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The genomic silencing of position-effect variegation in *Drosophila melanogaster*: interaction between the heterochromatin-associated proteins Su(var)3-7 and HP1

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SUMMARY

Position-effect variegation results from mosaic silencing by chromosomal rearrangements juxtaposing euchromatin genes next to pericentric heterochromatin. An increase in the amounts of the heterochromatin-associated Su(var)3-7 and HP1 proteins augments silencing. Using the yeast two-hybrid protein interaction trap system, we have isolated HP1 using Su(var)3-7 as a bait. We have then delimited three binding sites on Su(var)3-7 for HP1. On HP1, the C-terminal moiety, including the chromo shadow domain, is required for interaction. In vivo, both proteins co-localise not only in heterochromatin, but also in a limited set of sites in euchromatin and at telomeres. When delocalised to the sites bound by the protein Polycomb in euchromatin, HP1 recruits Su(var)3-7. Finally, and in contrast with euchromatin genes, a decrease in the amounts of both proteins enhances variegation of the *light* gene, one of the few genetic loci mapped within pericentric heterochromatin. This body of data supports a direct link between Su(var)3-7 and HP1 in the genomic silencing of position-effect variegation.

Key words: *Drosophila*, Position-effect variegation, Heterochromatin

INTRODUCTION

Heterochromatin is the fraction of the genome that remains condensed throughout the mitotic cell cycle, replicates late during S phase, comprises only a few genes and is rich in repetitive DNA (reviewed by Elgin, 1996). Chromosomal rearrangements, or transgene insertions that juxtapose euchromatic genes to constitutive heterochromatin frequently result in mosaic gene silencing, a phenomenon known as position effect variegation (PEV; Spofford, 1976; Weiler and Wakimoto, 1995). The extent of silencing depends both on the *cis*-DNA sequences and on *trans*-acting proteins. Several genetic screens have identified dominant suppressors or enhancers of PEV. Interestingly, some loci exhibit both a haplo-supressor and a triplo-enhancer effect on variegation, making them good candidates for being structural components of constitutive heterochromatin (Reuter and Spierer, 1992). Most genes are located in euchromatin and variegate when moved to heterochromatin. There are, however, a few genes (including essential genes) located within heterochromatin, and proximity to major blocks of heterochromatin appears to be required for their normal expression. Indeed, these genes variegate when moved to distal euchromatin (Wakimoto and Hearn, 1990). Effects of *trans*-acting modifiers on PEV of heterochromatic genes are generally opposite to the effects seen for PEV of euchromatic genes (Hearn et al., 1991).

The suppressor of variegation *Su(var)2-5* encodes HP1, a protein associated with centromeric heterochromatin in interphase and mitotic chromosomes but also with telomeres and some euchromatic sites (James et al., 1989; Eissenberg et al., 1992; Kellum et al., 1995; Fanti et al., 1998; for a recent review see Eissenberg and Elgin, 2000). HP1 contains two domains, the chromodomain and the chromo shadow domain, which are conserved in the HP1 homologues in human and mouse, HP1α, HP1β (MOD1/M31) and HP1γ (MOD2/M32) (Singh et al., 1991; Saunders et al., 1993; Wreggett et al., 1994). Recent studies with mammalian HP1 homologues indicate that transcription factors could recruit HP1-like proteins to establish a heterochromatin-like complex (LeDouarin et al., 1996; Seeler et al., 1998; Lehming et al., 1998; Ryan et al., 1999). Mammalian HP1γ was also shown to interact with the lamin B receptor, a nuclear membrane protein (Ye and Worman, 1996; Ye et al., 1997) and to co-immunoprecipitate with Suv39h1, a homologue of the *Drosophila* suppressor of variegation Su(var)3-9 (Tschiersch et al., 1994; Aagaard et al., 1999). Recently, mouse HP1 proteins were shown to interact with the p150 subunit of CAF-1, the chromatin assembly factor 1 (Murzina et al., 1999). The inner centromere protein (INCENP), a component of the mitotic chromosome scaffold interacts also with human HP1α and HP1γ in a yeast two-hybrid screen (Ainsztein et al., 1998). In *Drosophila*, HP1 was shown to interact with ORC proteins

(Origin Recognition Complex) and to colocalise with the Arp4 protein in pericentric heterochromatin (Pak et al., 1997; Frankel et al., 1997).

Another suppressor of variegation seeming to play a central role in heterochromatin formation is the *Su(var)3-7* gene of *Drosophila*. It encodes a large protein of 1169 amino acids containing seven widely spaced zinc fingers (Reuter et al., 1990; Cléard et al., 1995). The Su(var)3-7 protein is mainly associated with pericentromeric heterochromatin in embryonic nuclei and polytene chromosomes. We have previously shown that the effect of the dose of *Su(var)3-7* on the silencing of various variegating rearrangements is strikingly similar to the effect of dose of *Su(var)2-5* gene, which encodes the HP1 protein, and that both loci interact genetically (Cléard et al., 1997). In addition, both proteins co-immunoprecipitate from nuclear extract.

We report here that Su(var)3-7 and HP1 interact directly in a yeast two-hybrid assay, and we have delineated the domains required for this interaction. The relevance of this interaction in yeast is strongly supported by the fact that Su(var)3-7 and HP1 co-localise on chromosomes, primarily on heterochromatic regions. Moreover, we show that delocalised HP1 recruits Su(var)3-7 in vivo. The effect of Su(var)3-7 and HP1 on variegation, their co-localisation and their direct interaction in yeast strongly suggest that they are close partners.

MATERIALS AND METHODS

Two-hybrid vectors and screens

The two-hybrid screen was performed in the yeast strain EGY48 using a *Drosophila* cDNA library derived from embryos (0-21 hours) of the Canton S strain, and cloned into the pJG4-5 plasmid (Clontech). Fusion proteins for the two-hybrid screen were made with the pEG202 bait and PJG4-5 prey plasmids (Gyuris et al., 1993). All constructs were verified by sequencing.

