yeast.

Crosses

For details of the crosses see Fig. 1.

Fixation of embryos

Embryos were dechorionated in a 3% sodium hypochlorite solution for 3 minutes and fixed by the phase partition method (Zalokar and Erk, 1977) by shaking in a mixture of heptane, buffer A (buffer A: 60 mM KCl, 15 mM NaCl, 0.5 mM spermidine, 0.15 mM spermine, 2 mM Na-EDTA, 0.5 mM Na-EGTA, 15 mM Na-PIPES, pH 7.4) and 37% paraformaldehyde (9:0.9:0.1, by volume) for 30 minutes, and devitellinized by vigorous shaking in a 1:1 mixture of heptane and methanol. The embryos were then transferred into PBT (phosphate-buffered saline, 0.1% Tween 20) via a graded solvent series (methanol:buffer 70:30, 50:50, 30:70) and washed extensively in buffer.

Chromosome painting of chromosome 4

Embryos were fixed as described (see above) and digested with RNase A: 100 μg/ml for 3-5 hours at 37°C in PBTX (phosphate-buffered saline+0.02% Triton X-100). They were then stained with a combination of 0.1 μM YOYO-1 and 0.2 μM TOTO-3 (Molecular Probes, Eugene, Oregon) for 30 minutes in PBTX. Embryos were equilibrated and mounted in Mowiol 4-88 (Hoechst) (Heimer and Taylor, 1974). Whereas YOYO-1 stained all chromosomes, TOTO-3 selectively stained the chromosome number 4 (see results).

Antibodies

Anti-digoxigenin polyclonal Fab fragments from sheep were purchased from Boehringer Mannheim and coupled with the fluorescent dye Cy3 (see below). The anti-lamin monoclonal antibody producing cell line T40 (Risau et al., 1981) was purified from hybridoma cell supernatants by chromatography over a protein G Sepharose column (Pharmacia). The supernatant of the anti-Ultrabithorax monoclonal antibody producing cell line FP 3.38 was a kind gift of Dr M. Akam (White and Wilcox, 1985).

Secondary fluorescently labeled antibodies, FITC- and Cy5-conjugated F(ab′)$_2$ fragments of goat anti-mouse IgG (H+L), were purchased (Jackson Labs).

Coupling of fluorescent dyes to primary antibodies

The anti-DIG Fab fragments were labeled as previously described (Buchenau et al., 1993, 1997) with the succinimidyl-ester Cy-3-OSu (Cy3; Amersham). The molar dye:protein ratio was 4-5.

Fluorescence in situ hybridization in whole-mount embryos

The hybridization protocol as described earlier was followed

(Gemkow et al., 1996). Briefly: embryonic DNA was denatured for 15 minutes at 76-80°C in hybridization mixture (50% formamide, 4× SSC, 100 mM sodium phosphate, pH 7.0, 0.1% Tween 20). While the embryos were still at the denaturation temperature, as much buffer as possible was removed and the denatured DNA probe was added. The hybridization was incubated in a thermomixer at 37°C with gentle agitation for 14-17 hours. After stringent washing, the embryos were returned to PBT and stained stimultaneously for lamin protein with monoclonal antibody followed by Cy5-labeled goat anti-mouse $(Fab')_2$, for digoxigenin-labeled DNA with a Cy3-labeled sheep anti-digoxigenin Fab fragment and, when needed, by monoclonal anti-Ultrabithorax followed by FITC-labeled goat anti-mouse. DNA was counterstained with 3 μM 4,6-diamidino-2-phenylindole (DAPI).

DNA probes for the in situ hybridizations

P1 clones from the collection originally described by Hartl et al. (1994) and provided by the EMBL were used in this study. The following clones: DS03126, DS00846 and DS0769 represent parts of the *Ultrabithorax* (*Ubx*), *abdominal-A* (*abd-A*) and *Abdominal-B* (*Abd-B*) genes, respectively. All clones were checked by hybridization to polytene salivary gland chromosomes and by dot blot hybridization to the phage lambda clones for the Bithorax-complex (BX-C) locus mapped by chromosomal walking and described by Bender et al. (1983). In addition to the BX-C clones, the clones DS01440 and DS00464, localizing to 96F9-96F11 and 84B2-84C2, respectively, were used. Probes were labeled with digoxigenin by random priming as previously described (Gemkow et al., 1996).

Confocal microscopy and image processing

Optical sections from whole-mount embryos were recorded with a Zeiss confocal laser scanning microscope (model LSM 310) equipped with a high-precision Z-scanning galvanometric stage and a ×63, NA 1.4 Plan-Apochromat oil immersion, ×63, NA 1.2 C-Apochromat water immersion or ×63, NA 1.25 Plan-Neofluar Anti-flex oil immersion lens. The microscope was equipped with two internal lasers (HeNe, 633 nm; and argon ion, 488 and 514 nm) and three additional external lasers: (argon ion, tunable (364 nm); doubled Ni-YAG, 532 nm; HeNe, 594 nm). The external lasers were coupled into the microscope via fiber optics and collimators aligned to the internal lasers.

The different dyes were excited with the following laser lines: DAPI: 365 nm, fluorescein and YOYO-1: 488 nm, Cy3: 532 nm or 514 nm, Cy5 and TOTO-3: 633 nm. The following emission filters were used: DAPI: LP 418, fluorescein: BP530, YOYO-1: BP 535-580, Cy3: BP 535-580 (when excited at 514 nm), LP575 or LP610 (when excited at 532 nm), Cy5 and TOTO-3: LP665.

Images (8 bit/pixel) were acquired with an appropriate scanning time and frame averaging. Image stacks were limited to 20 μm Z axis depth to avoid problems with refractive index mismatches or quenching of fluorescence intensity (Hell et al., 1993). For doubly stained embryos, the images of the two fluorophore distributions were recorded separately and saved to separate channels of an RGB-image.

Image processing tasks, such as the determination of the distance between loci in image stacks at high resolution, were carried out in SCIL-Image (TNO Institute of Applied Physics, TU Delft, the Netherlands) and DeltaVision (Applied Precision, Mercer Island, WA) software. The SCIL-Image package was extended with routines, written in C language, to threshold the signals, determine their center of gravity and measure the distances between two signals in a nucleus in three dimensions. The number of hybridization signals per nucleus were determined by analyzing the data stacks in Imaris (Bitplane AG,

