Loss of the modifiers of variegation Su(var)3-7 or HP1 impacts male X polytene chromosome morphology and dosage compensation

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Summary

Loss of Su(var)3-7 or HP1 suppresses the genomic silencing of position-effect variegation, whereas over-expression enhances it. In addition, loss of Su(var)3-7 results in preferential male lethality. In polytene chromosomes deprived of Su(var)3-7, we observe a specific bloating of the male X chromosome, leading to shortening of the chromosome and to blurring of its banding pattern. In addition, the chromocenter, where heterochromatin from all polytene chromosomes fuses, appears decondensed. The same chromosomal phenotypes are observed as a result of loss of HP1. Mutations of *Su(var)3-7* or of *Su(var)2-5*, the gene encoding HP1, also cause developmental defects, including a spectacular increase in size of the prothoracic gland and its polytene chromosomes. Thus, although structurally very different, the two proteins cooperate closely in chromosome organization and development. Finally, bloating of the male X chromosome in the *Su(var)3-7* mutant depends on the presence of a functional dosage compensation complex on this chromosome. This observation reveals a new and intriguing genetic interaction between epigenetic silencing and compensation of dose.

Key words: Dosage compensation, *Drosophila*, HP1, Mitotic chromosome, Polytene chromosome, Su(var)3-7

Introduction

Euchromatic genes placed near or within heterochromatin by chromosomal rearrangements often become epigenetically silenced in some cells and not in others, a phenomenon called position-effect variegation (PEV). By contrast, heterochromatic genes can become silenced in euchromatin (reviewed by Weiler and Wakimoto, 1995). Because of the importance of epigenetics in gene regulation, studying the different partners involved in establishing large silent chromatin domains in PEV has become an extremely useful tool. Among these components, the proteins HP1, Su(var)3-9 and Su(var)3-7 are mainly associated with pericentric heterochromatin and play a key role (Wallrath, 1998; Li et al., 2002; Schotta et al., 2003). The genes coding for these three proteins are haplo-supressor, triplo-enhancer of PEV. HP1 and Su(var)3-9 have a central function in heterochromatin establishment and maintenance (Bannister et al., 2001), and in gene regulation (Nielsen et al., 2001; Hwang et al., 2001; Greil et al., 2003). The mode of action of HP1 and of the histone methyltransferase Su(var)3-9 has been demonstrated in part: Su(var)3-9 methylates lysine 9 of histone H3 (H3-MeK9), thus creating a binding site for HP1 (Rea et al., 2000; Bannister et al., 2001; Lachner et al., 2001). The cooperative action of Su(var)3-9 and HP1 is instrumental in forming silent heterochromatin (reviewed by Grewal and Rice, 2004).

Su(var)3-7 function is still poorly understood. It encodes a large protein associated with pericentric heterochromatin, telomeres and a few euchromatic sites on interphase polytene chromosomes. Seven widely spaced zinc fingers stand out in the sequence of the N-terminal half (Reuter et al., 1990; Cléard et al., 1995; Cleard et al., 1997). In vitro, the zinc finger region of Su(var)3-7 has affinity for DNA, and preferentially for some satellite sequences (Cléard and Spierer, 2001). There is also evidence for direct binding of Su(var)3-7 with DNA in vivo (Perrini et al., 2004). The N-terminal half of Su(var)3-7 interacts nonspecifically in vivo with heterochromatin and euchromatin, whereas the C-terminal half promotes interaction with itself, and with pericentric heterochromatin (Jaquet et al., 2002). Su(var)3-7 also interacts genetically and physically with HP1 (Cléard et al., 1997; Delattre et al., 2000) and with Su(var)3-9, as determined in yeast by the two-hybrid assay (Schotta et al., 2002) and in vivo (Delattre et al., 2004). To decipher the function of Su(var)3-7, we have generated mutants by homologous recombination (Seum et al., 2002), and have undertaken a detailed examination of their phenotype. Su(var)3-7 was shown to be essential, the maternal contribution being sufficient for viability. Interestingly, males are more sensitive than females to the lack of Su(var)3-7 (Seum et al., 2002). The cause of this lethality is unknown.