Su(var)3-7 constructs

A series of baits were generated using different segments of the Su(var)3-7 protein fused to the LexA DNA-binding domain in the vector pEG202 (Gyuris et al., 1993). Several controls verified that the fusion proteins fulfil the criteria for a two-hybrid assay (see below). Although well detectable on western blots at the expected size, the two constructs containing the largest Su(var)3-7 fusion proteins (amino acid 122 to 1169 and amino acid 189 to 1169) were not usable because of their inability to enter the nucleus or to bind the LexA binding sites. *Construct Su122-1169*: A 1.7 kb *Bam*HI/*Not*I fragment from Su(var)3-7 cDNA (Cléard et al., 1995) containing all the C-terminal part, from the *Bam*HI site (position 2593) until the end of the cDNA at the *Not*I site of pNB40 plasmid (Brown and Kafatos, 1988) was cloned into the *Bam*HI/*Not*I sites of pEG202. The *Bam*HI/*Bam*HI fragment (coordinates 753-2593) was inserted upstream into the *Bam*HI site of PEG202. *Construct Su189-1169*: a 3.3 kb *Eco*RI/*Not*I fragment containing all the protein from the *Eco*RI site (position 953) until the end of the cDNA (*Not*I site of the pNB40 plasmid; Brown and Kafatos, 1988) was inserted into the *Eco*RI/*Not*I sites of pEG202. *Construct Su189-844*: a 2.0 kb *Eco*RI/*Nru*I fragment (coordinates 953-2916) was inserted into the *Eco*RI site of PEG202. *Construct Su189-612*: a 1.3 kb *Eco*RI/*Xho*I fragment (coordinates 953-2223) was inserted into the *Eco*RI/*Xho*I sites of PEG202. *Construct Su189-485*: a 0.9 kb *Eco*RI/*Bst*YI fragment (coordinates 953-1836) was inserted into the *Eco*RI/*Bam*HI sites of PEG202. *Construct Su189-390*: a 0.6 kb *Eco*RI/*Nsi*I fragment (coordinates 953-1557) was inserted into the *Eco*RI/*Not*I sites of PEG202. *Construct Su189-334*: a 0.4 kb *Eco*RI/BspE1 fragment (coordinates 953-1387) was inserted into the *Eco*RI/*Bam*HI sites of PEG202. *Construct Su189-255*: a 0.2 kb *Eco*RI/*Hae*II fragment (coordinates 953-1150) was inserted into the *Eco*RI/*Not*I sites of PEG202. *Construct Su266-334*: a 0.2 kb *Fsp*I/*Bsp*EI fragment (coordinates 1184-1387) was inserted into the *Bam*HI/*Xho*I sites of PEG202. *Construct Su332-390*: a 0.2 kb *Apa*LI/*Nsi*I fragment (coordinates 1377-1557) was inserted into the *Eco*RI/*Xho*I sites of PEG202. *Construct Su390-736*: a 1.0 kb *Nsi*I/*Bam*HI fragment (coordinates 1557-2593) was inserted into the *Eco*RI/*Bam*HI sites of PEG202. *Construct Su485-736*: a 0.7 kb *Bst*YI/*Bam*HI fragment (coordinates 1836-2593) was inserted into the *Bam*HI site of PEG202. *Construct Su485-612*: this construct contains a 0.4 kb *Bst*YI/*Xho*I fragment (coordinates 1836-2223), and was made by *Xho*I digestion and religation of the construct AA(485-736*). Construct Su573-612*: a 0.1 kb *Fsp*I/*Xho*I fragment (coordinates 2106-2223) was inserted into the *Eco*RI/*Bam*HI sites of PEG202. *Construct Su390-736*: this construct contains a 0.4 kb *Xho*I/*Bam*HI fragment (coordinates 2223-2593) and was made by deleting the N-terminal part until the *Xho*I site of the *Nsi*I/*Bam*HI insert of the construct AA(390-736*). Construct Su736-1169*: a 1.7 kb *Bam*HI/*Not*I fragment containing all the C-terminal part of the protein from the *Bam*HI site (position 2593) until the end of the cDNA (*Not*I site of the pNB40 plasmid; Brown and Kafatos 1988) was inserted into the *Bam*HI/*Not*I sites of pEG202. *Construct Su736-845*: a 0.3 kb *Bam*HI/*Nru*I fragment (coordinates 2593-2916) was inserted into the *Bam*HI/*Not*I sites of PEG202. *Construct Su845-1169*: a 1.0 kb *Nru*I/*Hae*III fragment (coordinates 2916-3889) was inserted into the *Eco*RI/*Bam*HI sites of PEG202. *Construct Su845-971*: a 0.4 kb *Nru*I/*Bst*EII fragment (coordinates 2916-3295) was inserted into the *Eco*RI/*Bam*HI sites of PEG202. *Construct Su971-1169*: a 0.4 kb *Bst*EII/*Hae*III fragment (coordinates 3295-3889) was inserted into the *Bam*HI/*Not*I sites of PEG202.

HP1 constructs

In the *PJG4-5 HP1* construct, the almost complete sequence of HP1, namely from amino acids 5 to the end of the HP1 cDNA, was cloned in the *Eco*RI/*Xho*I sites of pJG4-5. Several deletions plasmids were made from this construct: *Constructs HP1(95)* and *HP1(152)* were made by using the *Sty*I and *Bgl*II sites in the HP1 sequence, and deleting all amino acids from 95 to the end, and from 152 to the end, respectively. *Construct HP1(95-206) and HP1(152-206)* were made by using the *Sty*I and *Bgl*II sites in the HP1 sequence and deleting all amino acids from 5 to 95 and from 5 to 152, respectively.

Two-hybrid tests

Yeast strains were constructed from the strain *EGY48 (Matα ura3 his3 trp1 6lexAop-LEU2)*(Gyuris et al., 1993). Plasmids were introduced into yeast by lithium acetate transformation with 1-5 μg of plasmid DNA. Two-hybrid tests were performed on indicator plates supplemented with 2% galactose and 1% raffinose to induce prey fusion protein expression. Activation of *lacZ* from the pSH18-34 reporter plasmid (Gyuris et al., 1993) was assayed by scoring blue color on medium lacking uracil, histidine and tryptophan, and containing 20 μg of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) per ml. Activation of the chromosomal LEU2 reporter was tested by scoring for growth on minimal medium lacking uracil, histidine, tryptophan and leucine. Control experiments were performed to test the expression and behaviour of the bait fusion proteins. Expression of bait fusion proteins containing the epitope for the anti-Su(var)3-7 antibody Ab212 (Cléard et al., 1997) was verified on a western blot of crude yeast extracts. Western blotting was performed as described (Cléard et al., 1997). Each bait construct detected by the Ab212 antibody was shown to migrate at approximately the predicted molecular mass. Nuclear localisation and ability to bind LexA operators of each bait fusion proteins were verified by testing ability to partially repress *lacZ* expression from pJK101 (Gyuris et al., 1993). Finally, we have verified that each bait

protein failed to activate the *lacZ* and *LEU2* reporters in absence of a prey protein.

Immunostaining of polytene chromosomes

Salivary glands from third instar larvae were dissected in PBS, 0.1% Triton X-100, fixed for 20 seconds in 2% formaldehyde, PBS, 0.25% Triton X-100 and squashed in 3.7% formaldehyde, 50% acetic acid. Slides were incubated in PBS, 3% bovine serum albumin (BSA), 10% non-fat dry milk, 0.2% Tween-20 and 0.2% NP-40. After 1 hour at room temperature, most of the blocking solution was removed and primary antibodies were added at the appropriate dilution (1:100 for crude anti-Su(var)3-7 Ab212 (Cléard et al., 1997), and 1:400 for C1A9 anti-HP1) in PBS. Slides were incubated overnight at 4°C in a humid chamber. After 3 washes in BBT (PBS, 100 mM NaCl, 0.1% BSA, 0.1% Tween-20), secondary antibodies were added: anti-rabbit biotinylated (1/400) and Cy3-conjugated anti-mouse (1/400). After 1 hour at room temperature, slides were washed and incubated 1 hour in PBT with avidine-FITC (1/200). Slides were washed in PBT, and mounted in Vectashield (Vector laboratory) with DAPI (0.05 mg/ml) or propidium iodide (1 mg/ml).

Effect of Su(var)3-7 on variegation of *light*

Flies homozygous for the lt^{x13} allele (kindly provided by B. Wakimoto) were used to assess the effect of the *Su(var)3-7* deficiency $Df(3R)Ace^{HD1}$ on *light* variegation as described (Hearn et al., 1991). lt^{x13}/lt^{x13} virgin females were crossed to $lt^{1}rl/Cyo;$ $Df(3R)Ace^{HD1}/TM3$ males. Pigment values of the F1 $lt^{x13}/lt^{1};$ $Df(3R)Ace^{HD1}/+$ progeny were measured and compared to control lt^{x13}/lt^{1}; *TM3*/+. Eye pigments extraction and measurement were as described (Réal et al., 1985).

RESULTS

Su(var)3-7 and HP1 interact in a yeast two-hybrid assay

Genetic and biochemical evidences suggest that heterochromatin proteins form multiprotein complexes (reviewed by Elgin, 1996). We therefore used the yeast two-hybrid system to search for proteins interacting with Su(var)3-7 (Fields and Song, 1989). A piece of Su(var)3-7 containing zinc fingers two to seven (amino acids 189 to 844) was used as a bait to screen a cDNA library derived from *Drosophila* embryos. Approximately three millions yeast transformants were screened, and 50 positives isolated. One of them encodes the almost complete sequence of the heterochromatin-associated protein HP1.