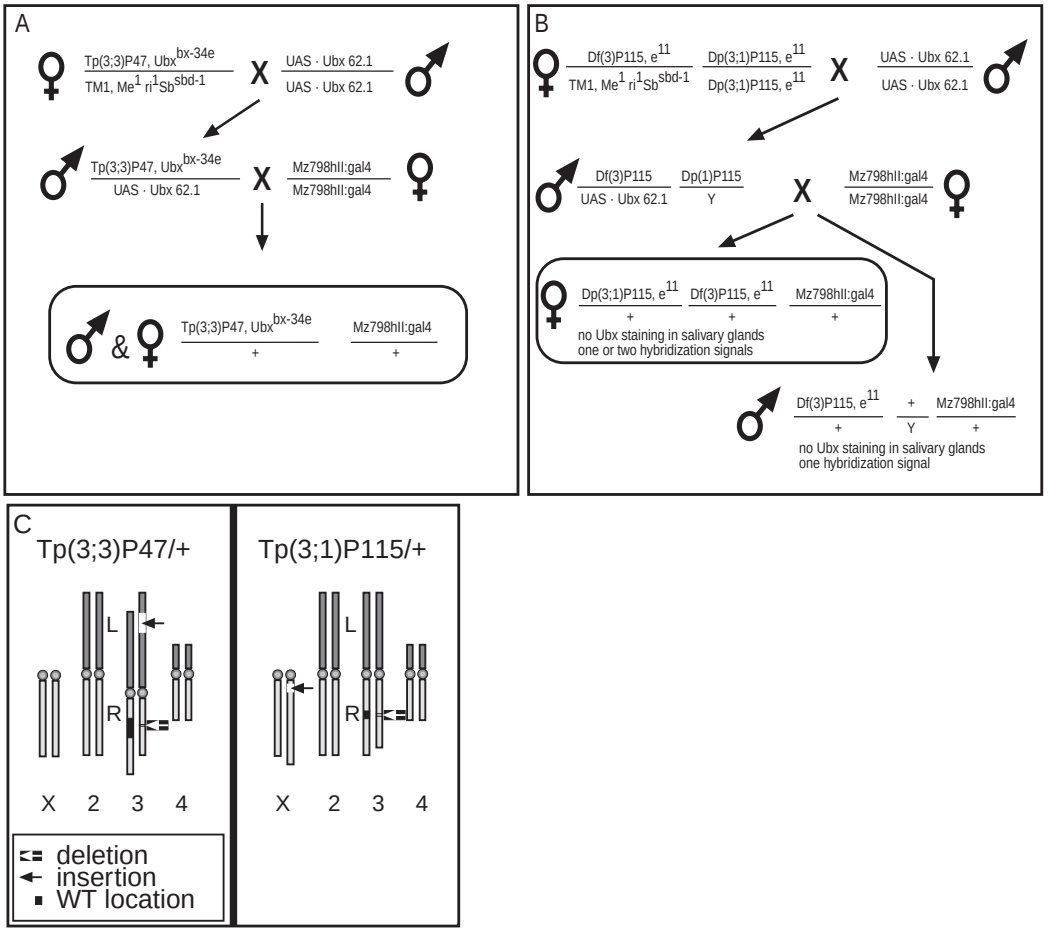

Fig. 1. Crossing strategy for the identification of embryos bearing a translocated copy of the BX-C. Embryos carrying the rearrangement on the third chromosome were identified by the absence of *Ubx* transcription in the salivary glands from the *UAS·Ubx 62.1* driven by *Mz798hII:gal4*. Only relevant genotypes are shown. (A) Translocation of one copy of the BX-C to the left arm of chromosome 3: *Tp(3;3)P47*. (B) Translocation of one copy of the BX-C to the X chromosome: *Tp(3;1)P115*. *Tp(3;1)P115*: is distinguished by the deletion of *Ubx* in the third chromosome (*Df(3)P115*) and its transposition to the X chromosome (*Dp(3;1)P115*). Heterozygotic females carrying the transposed and wild-type copies of the BX-C were identified by the absence of *Ubx* expression in salivary gland tissue and nuclei with both single and double FISH signals. (C) Schematic drawing of the resulting genotypes for female embryos showing the locations of the two copies of the BX-C in the genome.

Switzerland). Deconvolution algorithims (Verveer and Jovin, 1997) were performed in SCIL-Image. Figures were composed from images processed in Imaris for 3D views with Photoshop 4.0 and FreeHand 7.0 (Adobe Systems, Mountain View, CA; Macromedia).

RESULTS

The onset and stability of pairing of the homologous alleles of the BX-C during embryogenesis

Although the physical proximity of homologous alleles is invoked as the causal effect for transvection phenotypes in larvae and adults, the pairing of homologous chromosomes must occur much earlier since the transcriptional patterns of activatable homeotic genes are already determined in the embryo and maintained in the embryo, larvae and adult by the trithorax and Polycomb group genes (Simon, 1995). Thus, an understanding of the physical basis of transvection requires the examination of the disposition of the chromosomal elements in the embryo and the stability of the arrangements. We designate the blastoderm stages by the nuclear division cycles and the embryos after gastrulation according to the scheme introduced by Campos-Ortega and Hartenstein (1985).

We have determined the physical proximity (by means of FISH) of P1 clones to the individual genes in the BX-C in whole-mount embryos stained with antibody to nuclear lamin to delineate the nuclear boundaries. High-resolution CLSM images in multiple wavelengths were recorded and analyzed as described in Materials and Methods. Paired homologous alleles were defined as a single resolvable hybridization signal per nucleus and the pairing frequency as the percentage of the investigated nuclei that showed single hybridization signals. The X-Y resolution of the CLSM under the conditions of our experiments was ~400 nm. That is, hybridization signals at less than this distance apart appear as a single locus and were scored as paired. Deconvolution algorithms (Verveer and Jovin, 1997) seldom resolved paired signals into two individual signals (Gemkow et al., 1996) and thus, data without deconvolution are shown.

We found no pairing of the homologous alleles in the first twelve synchronous nuclear division cycles for any of the probes for the BX-C or the combination of all three. The first paired alleles were detectable in cycle 13 embryos and a pairing frequency of 10-14% was measured. This value increased in cycle 14 to 20-30% (Figs 2, 3), and reached a maximum of 46% by late cycle 14 (stage 5, defined by the appearance of the ventral furrow). Cycle 14 embryos arrest in G_2 as discussed below, and in most cases, the newly synthesized DNA strands remained associated with their respective homologues so that only rarely are 4 hybridization signals observed (see Discussion).

After gastrulation, the pairing frequency of the *Ubx* and *Abd-B* loci increased to 60-70%. We investigated nuclei of the lateral epidermis of stage 13 and stage 16 embryos after germband retraction, which are postmitotic G_1 (Edgar and O'Farrell, 1990). The results show that the pairing of homologous alleles is clearly established before hatching and larval development. The data for the frequency of pairing in wild-type embryos as a function of development are shown as a bar graph in Fig. 3 and in Table 1. Importantly, we never found 100% of the nuclei in a field with paired alleles for the genes of the BX-C. Even in late-stages, after gastrulation, the frequency was never >70% in any embryo.

The influence of transpositions of the *BX-C* on the pairing frequency in postgastrulating embryos

Two different transpositions were investigated, namely

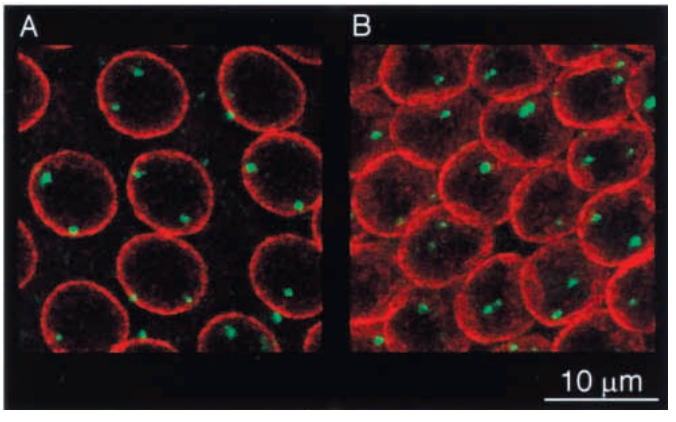

Fig. 2. Typical FISH identification of alleles of the BX-C in a field of nuclei from whole-mount blastoderm embryos presented as projections from 15 optical sections taken at 250 nm intervals. Signals resulting from DIG-labeled probes were detected with a Cy3 directly labeled polyclonal antibody and are shown in green. The nuclear envelope was delineated with the monoclonal antibody T40 shown in red. (A) Cycle 12 embryo hybridized with the P1 clone DS07696 to the *Abd-B* gene. (B) Cycle 14 embryo hybridized with the P1 clone DS03126 to the *Ubx* gene.

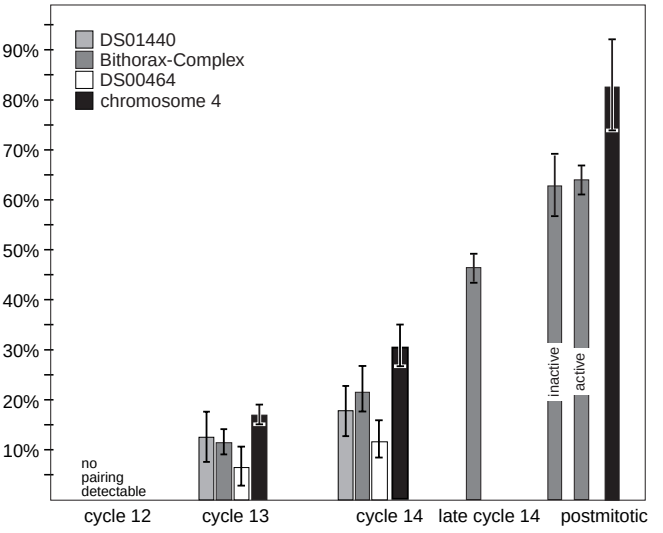

Fig. 3. Frequencies of homologue pairing for different stages of embryonic development. Pairing frequencies for probes in the *BX-C*, probe DS00464, probe DS01440, and chromosome 4 during embryogenesis. The pairing frequency is defined as the percentage of nuclei that contained a single hybridization signal. Shown are the frequencies for the onset in nuclear cycles 13 and 14 (blastoderm), as well as for late cycle 14 for the *BX-C*, and for the extent of pairing in postgastrulating embryos, stages 13 and 16. The values for the *BX-C* in postgastrulating embryos are shown separately for regions where the genes were active or inactive.