To delimit the domains of the Su(var)3-7 protein involved in the interaction with HP1, we have performed a deletion analysis illustrated in Fig. 1. First, the Su(var)3-7 protein was cut in three pieces containing amino acids 122 to 189, 189 to 844 and 845 to the end. Two of these three constructs interact with HP1 (Fig. 1), but fail to interact with unrelated proteins or empty pJG4-5 vector (data not shown). In a second step, several others fragments were tested and showed that Su(var)3-7 contains at least three HP1-binding domains localised between zinc finger 2 and 3 (construct AA266-334), between zinc finger 5 and 6 (construct AA573-612), and in the C terminal part of Su(var)3-7 (construct AA845-971). Zinc finger motifs and the so-called tryptophan boxes, which are found in front of each zinc finger (Cléard et al., 1995) seem not required for this interaction, since constructs Su189-255, Su332-390, Su612-736 or Su736-845 do not interact with HP1(Fig. 1).

To identify the HP1 sequences required for interaction with Su(var)3-7, several deletion mutants were examined in yeast (Fig. 2). Interaction between Su(var)3-7 and HP1 could not be detected with fusion proteins containing N-terminal forms of HP1 co-expressed with Su(var)3-7 fusion proteins (construct AA189-844 or construct AA845-end). This indicates that the N-terminal part of HP1, including the chromo-domain, is not sufficient to interact with Su(var)3-7. In contrast, an interaction was observed in the presence of a C-terminal fusion protein containing HP1 residues 95-206 (Fig. 2). Thus, the C-terminal

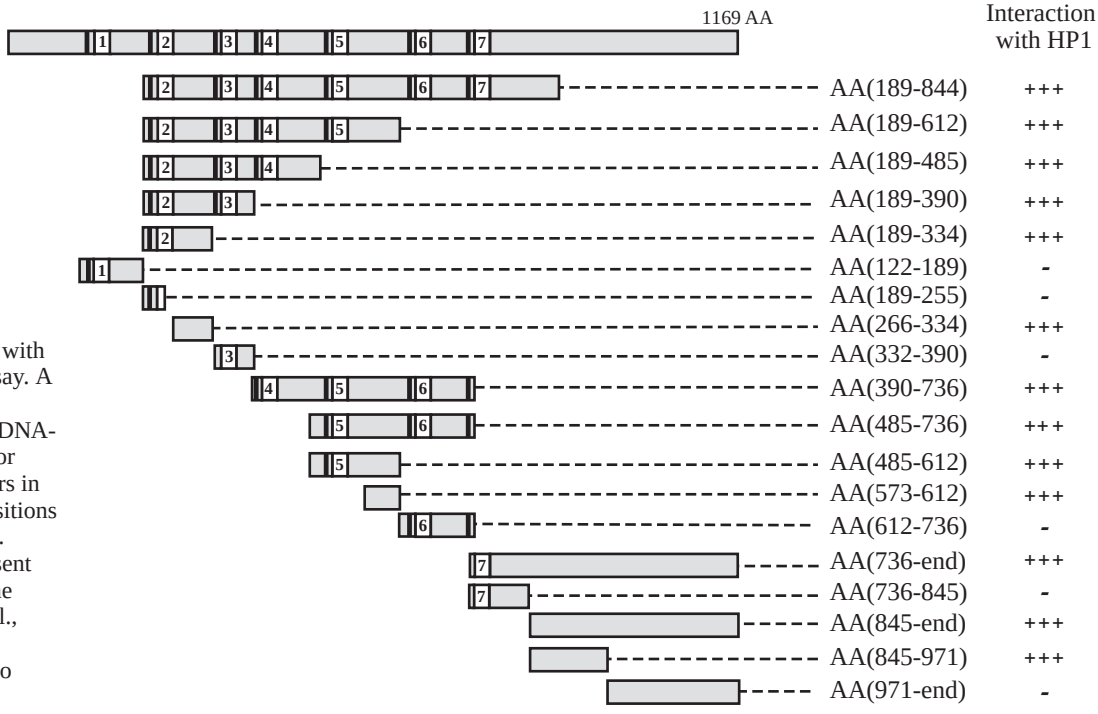

Fig. 1. Su(var)3-7 interaction with HP1 in a yeast two-hybrid assay. A series of Su(var)3-7 deletion constructs fused to the LexA DNA-binding domain were tested for interaction with HP1. Numbers in parentheses correspond to positions in the sequence of Su(var)3-7. White numbered boxes represent zinc fingers, and black bars the tryptophan boxes (Cléard et al., 1995). +, interaction between Su(var)3-7 bait and HP1; –, no interaction.

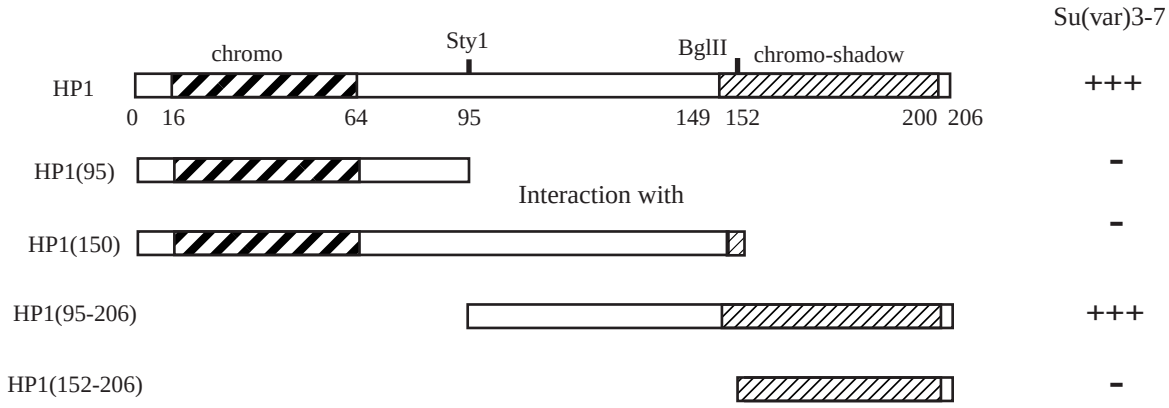

Fig. 2. Identification of the region of HP1 involved in the interaction with Su(var)3-7. A panel of deletion constructs of HP1 were tested for interaction with Su(var)3-7 (construct AA189-844 and AA736-end) in the yeast two-hybrid system. +, interaction between Su(var)3-7 bait and HP1; –, no interaction.

half of HP1, which contains the chromo shadow domain, can interact with Su(var)3-7 in yeast. This interaction necessitates at least the complete chromo shadow domain, since the construct harbouring HP1 residues 152-206 fails to interact with Su(var)3-7 (Fig. 2). It is important to note that the deleted forms of HP1 interact with each of the three minimal regions of Su(var)3-7 (construct AA266-334, construct AA573-612, construct AA845-971) exactly as the full length HP1 (data not shown). This suggests that the three regions of Su(var)3-7 interact each with the HP1 region containing the chromo shadow domain.