Table 1. Pairing frequencies for the BX-C, chromosome 4, the proximal probe DS00464, the distal probe DS01440, and for the BX-C in the translocations and the z^{a694} mutant embryos

Locus/probe	Cycle/stage	No. of nuclei	Pairing frequency/%
BX-C	cycle 12	216	2±2
BX-C	cycle 13	240	11±2
BX-C	cycle 14	469	25±8
BX-C	late cycle 14	129	46±3
BX-C	stage 13 and 16	458	66±4
BX-C	postmitotic/active	259	65±2
BX-C	postmitotic/inactive	199	65±6
chromosome 4	cycle 13	70	17±2
chromosome 4	cycle 14	257	30±4
chromosome 4	postmitotic	262	83±10
DS00464	cycle 12	128	4±3
DS00464	cycle 13	197	12±6
DS00464	cycle 14	222	20±5
DS01440	cycle 12	163	4±4
DS01440	cycle 13	121	8±4
DS01440	cycle 14	102	13±2
Tp(3;3)P47; BX-C	postmitotic	304	25±3
Tp(3;1)P115; BX-C	postmitotic	206	27±8
z^{a694} ct^{6}; BX-C	postmitotic	372	61±8

The values for the BX-C are combined from hybridizations against *Ubx*, *Abd-B* and a combination of all three clones against all BX-C genes. Given are the total number of nuclei counted from different embryos and different experiments. The pairing frequency represents the percentage of nuclei containing single hybridization signals of the total number of nuclei examined and is given as the mean between differnt embryonic regions.

Tp(3;3)P47 and *Tp(3;1)P115*. The experiments were designed such that only two copies of the BX-C were maintained in the genome (Fig. 1C). Both of these transpositions have been shown by Castelli-Gair and coworkers to completely disrupt transvection phenotypes at the *Ubx* locus (Castelli-Gair et al., 1990; Micol and García-Bellido, 1988). A strong reduction but not complete inhibition of transvection of the *abd-A* and the *Abd-B* genes was found for *Tp(3;1)P115* (Hopmann et al., 1995; Jijakli and Ghysen, 1992).

We investigated postgastrulating embryos by performing hybridizations to both the *Ubx* and *Abd-B* genes in different experiments. Surprisingly, homologue pairing was not abolished but only reduced by 50-60% for each of the transpositions. The pairing frequencies were 20-30% in embryos from both transpositions (Fig. 4; Table 1). Thus, transposition to the opposite arm of the same chromosome or to another chromosome have equal probabilities of pairing. In addition, the results show that there is no significant difference in the pairing frequency between *Tp(3;3)P47* and *Tp(3;1)P115* although the rearranged locus is at least twice as large in the former as in the latter case. Furthermore, there was no difference in the frequency of homologue pairing measured when either *Ubx* or *Abd-B* were used as probe although the effect on gene expression in adults is different for the two genes (Hopmann et al., 1995; Jijakli and Ghysen, 1992). Thus, the physical proximities of the homologues of the three genes of the BX-C are the same although the loci differ with respect to their transvection-sensitive phenotypes in these transpositions.

The influence of *zeste* mutations on the pairing of the BX-C

Certain gene products can influence the efficiency of transvection. One such gene, which affects the BX-C is *zeste* (*z*), located on the X chromosome (Gans, 1953; Lewis, 1954). The ZESTE protein is found in a distribution of ~100 bands on polytene chromosomes and has been proposed to play a role in the pairing process of chromosomes (Pirrotta, 1991; Wu and Goldberg, 1989). The z^{a}-type mutations influence transvection at several loci including *Ubx* and are ZESTE protein null (Wu and Goldberg, 1989). We measured the pairing frequency in postmitotic nuclei of the *Ubx* gene in z^{a694} mutant embryos by hybridization to the *Ubx* locus.

In postgastrulating embryos, we found a pairing frequency of 50-70%, values in the same range as in wild-type embryos but with a slightly higher variation. There was obvious decondensation of the locus in some cases in the mutant background, as can be seen in images of the FISH hybridizations, suggestive of a mild destabilization of the pairing at the BX-C. A comparison of hybridization loci in gastrulated embryos for wild-type and the z^{a694} mutants is shown in Fig. 5.

We also examined whether z^{a}-type mutations delay the onset of pairing in the early cycles of embryogenesis by

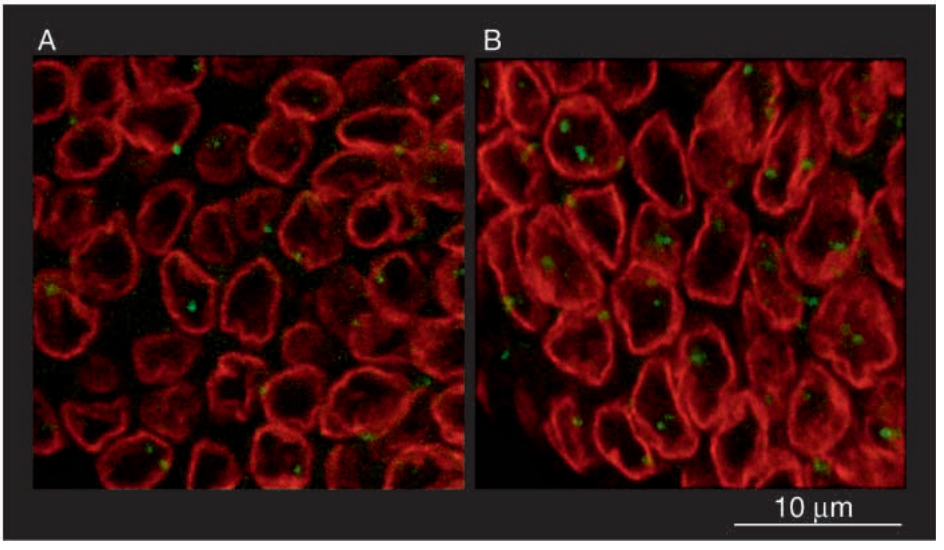

Fig. 4. Comparison of homologue pairing by FISH to genes in the *BX-C* in wild-type and mutant embryos carrying a translocation of the *BX-C* to the X-chromosome. FISH data for the *Abd-B* gene shown for postgastrulating embryos, stage 13, presented as projections of 6 optical sections taken at 250 nm intervals. The regions shown are derived from abdominal segment 5, the nuclei are postmitotic arrested in G_1. Signals resulting from DIG-labeled probes detected with a Cy3 directly labeled polyclonal antibody are shown in green and the nuclear envelope delineated with the monoclonal antibody T40 in red. (A) Hybridization to the *Abd-B* gene (P1 DS07696) in a wild type embryo. (B) Hybridization to the *Abd-B* gene (P1 DS07696) in an embryo with one allele of the *BX-C* translocated to the X-chromosome (Tp(3;1)P115, see Fig. 1C).

hybridizations to the *Ubx* gene. We found no differences in the time of onset nor in the pairing frequencies for nuclear division cycles 13 and 14 (data not shown). Thus, ZESTE protein is neither essential for chromosome recognition nor for final stabilization of pairing but has a local effect on transregulation of the *Ubx* gene.

The influence of transcription on the pairing frequencies of the BX-C

There are markedly conflicting ideas in the literature about the influence of transcription on homologue pairing. Peterson et al. (1994) argued that transcription should disrupt paired loci whereas according to another model, only transcribing genes should be paired (Cook, 1997). We therefore examined the pairing frequency in regions of the embryo where the BX-C genes are known to be active or inactive. We restricted the measurements to regions where all three genes were active or inactive to avoid compromising the results in cases where a neighboring gene might have the opposite transcriptional activity due to the finite optical resolution of the light microscope.

We found no difference for the onset of pairing in an early nuclear division cycle 14 embryos in three different regions along the anterior-posterior axis for which clear differences in transcriptional activity of the BX-C have been documented (Akam and Martinez-Arias, 1985; Jijakli and Ghysen, 1992). Representative data for a single cycle 14 embryo are as follows: in the repressed anterior region 13 of 61 nuclei (21%) showed paired alleles, in the posterior region 10 of 53 nuclei (19%), and in the middle region (where the genes are active) 29 of 115 nuclei (25%) were paired. In postgastrulating embryos, the pairing frequencies were always in the same range irrespective of the body segment investigated (data included in Fig. 3, Table 1). From these data, we conclude that homologous pairing is not a prerequisite for the onset of transcription, although our experiments would not detect subtle changes in transcriptional levels as observed by Goldsborough and Kornberg (1996).

How do the homologous alleles find each other?