As HP1, Su(var)3-7 is localised on the chomocenter but also on telomeres and a few euchromatic sites on polytene chromosomes

We wished to determine whether the protein-protein interaction in yeast corresponds to the subnuclear co-localisation of the proteins in the fly. We have previously shown that Su(var)3-7 is primarily associated with the chromocenter and the fourth chromosome in polytene chromosomes (Cléard et al., 1997). We have now re-examined the pattern using the more sensitive biotin-avidin system. Double stainings of polytene chromosomes from salivary glands with antibodies against Su(var)3-7 and HP1 were performed to determine the potential colocalisation. The results are in Fig. 3. On wild-type Canton S strain, Su(var)3-7 is mainly detected at the chromocenter, but also at a few less visible sites. Double staining reveals co-localisation of Su(var)3-7 and HP1 at the chromocenter. In addition, Su(var)3-7 is detected at some telomeres (most frequently telomeres of X and 2R) where it co-localises with HP1. A few sites in the euchromatic arms are also decorated by Su(var)3-7 although at a much lower intensity than the chromocenter. Among them, we have mapped the band 14AB on the X chromosome, a characteristic group of bands at 31, bands 42A and 60A on the second chromosome, and bands 62A and 70CD on the third chromosome (not shown). It is interesting to note that some but not all these sites were already described as HP1 binding sites (James et al., 1989). These results show that targets of Su(var)3-7 on polytene chromosomes are not restricted to centromeric heterochromatin but comprise also telomeres and specific bands in euchromatic arms. In most cases, the in vivo Su(var)3-7 binding regions are also targets of HP1.

Su(var)3-7 co-localises with HP1 on the heterochromatic *light* gene region in a variegating rearrangement

The heterochromatic *light* gene, localised in 40 on chromosome arm 2L, exhibits position-effect variegation when relocalised away from pericentric heterochromatin (Wakimoto and Hearn 1990). In *T(2;3)lt^{x13}*, the 2L chromosome arm is broken between the *light* gene and the centromere, and spliced to the chromosome arm 3R at 97C. In polytene nuclei of homozygous *lt^{x13}* larvae, we detect the Su(var)3-7 protein on the chromocenter and also on the block of heterochromatin at 97C (Fig. 4). Double-staining with antibodies against Su(var)3-7 and HP1 clearly shows that both proteins co-localise on the chromocenter, and on this second piece of heterochromatin originating from the centromeric heterochromatin of the arm 2L (Fig. 4). To establish the biological significance of this staining, we have tested the effect of the loss of a dose of *Su(var)3-7* gene on the *light* variegated allele *lt^{x13}*. Variegation was determined by eye pigments measurements of flies heterozygous for *lt^{x13}* and the hypomorphic *lt^{1}* mutation in the presence or absence of a deficiency uncovering the *Su(var)3-7* gene. The loss of a dose of *Su(var)3-7* enhances *light* variegation. For example, in females, we have measured a threefold decrease of eye pigments (data not shown). As already described for HP1, a suppressor of euchromatic gene variegation can act as an enhancer of variegation of the *light* heterochromatin-associated gene (Hearn et al., 1991). Therefore, Su(var)3-7 and HP1 bind the same heterochromatic region and affect in the same way variegation of the heterochromatic *light* gene.

As HP1, Su(var)3-7 binds to a large insertion of AAGAG satellite sequences

The *brown Dominant* (*bw^{D}*) allele is a null mutation caused by the insertion of a large block of AAGAG satellite DNA in the coding region of the *brown* gene at 59E (Slatis, 1955; Platero et al., 1998). In *bw^{D}/bw^{+}* heterozygous flies, the insertion acts in *trans* to inactivate the *bw^{+}* copy of *brown* on the homologous chromosome, resulting in a variegated eye. We have previously

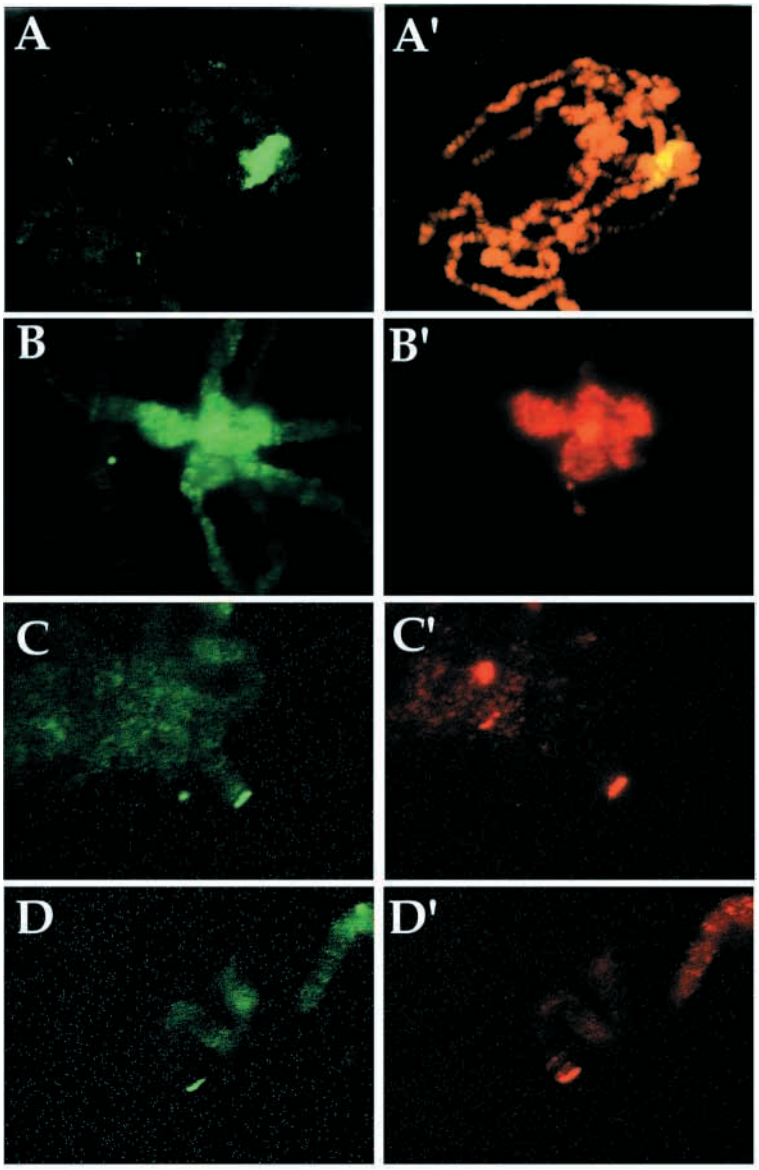

Fig. 3. Su(var)3-7 and HP1 immunolocalisation on wild-type polytene chromosomes. (A) Chromosomes stained with an anti-Su(var)3-7 antibody. Staining is enhanced by a biotinylated secondary antibody detected by fluorescein-conjugated avidin, (A′) same nucleus as in A double-staining for Su(var)3-7 (green) and for DNA with propidium iodide (red). (B to D′) Co-localisation of Su(var)3-7 and HP1 on chromocenter and telomeres. Green fluorescein-labelled anti-Su(var)3-7 on the left panels. Right panels: staining of same nuclei for HP1 in red.

shown that the loss of a dose of the *Su(var)3-7* gene suppresses *brown* variegation (Cléard et al., 1997). This implies that Su(var)3-7 plays a role in the variegation due to the proximity to the AAGAG satellite, and that this role should be reflected on chromosomes by the association of the protein with the insertion. In a line homozygous for the bw^D mutation, we detect indeed two sites bound by Su(var)3-7 on the 2R arm: the 60A site, already noted above in the wild type, and a second site, specifically stained in bw^D larvae, corresponding to the *brown* locus at 59E (Fig. 5). We also detect HP1 there, as previously described by others (Belyaeva et al., 1997; Platero et al., 1998). These results clearly show that Su(var)3-7 and HP1 are both able to bind an insertion of AAGAG satellite far away from the rest of the heterochromatin, and this confirms that the two proteins behave in a very similar manner.

Delocalised HP1 recruits Su(var)3-7 in vivo

We wanted to test whether ectopic binding of one partner would be followed by recruitment of the other. Platero and coworkers (Platero et al., 1995) have substituted the HP1 chromodomain by the chromodomain of Polycomb. This chimeric form of HP1 binds both to heterochromatin and to Polycomb binding sites in polytene chromosomes. The protein is encoded by a transgene under heat-shock control and is fused to β-galactosidase. Polytene chromosomes of flies expressing the HP1-Polycomb fusion were labelled simultaneously with antibodies against Su(var)3-7 and against the chimera (anti-β-galactosidase). Without heat-shock, the HP1-Polycomb chimera and Su(var)3-7 are mainly localised at the chromocenter (Fig. 6). After heat-shock induction, the HP1-Polycomb chimera is distributed on several euchromatic sites, corresponding to Polycomb binding sites (Platero et al.,1995). On the same nucleus, the localisation of the Su(var)3-7 protein is dramatically different from non heat shocked flies. The staining at the chromocenter has decreased while many bands on euchromatic arms are decorated (Fig. 6). These new sites of staining correspond to the same bands labeled by the antibody recognising HP1 fusion protein. This result shows that delocalised HP1 is able to recruit Su(var)3-7. This constitutes a strong argument for a close interaction in vivo between these proteins.

Over-expression of Su(var)3-7 does not modify HP1 localisation

Over-expression of the Su(var)3-7 protein leads to a strong enhancement of variegation (Reuter et al., 1990; Cléard et al., 1995; Cléard et al., 1997). Does the Su(var)3-7 distribution on polytene chromosomes expand in such conditions? To over-express the protein, we have used a transgenic line containing the complete Su(var)3-7 cDNA cloned downstream of the *hsp70* promoter (the P{HS-Su(var)3-7} line of Cléard et al., 1995). This transgene dramatically enhances *white* variegation in a *white mottled* rearrangement even in the absence of heat shock (Cléard et al., 1995). Without heat-shock, the Su(var)3-7 immunostaining is restricted to the chromocenter and the few euchromatic sites which correspond to sites already seen in wild-type condition (Fig. 7). After 2 minutes of heat-shock at 37°C, several other euchromatic binding sites appear, and this number increases with longer induction. After 45 minutes of heat-shock, chromosomes seem to be covered by the protein in a banding pattern which is different from the banding pattern obtained with propidium iodide staining of DNA (Fig. 8). It appears that these banding patterns are neither identical nor complementary (Fig. 8), meaning that Su(var)3-7 does not bind only bands or interbands. Interestingly, the endogenous HP1 protein localisation does not change in a context of strong over-expression of Su(var)3-7: HP1 is restricted to the chromocenter

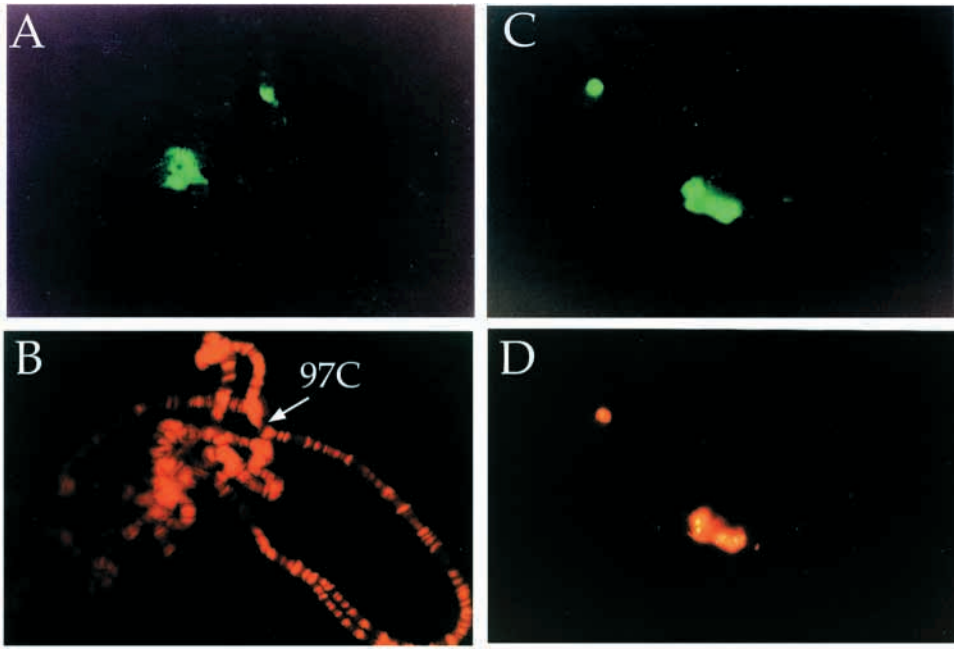

Fig. 4. Su(var)3-7 and HP1 co-localise in the chromosome rearrangement *T(2;3)lt^{x13}*. (A) Chromosomes stained with anti-Su(var)3-7; detection by a fluorescein-conjugated secondary antibody (green). The large spot corresponds to the chromocenter, and the second region stained by Su(var)3-antibody corresponds to position 97C at the junction of 2L and 3R in the *lt^{x13}* translocation. (B) Same chromosomes stained for DNA (red). (C and D) Double-staining for Su(var)3-7 (green) and HP1 (red) on another *lt^{x13}* nucleus.

and telomeres (Fig. 7). Hence, new sites bound by Su(var)3-7 when over-expressed are not able to displace endogenous HP1.

DISCUSSION

Interaction between Su(var)3-7 and HP1 in the yeast two-hybrid assay

We have shown that the C-terminal moiety of HP1, including the chromo shadow domain, interacts with Su(var)3-7 in a two-hybrid assay. The chromo shadow domain of HP1 is also involved in the interaction with SP100 (Seeler et al., 1998; Lehming et al., 1998), with TIF1α (Le Douarin et al., 1996) and with the p150 subunit of CAF-1 (Murzina et al., 1999). For the HP1 interaction with ORC proteins, both the chromo and the chromo shadow domains are required (Pak et al., 1997). The interaction of HP1 homologues with INCENP requires the region of HP1 connecting the chromo and chromo-shadow domains (Ainsztein et al., 2000). Recently also, Smothers and Henikoff (Smothers and Henikoff, 2000) have deduced a consensus peptide for binding to the HP1 chromo-shadow domain by probing a random peptide phage display library with chromo domains. Interestingly, two of the three regions of Su(var)3-7 we have determined to interact with HP1 contain a motif which fits well with this consensus (PVVMM in construct Su266-334 and PSVTV in construct Su845-971). The absence of consensus in the third region Su573-612 implicates that other protein motive could be involved in the interaction with the HP1 chromo shadow domain, as proposed for proteins devoid of this motif but which are, however, reported to interact with HP1 (Smothers and Henikoff, 2000). It is interesting to note also that Powers and Eissenberg (1993) have reported that residues 95 to 206 of HP1 are necessary for heterochromatin association. This is the same region we find to interact with Su(var)3-7. As Su(var)3-7 is itself a hetero-