It is not known how homologous chromosomal loci find each other nor how the pairing is established. From his original observations, Lewis proposed that the pairing of homologous chromosomes starts at the centromere and proceeds linearly towards the telomere (the zipper-model). Such a model is supported by translocation experiments, which show a higher frequency of transvection disruption for breakpoints proximal to the BX-C. Our translocation results, however, show that pairing is not abrogated under these circumstances (see above). Assuming a linearity of the onset of pairing along the chromosomal axis, one would expect different pairing frequencies for different loci on the chromosome. We compared the onset of pairing of the BX-C with two other loci on the right arm of chromosome 3. The cytological locations were 84B2-84C2 (close to the centromere) and 96F9-96F11 (close to the telomere) and the BX-C, 89E. None of the loci showed paired homologues before nuclear division cycle 13. The data for these probes are combined with those for BX-C in Fig. 3. No significant differences were found between the loci in the onset of pairing.

Using a whole chromosome painting technique that does not require denaturation of the DNA (Fig. 6), we measured the onset and extent of pairing for the whole chromosome 4 which carries the transvecting gene *cubitus interuptus*. The pairing frequency data for this chromosome are given in Fig. 3 and Table 1. Chromosome 4 contains approximately 5 Mb of DNA (Locke and McDermid, 1993) and is therefore 50 to 100 times larger than the P1 probes used for the hybridizations. We measured 17 ± 2% pairing (70 nuclei) in cycle 13, increasing to 30 ± 3% (257 nuclei) in cycle 14 and 70-90% (262 nuclei) in postmitotic stage 12 and 13 embryos. The values for the onset of pairing of chromosome 4 were only slightly higher than those found for the chromosome 3 probes (see Table 1). We believe this to be an effect of the larger size of the signal, which reduces the resolution by which we can distinguish individual loci in the light microscope. These results suggest uniform kinetics and a common mechanism of homology search for all chromosomes.

To determine whether the homologous alleles are randomly distributed or if they occupy specific sites inside the nucleus, we measured the distances between the unpaired homologous alleles in nuclear division cycle 14 embryos. We used data sets from hybridizations with all three P1 clones against the BX-C. Hybridization signals were segregated from the images and binary labeled, and measurements were made in 3D for the distance between the centers of gravity of the FISH signals (using a program written in SCIL-Image). The measured distances from this analysis are shown in Fig. 7. The data for paired loci are omitted from the graph (a pairing frequency of ~20% was found in these data sets). There was a preference for distances somewhat shorter than the radius of the nuclei, an indication that the pairing process is kinetically determined. The absence of multiple maxima indicates the lack of a preference for specific locations inside the nucleus of the alleles, or for nuclear membrane association. No distances >5 μm (approximately the diameter of the nuclei) were found in the cycle 14 embryos, although the nuclei had an extension in Z of ~8 μm (see Discussion).

To test various models for chromosome pairing and stabilization, we compared the interallelic distances for the unpaired BX-C loci in cycle 14 embryos with the corresponding distribution in postmitotic cells of the gastrulated embryo, in which ~70% of the alleles were paired. The distribution was strikingly different from that in cycle 14 embryos (Fig. 7). The largest distance approximated the radius of the nucleus, rather than the diameter as in cycle 14 embryos. Additionally, the distribution was skewed toward smaller distances and the mean was ~1 μm, a value close to twice the lateral resolution of our measurements. A curve for the expected random distribution of interallelic distances for two points in a sphere of radius 2.5 μm is superimposed on the data in Fig. 7.

DISCUSSION

Onset and extent of homologue pairing

None of the loci investigated on chromosome 3 or chromosome 4 showed pairing in the embryo throughout the first 12 synchronous nuclear divisions. We first observed homologue pairing in cycle 13, for which a frequency of 10-14% pairing was calculated. This value increased in early cycle 14, which is asynchronous, to 20-30% and by late cycle 14 to 46%. Cycle 14 has a duration of 50-160 minutes (Edgar and O'Farrell, 1990). There is a positive correlation between the length of the cell cycle and the pairing frequency for the onset and extent of

pairing as shown in Fig. 3 and Table 1. We assume that no homologue pairing persists through mitosis and anaphase as we can distinguish the unpaired signals in the anaphase and telophase cells observed.

The clear lack of pairing in the early synchronous cell divisions of the embryonic syncytial blastoderm may be explained by the strongly polarized position of the chromosomes within the nuclear envelope (Hiraoka et al., 1990), the rapid rate of DNA replication and concomitantly short cell cycle consisting only of S and M phases (division cycles being only ~10 minutes long up to cycle 13) (Foe and Alberts, 1983). In division cycle 13, the cell cycle doubles to approximately 20 minutes. The elegant work of Lehner, Edgar and O'Farrell on cyclins give us a very detailed picture of the cell cycle control in *Drosophila* embryogenesis (Edgar and O'Farrell, 1990; Lehner and O'Farrell, 1989). After the last rapid synchronous 13th cell cycle, all cells in the embryo undergo a complete S-phase in cycle 14 within a period of 35-45 minutes, which is followed by a 30-130 minutes G_2 interphase. The final asynchronous two mitoses and subsequent S phases (for all but a few cells in the neurogenic pathway) also occur in 35-45 minutes followed by G_2 arrest. At 6-7 hours after egg deposition, the final mitosis in these cells occurs, followed by arrest in G_1.

We restricted our measurements of late gastrulation stage 13 and 16 embryos to the lateral epidermis. These cells are arrested in G_1 and do not undergo mitosis (determined by BrdU incorporation, data of the authors not shown, reported by Bodmer et al., 1989). Pairing plateaued at a frequency of 70% for the genes of the BX-C. We never observed 100% pairing frequencies, indicative that the homologous associations are in equilibrium with a dissociated state. This latter point is supported by our measurements of the interallelic distances for unpaired loci measured during the onset of pairing and in postmitotic embryos (Fig. 7). The initial distribution of distances for cycle 14 embryos closely approximates the expected random Gaussian distribution for two points in a sphere of the dimensions of the nuclei in this stage as seen from the theoretical curve plotted with the data in Fig. 7. Results from mathematical simulations suggest that the data for postmitotic embryos are better fit by a Poissonian distribution with a mean distance approximately equal to twice the lateral resolution of the measurements (details of the simulations will be presented elsewhere, data not shown). Our findings are consistent with an initial random distribution of alleles with respect to the short axis of the elongated nucleus whose diffusional range is constrained as pairing ensues. In late embryogenesis, the unpaired alleles appear to represent a local unpairing or 'breathing' of the homologous chromosomes whereby the alleles are strongly constrained in diffusion presumably due to paired loci on adjacent segments of the chromosome.

Any mechanism that we invoke for homologous recognition and pairing for single-copy genes requires time for both chromatin movement and the homology search. A similar argument is invoked by Golic and Golic (1996) in their study of recombination frequencies for structurally rearranged FRT-containing P elements. The authors found that FRTs that lie far apart on structurally normal chromosomes recombine infrequently but that the frequency increases in mutant backgrounds that prolong the cell cycle length.

Chromosome 4 is the smallest chromosome of *D. melanogaster*, it has 5 Mb of DNA (Locke and McDermid, 1993), contains middle repetitive DNA repeats throughout its euchromatin (Miklos et al., 1988), binds the heterochromatin-binding protein HP1 over most of its length (James et al., 1989) and is associated with the chromocenter in polytene salivary glands. We investigated the onset of pairing of this chromosome as a model for centromeric and heterochromatin behavior during pairing using a whole chromosome painting technique that did not require DNA denaturation (Fig. 6). The data, summarized in Table 1 and Fig. 3, are very similar to those found for the euchromatic BX-C locus. Pairing started in both euchromatin and heterochromatin in cycle 13 embryos. The very similar frequencies of pairing in cycles 13 and early 14 for this whole chromosome compared to the single copy genes in the BX-C support the model that the length of the interval between mitoses is one of the important determinants for the pairing process. The data also make a mechanism by which centromeres nucleate the homologue search unlikely.

Prior to this manuscript, the only reports on physical pairing frequencies of homologous alleles in embryogenesis were for the multicopy histone gene locus (Hiraoka et al., 1993), for which the onset of pairing was observed already in nuclear cycle 12 and 74% pairing in cycle 14 was reported. This locus consists of a gene cluster of 120-150 copies of a 5 kb array of the histone genes with conserved intergenomic sequences (Lifton et al., 1978; Pardue et al., 1977). Such a cluster is over 700 kb and the repetitive nature of the array could allow it to assume a structure unlike that of single-copy dispersed genes or of untranscribed heterochromatin. In fact, the intergenic sequences in the histone repeat have been shown to promote ectopic pairing in polytene chromosomes (Pardue et al., 1977) and the locus is important for meiotic pairing (McKee, 1996). In addition, the constitutive transcription from the histone genes once cellularization has ensued and replication rates are still rapid, could also account for an unusual behavior. The histone locus constitutes a large target for FISH, 10-20 times bigger than the P1 clones used in this study. Thus, it is not surprising that the pairing frequency and the onset of pairing for this locus deviate strongly from our data.