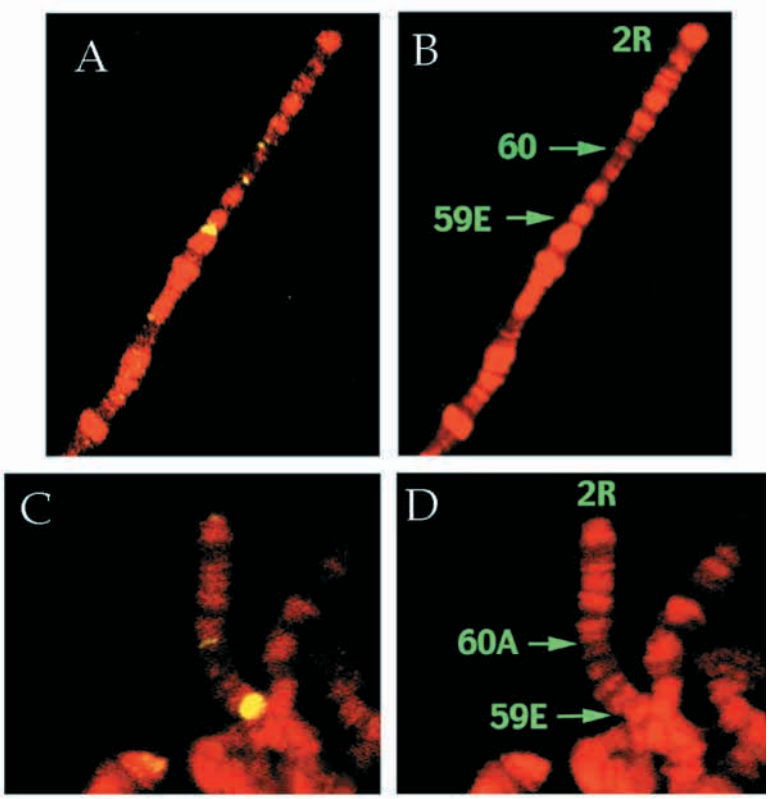

Fig. 5. Su(var)3-7 and HP1 associate with the region 59E in the *bw^{D}/bw^{D}* line. (A) Polytene chromosomes stained for DNA with propidium iodide (red) and with a rabbit anti-Su(var)3-7 antibody, detected by a fluorescein-conjugated secondary antibody (green). (B) Same chromosome stained only for DNA (red); an arrow indicates the *brown* locus at 59E. (C and D) Polytene chromosomes stained for DNA (red). Left panel, double staining with a mouse anti-HP1 antibody (green).

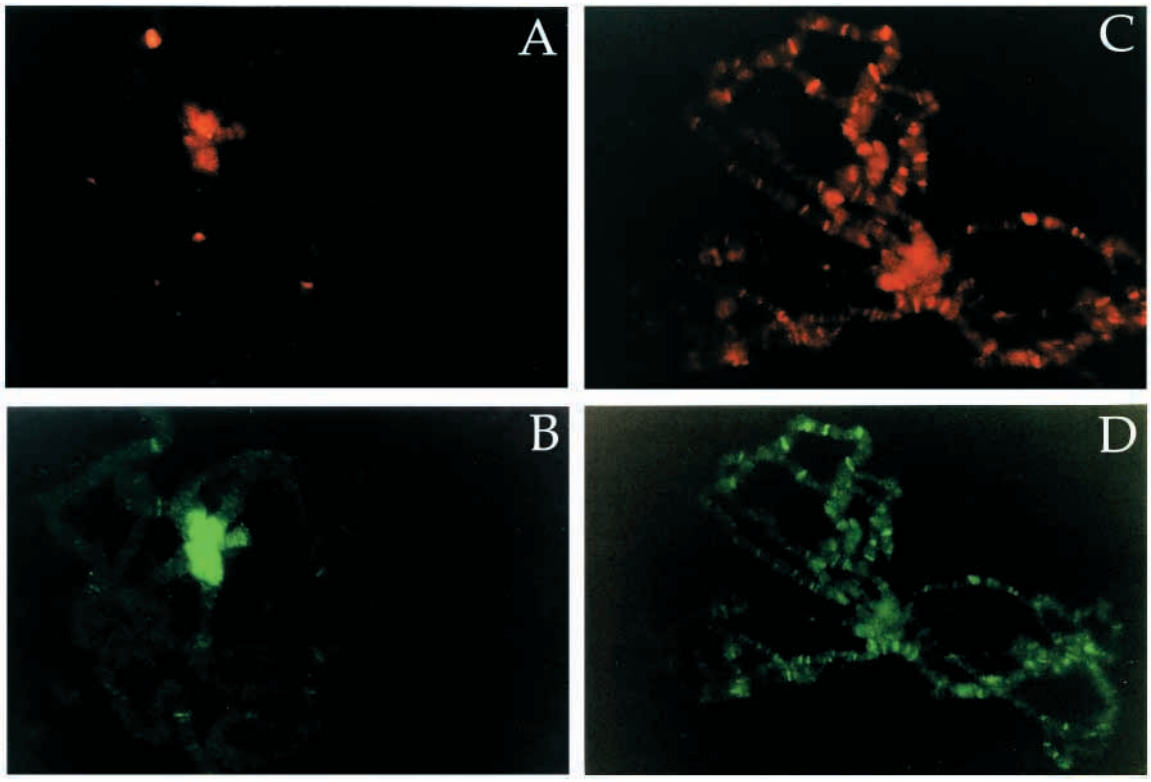

Fig. 6. The chimeric HP1-Polycomb protein recruits Su(var)3-7 on Polycomb euchromatic sites. (A) Polytene chromosomes from a transgenic line expressing a chimeric HP1/Polycomb/β-galactosidase fusion protein under heat-shock promotor. Weak expression without heat shock detected by an anti-β-galactosidase antibody, and a CY3-conjugated secondary antibody. (B) Same chromosome stained with a rabbit anti-Su(var)3-7 antibody, detected by a fluorescein-conjugated secondary antibody (green). (C and D) Immunodetection of Su(var)3-7 (green) and HP1-Polycomb chimera (red) on the same nuclei after heat-shock induction.

chromatin-associated protein, one could speculate that it mediates the binding of HP1 to heterochromatin.

Binding of Su(var)3-7 to AAGAG satellite DNA into the *brown* locus

The *brown Dominant* (bw^D) allele is a null mutation caused by an insertion of a large block of AAGAG satellite DNA in the coding region of the gene (Slatis, 1955; Platero et al., 1998). This allele has the unique property of causing variegated inactivation of a normal copy of the gene present on the homologous chromosome. Cytological studies have shown that the satellite sequences present on the mutant allele can associate with centric heterochromatin causing the mutant allele to drag the wild-type homologue at close proximity of pericentric heterochromatin (Csink and Henikoff, 1996; Dernburg et al., 1996; Henikoff, 1997). This unusual localisation in the

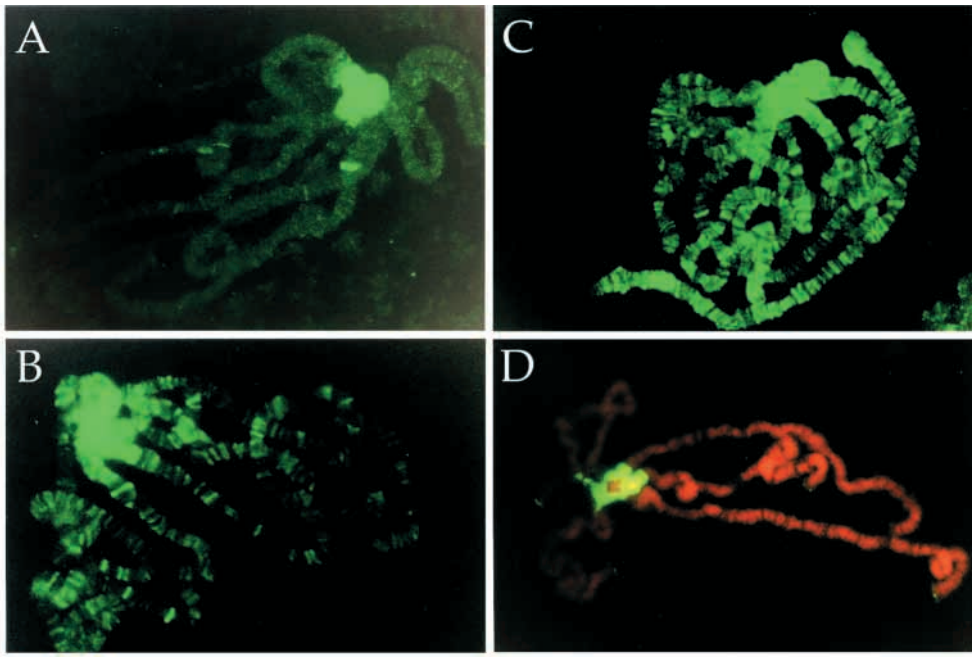

Fig. 7. Polytene chromosomes from a transgenic line expressing Su(var)3-7 protein under the control of a heat shock promoter. (A) Chromosomes stained with anti-Su(var)3-7 antibody (green) without heat shock. (B) moderate heat shock (5 minutes at 37°C). (C) Strong heat shock (45 minutes at 37°C). (D) Strong heat shock. Double staining for DNA with propidium iodide (red) and with mouse anti-HP1 antibody (green).

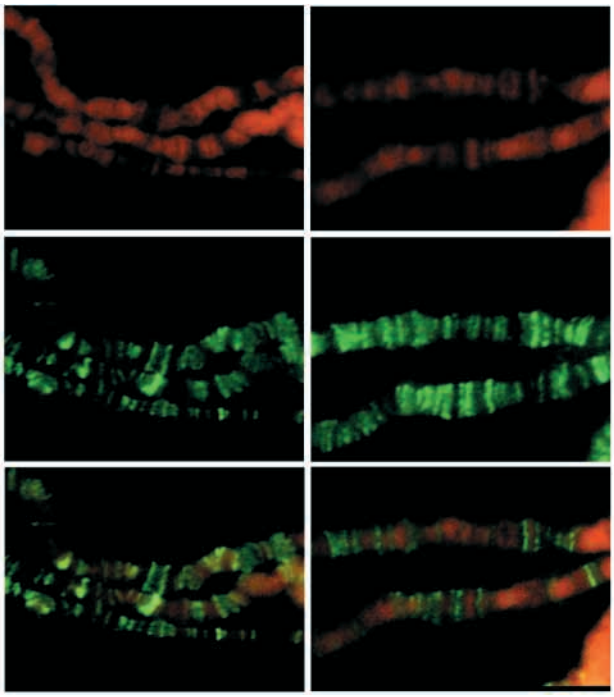

Fig. 8. Details of the pattern of over-expressed Su(var)3-7 on polytene chromosomes. Top: chromosomes stained for DNA with PI (red). Middle: same chromosomes stained for Su(var)3-7 (green). Bottom: merge of Su(var)3-7 and DNA staining, regions of overlapping appear yellow.

heterochromatin nuclear compartment, is proposed to mediate silencing of the bw^+ gene. Proteins associated with both the *brown Dominant* allele and centromeric heterochromatin could establish this reorganisation. HP1 is involved (Csink and Henikoff, 1996) and we proposed here that Su(var)3-7 is another crucial partner.

Recruitment of Su(var)3-7 by HP1

We have observed a modification of the distribution of endogenous Su(var)3-7 when the HP1-Polycomb chimera is expressed (Fig. 6). The Su(var)3-7 protein is less abundant at the chromocenter and is delocalised on Polycomb binding sites. On the other hand, Su(var)3-7 associates with many discrete sites on euchromatic arms when over-expressed (see Figs 7 and 8), but localisation of endogenous HP1 is not changed. We see two possibilities to explain the apparent inability of Su(var)3-7 to recruit HP1 ectopically, and the contrasting ability of HP1 to do so. The first one is that HP1 can recruit Su(var)3-7 at ectopic sites, but that Su(var)3-7 would need other factors to recruit HP1. The other possibility is suggested by the observation of Platero et al. (Platero et al., 1995) that in presence of the HP1-Polycomb chimera, not only does the chimera bind to Polycomb sites, but also that the endogenous HP1 is itself recruited to these sites. This leads us to speculate that this recruitment results in a decrease of the amount of endogenous HP1 at the chromocenter. Su(var)3-7 may then have no choice but to follow its partner on euchromatin arms. Inversely, we have seen that when Su(var)3-7 is over-expressed, and that some of the protein is ectopically bound on euchromatic arms, Su(var)3-7 prevails largely on pericentric heterochromatin and prevents HP1 from being displaced.

A comprehensive model of position-effect variegation

In addition to its presence in pericentric heterochromatin, and to a lesser extent in the fourth chromosome and at telomeres, Su(var)3-7 invades progressively the euchromatin arms at discrete sites when over-expressed (Fig. 7). The ability of the protein to bind to new sites on chromosomes could be due to affinity to DNA. The protein does indeed show affinity to DNA in vitro, with a preference for some satellite DNAs (F. Cléard and P. Spierer, unpublished). As reported here, however, the Su(var)3-7 pattern on chromosomes arms does not simply correspond to DNA staining. Su(var)3-7 should have a specificity for a number of DNA sequences scattered through the genome, or it can bind to a partner at these sites. The specific affinity of Su(var)3-7 for repeated DNA in vivo should now be tested with satellite DNA sequences, and with middle-repeated elements scattered in the genome. In contrast, when over-expressed, HP1 seems to associate non specifically with euchromatic arms (M. Delattre, unpublished). HP1 was recently shown to bind nucleosomes and DNA without sequence specificity (Zhao et al., 2000). This could explain, in an overexpression context, the expansion of HP1 on the polytene chromosomes in a pattern superimposable to DNA staining.

Another point stands out. Genetic evidence and immunostaining of polytene chromosomes suggest, in all of the limited number of cases examined, that Su(var)3-7, together with its companion HP1, are present wherever heterochromatin-promoted silencing is established, with the exception of telomeres (Cléard et al., 1997; this report). By binding DNA elements, namely satellites and possibly also middle repetitive DNA elements scattered in euchromatin and in heterochromatin, Su(var)3-7, in association with HP1, could both drag particular regions into the heterochromatin compartment, and drive expansion of heterochromatin by providing anchoring points in euchromatin. The spreading effect noted for a number of rearrangements could result from the high concentration of Su(var)3-7 and HP1 around large blocks of heterochromatin, favouring nucleation at the closest potential binding sites in euchromatin. This would explain both the spreading effect, the possibility of skipping (Belyaeva and Zhimulev, 1991; Talbert and Henikoff, 2000), the long distance effects (Dorer and Henikoff 1994; Csink and Henikoff, 1996; Dernburg et al., 1996) and the haplo-suppressor triplo-enhancer effect of modifiers of position-effect variegation.

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REFERENCES

Aagaard, L., Laible, G., Selenko, P., Schmid, M., Dorn, R., Schotta, G., Kuhfittig, S., Wolf, A., Lebersorger, A., Singh, P. B., Reuter, G. and

Jenuwein, T. (1999). Functional mammalian homologues of the Drosophila PEV-modifier Su(var)3-9 encode centromere-associated proteins which complex with the heterochromatin component M31. *EMBO J.* **18**, 1923-1938.

Ainsztein, A. M., Kandels-Lewis, S. E., Mackay, A. M. and Earnshaw, W. C. (1998). INCENP centromere and spindle targeting: identification of essential conserved motifs and involvement of heterochromatin protein HP1. *J. Cell Biol.* **143**, 1763-1774.

Belyaeva, E. S. and Zhimulev, I. F. (1991). Cytogenetic and molecular aspects of position effect variegation in Drosophila. III. Continuous and discontinuous compaction of chromosomal material as a result of position effect variegation. *Chromosoma* **100**, 453-466.

Belyaeva, E. S., Koryakov, D. E., Pokholkova, G. V., Demakova, O. V. and Zhimulev, I. F. (1997). Cytological study of the brown dominant position effect. *Chromosoma* **106**, 124-132.