Very recently Fung et al. (1998) published pairing data for 10 other probes on the second chromosome using primarily P1 clones. If we exclude their data for the histone locus and another tandem-repeat locus (*Responder*) in the heterochromatin, their results show strikingly similar kinetics (onset of pairing) to that which we found for the probes on chromosome 3 and 4. Unfortunately, the authors treated only the histone gene pairing data in their modeling of homologue pairing. We conclude from the unusual behaviour of this locus that it is more appropriate to consider loci without long tandem repeat sequences in order to generalize a mechanism for homologue recognition.

Investigation of transpositions and mutations that affect transvection

Although the transvection phenotype is defined in larval and adult *Drosophila*, the spatial pattern of the transcriptional state of the BX-C genes in the embryo predetermines whether or not the genes are transcriptionally competent in the larvae and adult (Fauvarque et al., 1995; Paro, 1993; Pirrotta, 1995; Simon, 1995). The RNA (or macromolecular transport) model (Castelli-Gair et al., 1990; Mathog, 1990; Lewis, 1954)

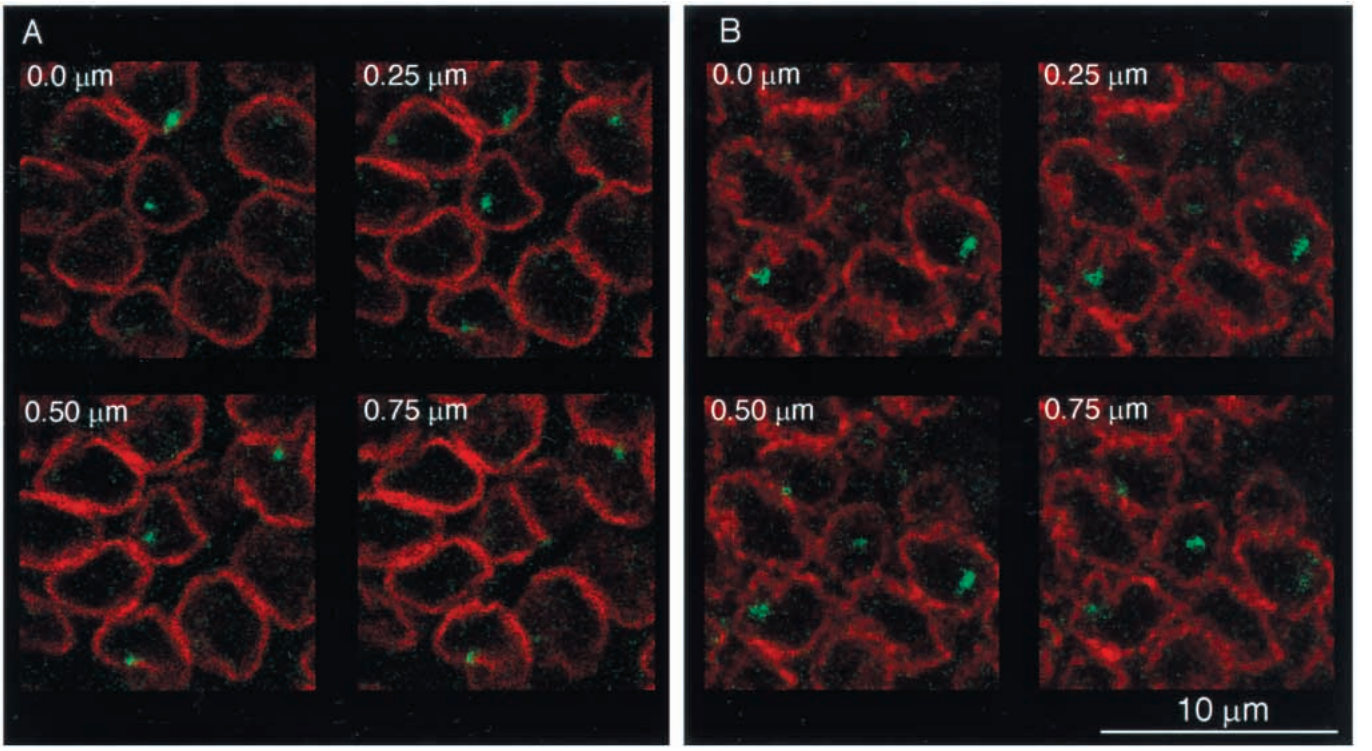

Fig. 5. Comparison of the FISH signals in wild-type and mutant *zeste* postmitotic embryos which show abrogation of transvection. Hybridization to the *Ubx* gene (P1 DS03126) in (A) wild type and (B) z^{a694} ct^{6} embryos. Shown are examples of postmitotic nuclei in stage 13 embryos. Four consecutive optical sections taken at a distance of 250 nm are presented at identical magnifications and were recorded with the same CLSM set-up in both cases. Again hybridization signals are presented in green and the nuclear envelope in red. Note: the complete nuclear volume is not included in these four sections. The loss of the ZESTE protein leads to a slightly decondensed hybridization signal as compared to the wild type.

postulates the obligatory production of a short-lived effector that interacts with the promoter. The transvection phenotype is then determined by the proximity of homologous loci during the lifetime of the effector whereby the effector can interact with the promoter in *trans*. Our data argue against this model. We found a reduction in the pairing frequency of only 50-60% to 20-30% (compared with 70% in wild-type embryos) in translocations that completely abolished the transvection phenotype. In addition, z^{a694} mutants, which also abolish the transvection phenotype at the *Ubx* locus, showed the same mean pairing frequency as wild-type embryos. In both cases, a large number of nuclei have loci in proximity that should allow transfer of the effector in this model to the *trans* promoter. In the case of the BX-C, we would expect gene transcription in the embryo and the concomitant adult transvection phenotype, which does not occur.

Our data on translocations help explain the observations of the persistence of mitotic recombination for inversions in the 3 and X chromosomes (Garcia-Bellido and Wandosell, 1978; Golic and Golic, 1996; Merriam and Garcia-Bellido, 1972). Sigrist and Pirrotta (1997) have recently demonstrated that different Polycomb Recognition Elements (PREs) inserted on homologous chromosomes interact in *trans* to suppress reporter genes. They infer from their phenotypes that the same PRE element inserted on heterologous chromosomes can also interact. The pairing of such small translocated insertions requires a model involving flexibility in both unpaired and paired chromosomes, such that homologue searching occurs over a large volume dominated by random interactions leading to a dynamic equilibrium at local pairing sites between association and disassociation. PREs and their multiprotein complexes may be a type of recognition and association site stabilizing homologue pairing.

It is not known how enhancers actually communicate with promoters, especially enhancers as distant as those in the BX-C. There is some evidence for a model of DNA looping in *cis* for distant promoters and enhancers (Ptashne, 1988). This model is supported by experiments investigating the LCR of the β-globin gene cluster in mammals (Dillon and Grosveld, 1993; Hanscombe et al., 1991; Strouboulis et al., 1992; Wijgerde et al., 1995). Grosveld and coworkers demonstrated that the probability of transcription of the developmentally regulated globin genes in the β-globin cluster is dependent upon the distance from the LCR and can be reversed by transposition of the genes. Transcription efficiency is then determined by looping probability and the complex stability. If homologous chromosomes are paired, it is plausible that looping could occur between an enhancer on one homologue and a promoter on the *trans* allele. The *trans*-interaction has a probability that is much lower than that for the *cis*-interaction as has been shown for the *yellow* locus (Geyer et al., 1990). If we assume that *trans*-looping is at least an order of magnitude less efficient than *cis* looping, as can be deduced from the data of Castelli-Gair et al. (1990) then a reduction of pairing to 20-30% could lead to a very low probability of a competent transcription complex being assembled. It is assumed that the

initiation complex would have to be formed anew for each transcript, as is the case at the β-globin locus (Wijgerde et al., 1995). One can extend this model to explain the differences in transvection penetrance observed between the three loci of the BX-C by assigning individual loci-specific probabilities for *trans*-interactions or for the lifetimes of the interaction.

Effect of transcription on the frequency of pairing

We were unable to detect any influence of transcription on the pairing frequencies of the homologous alleles of the BX-C. The onset of pairing occurred in all regions examined along the anterior-posterior axis of the embryo with the same probability and the extent of pairing did not differ in regions in which all three genes of the BX-C were inactive or expressed. These data would exclude homologue pairing models such as that proposed by Cook (1997), proposing an obligatory coupling of pairing to transcription. On the contrary, it is conceivable that very strong associations along the chromatids could in fact inhibit the *cis* interactions necessary for enhancer/promoter activation and be inhibitory to transcription, as has been proposed by Wu (1993).

Models and mechanisms for *trans*-sensing phenomena

Our data support a model for *trans*-sensing based on physical proximity and association of the homologous alleles. The translocation mutants that we investigated demonstrated that pairing can occur between segments of non-homologous chromosomes, albeit with a reduced frequency compared to that for the sites on homologous chromosomes. We can conclude that the efficacy of the *trans*-sensing effect is dependent upon the sensitivity of the phenotype to the transcriptional level of the gene in question and to the stability or affinity of the specific paired locus.

What model might be consistent with our data and those of others? In particular, what mechanism is responsible for the stable pairing of chromosomes? The information that loci on extreme positions of the 3R chromosome (near the centromere, central to the chromosome arm and close to the telomere) show

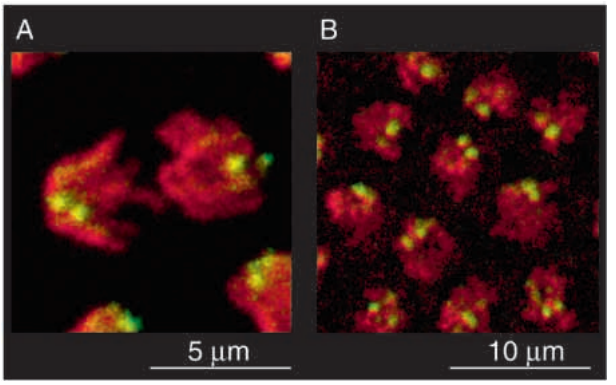

Fig. 6. Chromosomal painting of chromosome 4 in blastoderm embryos. The embryos were stained simultaneously with YOYO-1 (red) and TOTO-3 (green). TOTO-3 selectively stained chromosome 4 as can be seen in the cycle 13 telophase shown in A. In the interphase of cycle 14, shown in B, unpaired and paired chromosomes can be distinguished. The images represent projections of five consecutive optical sections taken at a distance of 400 nm.

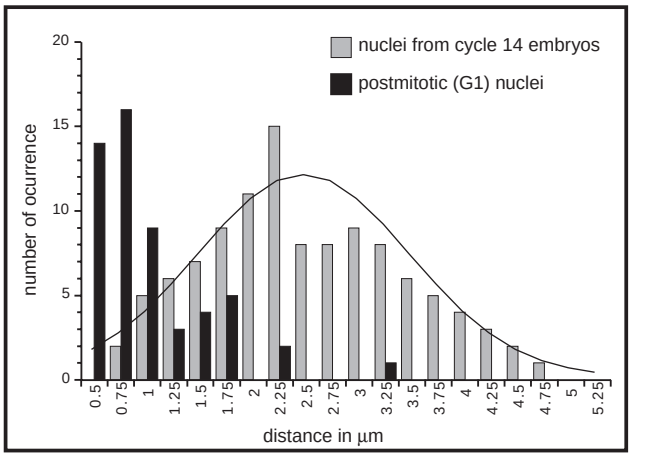

Fig. 7. Distributions of the interallelic distances for unpaired genes of the BX-C in cycle 14 embryos (shown in grey) and in postmitotic nuclei (stages 13 and 16) shown in black. The paired alleles are not included in this graph. Pairing frequencies were ~20% for cycle 14 and 60-70% for the postmitotic data. The theoretical curve (solid line) for the distribution of distances between two loci randomly dispersed in a sphere of diameter 5 μm is overlaid on the measured data.

the same frequency and onset of pairing argues against a zipper mechanism by which a centromeric association leads to a linear pairing along the chromosome to the telomere. The equal pairing frequencies observed for the translocation to the X chromosome and the opposite arm of the chromosome 3 also argue against the centromere having a dominant role in the pairing mechanism and furthermore require flexibility in the chromosomal arms with respect to each other. In addition, the pairing properties of chromosome 4 argue against an earlier association of centromeric heterochromatin than euchromatic loci or a dominant role of heterochromatin-binding proteins in the recognition process. The heterochromatin-binding protein, HP1, does not appear in embryos until nuclear cycle 10, increasing dramatically in cycle 14 (James et al., 1989). Although HP1 has been implicated as a protein causing heterochromatin aggregation, our data do not support a model by which heterochromatin regions would preferentially interact and drive homologue recognition and pairing. The HP1-binding loci remain independent in diploid embryonic nuclei even as late as stage 12. Distinct centromeres can be discerned in many stage 14 diploid nuclei in which chromosome 4 lies outside the regions occupied by the other centromeres.

Several conclusions concerning the mode of pairing can be deduced from the data presented in Fig. 7. Firstly, we see no preferential disposition of the unpaired BX-C in the lateral dimension of the nucleus in cycle 14 embryos. In cycle 14 embryos the largest interallelic distance measured for probes from the BX-C is close to the diameter of the nucleus but is not as large as the axial dimension of the nucleus. This is most easily explained by the fact that, following the rapid early division cycles, the chromosomes are all still oriented with their centromeres at the apical surface (Rabl, 1885), although no chromocenter exists. Decondensation of the chromosomes in this orientation predetermines a preferred axial disposition of the locus. That this interpretation is probably correct is supported by our results for chromosome 4, which always lies on the apical surface in blastoderm embryos (unpublished data of the authors).

Our observations are compatible with a multipoint recognition of sequences dispersed along the chromosome, resulting in globally stable interactions despite relatively unstable (short lifetime) individual associations. The combination of numerous such associations would lead to a sudden increase in the overall stability of the paired chromosome, i.e. to a highly cooperative pairing along the whole chromosome once a threshold number of interactions was established. If the pairing is driven by associations of the chromosomes through protein-protein interactions then each individual paired site will have a finite binding constant and be in equilibrium with its unpaired state. Thus, if we probe any individual locus (in our case the genes of the BX-C) there will be a probability (or frequency) of dissociation (in our case about 30-35%, see Fig. 3). The fact that the paired state predominates and that the distance between unpaired loci becomes greatly reduced at later times during embryogenesis is consistent with this model (Fig. 7). We might then liken the paired alleles on the chromosome to the buttons on a shirt or cardigan. Any one button may become unfastened yet the whole cardigan will not open. In addition, the probability of the unpaired region to pair again will be much higher than in the original recognition step since the local concentration, i.e. the total volume available to the homologous loci is reduced by some orders of magnitude due to the associations of sites flanking the test locus. A similar model for chromosome association during leptotene in meiosis has been proposed (Kleckner and Weiner, 1993).

Several conclusions can be derived from our data: (a) the homology search occurs with approximately the same kinetics for the BX-C as for chromosome 4, as well as other sites on chromosome 3 as soon as the cell cycle is lengthened beyond successive S and M phases, (b) the frequency of pairing in postmitotic embryos reflects the affinity of the recognition elements scaled by the size of the chromosomal target locus, and (c) pairing or association of the chromosomes may be mediated through protein-protein interactions.

To address this latter point, we investigated a protein that influences transvection of some genes (in particular *white*, *yellow*, *Ubx* and *decapentaplegic*) shows self-association (Wu and Goldberg, 1989), and binds at several hundred loci on polytene chromosomes (Benson and Pirrotta, 1988), that is, ZESTE. It has been argued that the self-association tendency of this protein promotes associations of ZESTE-binding sites in both *cis* and *trans* (Bickel and Pirrotta, 1990; Chen et al., 1992; Chen and Pirrotta, 1993a,b; Gelbart and Wu, 1982; Pirrotta, 1991). In addition, there are a number of clustered ZESTE-binding sites in the upstream control region for the *Ubx* gene (Benson and Pirrotta, 1988; Biggin et al., 1988), the locus that we investigated. The effect of $zeste^{a694}$, a protein null mutation, on pairing at the *Ubx* locus was of interest, since this mutant changes the *Ubx* phenotypes in transvection-sensitive experiments. We observed a higher variation of the pairing frequencies but a similar mean for both the onset and extent of pairing compared to wild type, that is mutant embryos were detected with pairing frequencies as low as 50%. We also detected local decondensation of the FISH signals in some of the chromosomes at the BX-C in z^a mutants, see Fig. 5. It is possible that the Polycomb group protein complexes themselves, which assemble at the PREs in the BX-C (Strutt et al., 1997), are a stronger determinant of the local pairing affinity at this locus. Such a possibility is supported by the observation of the *trans* interactions of PREs quoted above (Sigrist and Pirrotta, 1997).