Brown, N. H. and Kafatos, F. C. (1988). Functional cDNA libraries from Drosophila embryos. *J. Mol. Biol.* **203**, 425-437.

Cléard, F., Matsarskaia, M. and Spierer, P. (1995). The modifier of position-effect variegation Suvar(3)7 of Drosophila: there are two alternative transcripts and seven scattered zinc fingers, each preceded by a tryptophan box. *Nucl. Acids Res.* **23**, 796-802.

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

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

Dorer, D. R. and Henikoff, S. (1994). Expansions of transgene repeats cause heterochromatin formation and gene silencing in Drosophila. *Cell* **77**, 993-1002.

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

Eissenberg, J. C. and Elgin, S. C. (2000). The HP1 protein family: getting a grip on chromatin. *Curr. Opin. Genet. Dev.* **10**, 204-210.

Elgin, S. C. (1996). Heterochromatin and gene regulation in Drosophila. *Curr. Opin. Genet. Dev.* **6**, 193-202.

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

Fields, S. and Song, O. (1989). A novel genetic system to detect protein-protein interactions. *Nature* **340**, 245-246.

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

Gyuris, J., Golemis, E., Chertkov, H. and Brent, R. (1993). Cdi1, a human G1 and S phase protein phosphatase that associates with Cdk2. *Cell* **75**, 791-803.

Hearn, M. G., Hedrick, A., Grigliatti, T. A. and Wakimoto, B. T. (1991). The effect of modifiers of position-effect variegation on the variegation of heterochromatic genes of Drosophila melanogaster. *Genetics* **128**, 785-797.

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

James, T. C., Eissenberg, J. C., Craig, C., Dietrich, V., Hobson, A. and Elgin, S. C. (1989). Distribution patterns of HP1, a heterochromatin-associated nonhistone chromosomal protein of Drosophila. *Eur. J. Cell Biol.* **50**, 170-80.

Kellum, R., Raff, J. W. and Alberts, B. M. (1995). Heterochromatin protein 1 distribution during development and during the cell cycle in Drosophila embryos. *J. Cell Sci.* **108**, 1407-1418.

Le Douarin, B., Nielsen, A. L., Garnier, J. M., Ichinose, H., Jeanmougin, F., Losson, R. and Chambon, P. (1996). A possible involvement of TIF1 alpha and TIF1 beta in the epigenetic control of transcription by nuclear receptors. *EMBO J.* **15**, 6701-6715.

Lehming, N., Le Saux, A., Schuller, J. and Ptashne, M. (1998). Chromatin components as part of a putative transcriptional repressing complex. *Proc. Nat. Acad. Sci. USA* **95**, 7322-7326.

Murzina, N., Verreault, A., Laue, E. and Stillman, B. (1999). Heterochromatin dynamics in mouse cells: interaction between chromatin assembly factor 1 and HP1 proteins. *Mol. Cell* **4**, 529-540.

Pak, D. T., Pflumm, M., Chesnokov, I., Huang, D. W., Kellum, R., Marr, J., Romanowski, P. and Botchan, M. R. (1997). Association of the origin recognition complex with heterochromatin and HP1 in higher eukaryotes. *Cell* **91**, 311-323.

Platero, J. S., Hartnett, T. and Eissenberg, J. C. (1995). Functional analysis of the chromo domain of HP1. *EMBO J.* **14**, 3977-3986.

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

Powers, J. A. and Eissenberg, J. C. (1993). Overlapping domains of the heterochromatin-associated protein HP1 mediate nuclear localization and heterochromatin binding. *J. Cell Biol.* **120**, 291-299.

Réal, M. D., Ferré, J. and Mensua, J. L. (1985). Quantitative estimation of the red (drosopterins) and brown (xanthommatins) eye pigments of Drosophila melanogaster. *DIS* **61**, 198-199.

Reuter, G., Giarre, M., Farah, J., Gausz, J., Spierer, A. and Spierer, P. (1990). Dependence of position-effect variegation in Drosophila on dose of a gene encoding an unusual zinc-finger protein. *Nature* **344**, 219-223.

Reuter, G. and Spierer, P. (1992). Position effect variegation and chromatin proteins. *BioEssays* **14**, 605-612.

Ryan, R. F., Schultz, D. C., Ayyanathan, K., Singh, P. B., Friedman, J. R., Fredericks, W. J. and Rauscher, F. J. (1999). KAP-1 corepressor protein interacts and colocalizes with heterochromatic and euchromatic HP1 proteins: a potential role for Kruppel-associated box-zinc finger proteins in heterochromatin-mediated gene silencing. *Mol. Cell Biol.* **19**, 4366-4378.

Saunders, W. S., Chue, C., Goebl, M., Craig, C., Clark, R. F., Powers, J. A., Eissenberg, J. C., Elgin, S. C., Rothfield, N. F. and Earnshaw, W. C. (1993). Molecular cloning of a human homologue of Drosophila heterochromatin protein HP1 using anti-centromere autoantibodies with anti-chromo specificity. *J. Cell Sci.* **104**, 573-582.

Seeler, J. S., Marchio, A., Sitterlin, D., Transy, C. and Dejean, A. (1998). Interaction of SP100 with HP1 proteins: a link between the promyelocytic leukemia-associated nuclear bodies and the chromatin compartment. *Proc. Nat. Acad. Sci. USA* **95**, 7316-7321.

Singh, P. B., Miller, J. R., Pearce, J., Kothary, R., Burton, R. D., Paro, R., James, T. C. and Gaunt, S. J. (1991). A sequence motif found in a Drosophila heterochromatin protein is conserved in animals and plants. *Nucl. Acids Res.* **19**, 789-794.

Slatis, H. M. (1955). A reconsideration of the *brown*-dominant position effect. *Genetics* **40**, 246-251.

Smothers, J. F. and Henikoff, S. (2000). The HP1 chromo shadow domain binds a consensus peptide pentamer. *Curr. Biol.* **10**, 27-30.

Spofford J. B. (1976). Position-effect variegation in *Drosophila*. In *The Genetics and Biology of* Drosophila, vol. 1c (ed. M. Ashburner and E. Novitski), pp. 955-1018. Academic Press, London.

Talbert, P. B. and Henikoff, S. (2000). A reexamination of spreading of position-effect variegation in the white-roughest region of Drosophila melanogaster. *Genetics* **154**, 259-272.

Tschiersch, B., Hofmann, A., Krauss, V., Dorn, R., Korge, G. and Reuter, G. (1994). The protein encoded by the Drosophila position-effect variegation suppressor gene Su(var)3-9 combines domains of antagonistic regulators of homeotic gene complexes. *EMBO J.* **13**, 3822-3831.

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

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

Wreggett, K. A., Hill, F., James, P. S., Hutchings, A., Butcher, G. W. and Singh, P. B. (1994). A mammalian homologue of Drosophila heterochromatin protein 1 (HP1) is a component of constitutive heterochromatin. *Cytogenet. Cell Genet.* **66**, 99-103.

Ye, Q. and Worman, H. J. (1996). Interaction between an integral protein of the nuclear envelope inner membrane and human chromodomain proteins homologous to Drosophila HP1. *J. Biol. Chem.* **271**, 14653-14656.

Ye, Q., Callebaut, I., Pezhman, A., Courvalin, J. C. and Worman, H. J. (1997). Domain-specific interactions of human HP1-type chromodomain proteins and inner nuclear membrane protein LBR. *J. Biol. Chem.* **272**, 14983-14989.

Zhao, T., Heyduk, T., Allis, C. D. and Eissenberg, J. C. (2000). Heterochromatin protein 1 (HP1 binds to nucleosomes and DNA in vitro. *J. Biol Chem*. **275**, 28332-28338.