Our present hypothesis is that many specific protein-protein interactions are responsible for recognition and pairing along the chromosomes and that they comprise proteins that have other functions such as enhancers or repressors. This hypothesis is presently being tested in our laboratory.

There is clear indication from our data and that of Golic and Golic (1996) on mitotic recombination, that the length of time between mitoses sets the lower limit on the frequency of pairing. A number of groups have considered the diffusion rate for macromolecules in the cell (Fung et al., 1998; Kubitscheck et al., 1994; Swedlow et al., 1993; Wedekind et al., 1996), and specifically for DNA segments within the cell nucleus (Buchenau et al., 1997; Marshall et al., 1997; Robinett et al., 1996). Calculated diffusion constants are of the order of 10^{-10}-10^{-11} cm^2/second. In the case of the lac operator sequence insert in a mammalian chromosome (Robinett et al., 1996), no movement was observed over many hours whereas movements of whole chromosome loci exceeding >2 μm/minute were seen in another study (Buchenau et al., 1997). This latter rate of movement as well as the estimated diffusion constants are compatible with the fact that pairing first occurs when the cell cycle lengthens beyond 20 minutes. According to our model, sites with strong protein-protein interactions would act as nucleating sites but stable homologue associations along the length of the chromatids would only occur by multipoint recognition and interaction. We would expect that, at higher resolution than that achievable in the light microscope, the actual associations of the chromatids would be selective, i.e. with a sufficient number of open regions without homologous interaction to allow *cis* looping and movements required during transcriptional processes.

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REFERENCES

Akam, M. E. and Martinez-Arias, A. (1985). The distribution of *Ultrabithorax* transcripts in *Drosophila* embryos. *EMBO J.* **4,** 1689-1700.

Allis, C. D., Waring, G. L. and Mahowald, A. P. (1977). Mass isolation of pole cells from *Drosophila melanogaster*. *Dev. Biol.* **56,** 372-381.

Babu, P., Selvakumar, K. S. and Bhosekar, S. (1987). Studies on transvection at the Bithorax Complex in *Drosophila melanogaster*. *Mol. Gen. Genet.* **210,** 557-563.

Bender, W., Akam, M., Karch, F., Beachy, P. A., Peifer, M., Spierer, P., Lewis, E. B. and Hogness, D. S. (1983). Molecular Genetics of the Bithorax Complex in *Drosophila melanogaster*. *Science* **221,** 23-29.

Benson, M. and Pirrotta, V. (1988). The *Drosophila* zeste protein binds cooperatively to sites in many gene regulatory regions: implications for transvection and gene regulation. *EMBO J.* **12,** 3907-3915.

Bickel, S. and Pirrotta, V. (1990). Self-association of the *Drosophila* zeste protein is reponsible for transvection effects. *EMBO J.* **9,** 2959-2967.

Biggin, M. D., Bickel, S., Benson, M., Pirrotta, V. and Tjian, R. (1988). *zeste* encodes a sequence-specific transcription factor that activates the *Ultrabithorax* promoter in vitro. *Cell* **53,** 713-722.

Bodmer, R., Carretto, R. and Nung Jan, Y. (1989). Neurogenesis of the peripheral nervous system in *Drosophila* Embryos: DNA replication patterns and cell lineages. *Neuron* **3,** 21-32.

Buchenau, P., Arndt-Jovin, D. J. and Saumweber, H. (1993). In vivo observation of the puff-specific protein no-on transient A (NONA) in nuclei of *Drosophila* embryos. *J. Cell Sci.* **106,** 189-199.

Buchenau, P., Saumweber, H. and Arndt-Jovin, D. J. (1997). The dynamic nuclear redistribution of an hnRNP K-homologous protein during *Drosophila* embryo development and heat shock. Flexibility of transcription sites in vivo. *J. Cell Biol.* **137,** 291-303.

Campos-Ortega, J. A. and Hartenstein, V. (1985). The embryonic development of *Drosophila melanogaster*. Berlin: Springer Verlag.

Castelli-Gair, J., Greig, S., Micklem, G. and Akam, M. (1994). Dissecting the temporal requirements for homeotic gene function. *Development* **120,** 1983-1995.

Castelli-Gair, J. E., Micol, J. L. and García-Bellido, A. (1990). Transvection in the *Drosophila Ultrabithorax* gene: a *Cbx1* mutant allele induces ectopic expression of a normal allele in trans. *Genetics* **126,** 177-184.

Chen, J. D., Chan, C. S. and Pirrotta, V. (1992). Conserved DNA binding and self-association domains of the *Drosophila* zeste protein. *Mol. Cell. Biol.* **12,** 598-608.

Chen, J. D. and Pirrotta, V. (1993a). Multimerization of the *Drosophila* zeste protein is required for efficient DNA binding. *EMBO J.* **12,** 2075-2083.

Chen, J. D. and Pirrotta, V. (1993b). Stepwise assembly of hyperaggregated forms of *Drosophila* zeste mutant protein suppresses *white* gene expression *in vivo*. *EMBO J.* **12,** 2061-2073.

Cook, P. R. (1997). The transcriptional basis of chromosome pairing. *J. Cell Sci.* **110,** 1033-1040.

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

Dernburg, A. F., Broman, K. W., Fung, F. 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.

Dillon, N. and Grosveld, F. (1993). Transcriptional regulation of multigene loci: multilevel control. *Trends Genet.* **9,** 134-137.

Edgar, B. and O'Farrell, P. (1990). The three postblastoderm cell cycles of *Drosophila* embryogenesis are regulated in G_2 by *string*. *Cell* **62,** 469-480.

Fauvarque, M.-O., Zuber, V. and Dura, J.-M. (1995). Regulation of *polyhomeotic* transcription may involve local changes in chromatin activity in *Drosophila*. *Mech. Dev.* **52,** 343-355.

Foe, V. E. and Alberts, B. M. (1983). Studies of nuclear and cytoplasmic behaviour during the five mitotic cycles that precede gastrulation in *Drosophila* embryogenesis. *J. Cell Sci.* **61,** 31-70.

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

Gans, M. (1953). Étude génétique et physiologique du mutant *z* de *Drosophila melanogaster*. *Bull. Biol. Fr. Belg., Suppl.* **38,** 1-90.

García-Bellido, A. and Wandosell, F. (1978). The effect of inversions on mitotic recombination in *Drosophila melanogaster*. *Mol. Gen. Genetics* **161,** 317-321.

Gelbart, W. M. (1982). Synapsis-dependent allelic complementation at the *decapentaplegic* gene complex in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **79,** 2636-2640.

Gelbart, W. M. and Wu, C.-T. (1982). Interactions of *zeste* mutations with loci exhibiting transvection effects in *Drosophila melanogaster*. *Genetics* **102,** 179-189.

Gemkow, M. J., Buchenau, P. and Arndt-Jovin, D. J. (1996). FISH in whole-mount *Drosophila* embryos. RNA: activation of a transcriptional locus, DNA: gene architecture and expression. *Bioimaging* **4,** 107-120.

Geyer, P. K., Green, M. M. and Corces, V. G. (1990). Tissue-specific transcriptional enhancers may act in *trans* on the gene located in the homologous chromosome: the molecular basis of transvection in *Drosophila*. *EMBO J.* **9**, 2247-2256.

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

Golic, M. M. and Golic, K. G. (1996). A quantitative measure of the mitotic pairing of alleles in *Drosophila melanogaster* and the influence of structural heterozygosity. *Genetics* **143,** 385-400.

Gubb, D., Ashburner, M., Roote, J. and Davis, T. (1990). A novel transvection phenomenon affecting the white gene of *Drosophila* melanogaster. *Genetics* **126,** 167-176.

Gubb, D., Roote, J., Trenear, J., Coulson, D. and Ashburner, M. (1997). Topological constrains on transvection between white genes within the transposing element TE35B in *Drosophila melanogaster*. *Genetics* **146,** 917-937.

Hanscombe, O., Whyatt, D., Fraser, P., Yannoutsos, N., Greaves, D., Dillon, N. and Grosveld, F. (1991). Importance of globin gene order for correct developmental expression. *Genes Dev.* **5,** 1387-1394.

Hartl, D., Nurminsky, D., Jones, R. and Lozovskaya, E. (1994). Genome structure and evolution in *Drosophila*: applications of the framework P1 map. *Proc. Natl. Acad. Sci. USA* **91,** 6824-6829.

Heimer, G. V. and Taylor, C. E. D. (1974). Improved mountant for immunofluorescence preparations. *J. Clin. Path.* **27,** 254-256.

Hell, S., Reiner, G., Cremer, C. and Stelzer, E. H. K. (1993). Aberrations in confocal fluorescence microscopy induced by mismatches in refractive index. *J. Microsc.* **169,** 391-405.

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

Hiraoka, Y., Agard, D. and Sedat, J. (1990). Temporal and spatial coordination of chromosome movement, spindle formation, and nuclear envelope breakdown during prometaphase in *Drosophila melanogaster* embryos. *J. Cell Biol.* **111,** 2815-2828.

Hiraoka, Y., Dernburg, A. F., Parmelee, S. J., Rykowski, M. C., Agard, D. A. and Sedat, J. W. (1993). The onset of homologous chromosome pairing during *Drosophila melanogaster* embryogenesis. *J. Cell Biol.* **120,** 591-600.

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.

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.

Jijakli, H. and Ghysen, A. (1992). Segmental determination in *Drosophila* central nervous system: analysis of the *abdominal-A* region of the Bithorax Complex. *Int. J. Dev. Biol.* **36,** 93-99.

Kleckner, N. and Weiner, B. M. (1993). Potential advantages of unstable interactions for pairing of chromosomes in meiotic, somatic, and premeiotic Cells. *Cold Spring Harbor Symp. Quant. Biol.* **58,** 553-565.

Kubitscheck, U., Wedekind, P. and Peters, R. (1994). Lateral diffusion measurement at high spatial resolution by scanning microphotolysis in a confocal microscope. *Biophys. J.* **67,** 948-956.

Lehner, C. and O'Farrell, P. (1989). Expression and function of *Drosophila* cyclin A during embryonic cell cycle progression. *Cell* **56,** 957-968.

Leiserson, W.M., Bonini, N. M. and Benzer, S. (1994). Transvection at the *eyes absent* gene of *Drosophila*. *Genetics* **138,** 1171-1179.

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

Lifton, R., Goldberg, M., Karp, R. and Hogness, D. (1978). The organization of the histone genes in *Drosophila melanogaster:* Functional and evolutionary implications. *Cold Spring Harbor Symp. Quant. Biol.* **43,** 1047-1051.

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

Locke, J. and McDermid, H. E. (1993). Analysis of *Drosophila* chromosome 4 using pulsed field gel electrophoresis. *Chromosoma* **102,** 718-723.

Marshall, W., Straight, A., Marko, J., Swedlow, J., Dernburg, A., Belmont, B., Murray, A., Agard, D. and Sedat, J. (1997). Interphase chromosomes undergo constrained diffusional motion in living cells. *Curr. Biol.* **7,** 930-939.

Martinez-Laborda, A., González-Reyes, A. and Morata, G. (1992). Trans regulation in the *Ultrabithorax* gene of *Drosophila*: alterations in the promoter enhance transvection. *EMBO J.* **11,** 3645-3652.

Mathog, D. (1990). Transvection in the *Ultrabithorax* domain of the Bithorax complex of *Drosophila melanogaster*. *Genetics* **125,** 371-382.

McKee, B. D. (1996). The license to pair: Identification of meiotic pairing sites in *Drosophila*. *Chromosoma* 135-141.

Merriam, J. and García-Bellido, A. (1972). A model for somatic pairing derived from somatic crossing over with third chromosome rearrangements in *Drosophila melanogaster*. *Mol. Gen. Genet.* **115,** 302-313.

Metz, C. W. (1916). Chromosome studies of the Diptera. II: the paired association of chromosomes in the Diptera, and its significance. *J. Exp. Zool.* **21,** 213-279.

Micol, J.-L. and García-Bellido, A. (1988). Genetic analysis of 'transvection' effects involving *Contrabithorax* mutations in *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **85,** 1146-1150.

Miklos, G. L. G., Yamamoto, M. T., Davies, J. and Pirrotta, V. (1988). Microcloning reveals a high frequency of repetitive sequences characteristic of chromosome 4 and the beta heterochromatin of *Drosophila melanogaster*. *Proc. Natl. Acad. Sci. USA* **85,** 2051-2055.

Pardue, M., Kedes, L., Weinberg, E. and Birnstiel, M. (1977). Localization of sequences coding for histon messenger RNA in the chromosomes of *Drosophila melanogaster*. *Chromosoma* **63,** 135-151.

Paro, R. (1993). Mechanisms of heritable gene repression during development of *Drosophila*. *Curr. Opin. Cell Biol.* **5,** 999-1005.

Peterson, K. M., Davis, P. S. and Judd, B. H. (1994). The determined state of *white* expression in the *Drosophila* eye is modified by *zeste*^{1} in the *wzm* family of mutants. *Mol. Gen. Genet.* **242,** 717-726.

Pirrotta, V. (1991). The genetics and molecular biology of *zeste* in *Drosophila melanogaster*. *Adv. in Genetics* **29,** 301-348.

Pirrotta, V. (1995). Chromatin complexes regulating gene expression in *Drosophila*. *Curr. Opin. Genet. Dev.* **5,** 466-472.

Pirrotta, V., Bickel, S. and Mariani, C. (1988). Developmental expression of the *Drosophila zeste* gene and localization of zeste protein on polytene chromosomes. *Genes Dev.* **2,** 1839-1850.

Ptashne, M. (1988). How eukaryotic transcriptional activators work. *Nature* **335,** 683-689.

Rabl, C. (1885). Über Zelltheilung. *Morphol. Jahrbuch* **10,** 214-330.

Risau, W., Saumweber, H. and Symmons, P. (1981). Monoclonal antibodies against a nuclear membrane protein of *Drosophila*. *Exp. Cell Res.* **133,** 47-54.

Robinett, C. C., Straight, A., Li, G., Willhelm, C., Sudlow, G., Murray, A. 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.

Sigrist, C. J. A. 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.

Simon, J. (1995). Locking in stable states of gene expression: transcriptional control during *Drosophila* development. *Curr. Opin. Cell Biol.* **7.** 376-385.

Smolik-Utlaut, S. and Gelbart, W. (1987). The effects of chromosomal rearrangements on the *zeste-white* interaction in *Drosophila melanogaster*. *Genetics* **116,** 285-298.

Strouboulis, J., Dillon, N. and Grosveld, F. (1992). Developmental regulation of a complete 70 kb human β-globin locus in transgenic mice. *Genes Dev.* **6,** 1857-1864.

Strutt, H., Cavalli, G. and Paro, R. (1997). Co-localization of Polycomb protein and GAGA factor on regulatory elements responsible for the maintenance of homeotic gene expression. *EMBO J.* **16,** 3621-3632.

Swedlow, J. R., Sedat, J. W. and Agard, D. A. (1993). Multiple chromosomal populations of topoisomerase II detected in vivo by time-lapse, three-dimensional wide-field microscopy. *Cell* **73,** 97-108.

Tartof, K. D. and Henikoff, S. (1991). Trans-sensing effects from *Drosophila* to Humans. *Cell* **65,** 201-203.

Verveer, P. J. and Jovin, T. M. (1997). Efficient superresolution restoration algorithms using maximum a posteriori estimations with application to fluorescence microscopy. *J. Opt. Soc. Am.* **14,** 1696-1706.

Wedekind, P., Kubitscheck, U., Heinrich, O. and Peters, R. (1996). Linescanning microphotolysis for diffraction-limited measurements of lateral diffusion. *Biophys. J.* **71,** 1621-1632.

White, R. A. H. and Wilcox, M. (1985). Distribution of Ultrabithorax proteins in *Drosophila*. *EMBO J.* **4,** 2035-2043.

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

Wu, C.-t. (1993). Transvection, nuclear structure, and chromatin Proteins. *J. Cell Biol.* **120,** 587-590.

Wu, C.-T. and Goldberg, M. L. (1989). The *Drosophila zeste* gene and transvection. *Trends Genet.* **5,** 189-194.

Zalokar, M. and Erk, I. (1977). Phase-partition fixation and staining of *Drosophila* eggs. *Stain Technol.* **52,** 89-95.