Here, we report the building of a new mutant of *Su(var)3-7* by homologous recombination, and describe the phenotypes of mutations on polytene chromosome morphology and on the organism, which we find similar to phenotypes resulting from mutational loss of HP1. The male X chromosome is more sensitive to these effects, leading us to unravel an interaction

between the modifier of PEV Su(var)3-7 and the dosage compensation machinery. We conclude that the importance of the roles and partnership of Su(var)3-7 and HP1 extend beyond genomic silencing in the maintenance of chromosome integrity and function, including the male X-specific chromosome-wide mechanism of dosage compensation.

Materials and Methods

Genetic crosses

Crosses and culture were at 25°C on standard media. Homozygous *mle*^{1}/*Su(var)3-7*^{14} male larvae were selected from the strain *w*; *mle*$^{1\ or\ 9}$/*CyO GFP; Su(var)3-7*^{14}/*TM6B Tb*. The GFP transgene of the *CyO* balancer chromosome allows the scoring of the larvae containing this marker under a fluorescent dissecting microscope. Homozygous *mle*$^{1\ or\ 9}$/*Su(var)3-7*^{14} males were selected by the absence of fluorescence and of the dominant *Tb* mutation. Salivary glands were dissected for polytene chromosome squashing, and the remains of the larva were used for DNA extraction to verify by PCR the absence of the *Su(var)3-7* wild-type gene. Trans-heterozygous *Su(var)2-5* mutants were generated by crossing females *w*^{m4}; *Su(var)2-5*^{05}/*Cy* with males *w*^{m4}/*Y*; *Su(var)2-5*$^{04\ or\ 02}$/*CyO*. Homozygous *Su(var)2-5*^{05}/*Su(var)2-5*$^{04\ or\ 02}$ mutants were identified, after squashing of polytene chromosomes, either by the absence of the multiply rearranged balancer chromosome, or by the absence of HP1 immunostaining [at third-instar larval stage, the maternal HP1 product is not detectable (Lu et al., 2000)].

Generation of a Su(var)3-7 mutant by site-directed mutagenesis

Strategy

The procedure is adapted from Rong and Golic (Rong and Golic, 2001), and Xie and Golic (Xie and Golic, 2004). The *Su(var)3-7* locus is flanked upstream (according to transcription orientation) by *Ravus* and downstream by *CG8449*. *Su(var)3-7* and *CG8449* transcripts overlap as seen by the position of the two *Su(var)3-7* polyA sites on the *CG8449* locus (Fig. 7A, vertical arrows). We designed a donor construct comprising 2.6 kb upstream of the Su(var)3-7 protein coding sequence, and including *Ravus*, and 4.0 kb downstream of *Su(var)3-7* coding sequence, and including *CG8449*. The I-SceI site was inserted in the middle of downstream sequence in order to cause homologous recombination in the third exon of *CG8449*. We obtained 24 independent donor transgenic lines, and selected four different lines on the X or on the second chromosome for the targeting protocol. After crossing the different donors with flies expressing FLP and I-SceI under the heat shock promoter, we screened for *w*$^{+}$ non-mosaic flies for further analysis. 29 recombinants were obtained, 18 of which were mapped to the third chromosome. These were amplified by PCR using oligos 1 and 2 (see below) to confirm their targeting (Fig. 7C). They were also crossed with *bw*^{VDe2} to verify that they had no effect on PEV, showing that one copy of *Su(var)3-7* was intact. The reduction step is also shown schematically in Fig. 7. The targeted allele used for the reduction was not intact. We chose a homologous recombinant bearing a 1033 bp deletion in the *CG8449* gene and deleting the second *Su(var)3-7* polyA site (Fig. 7D) in order to displace the equilibrium towards a recombination event in the 5′ region of *Su(var)3-7* (Fig. 7F). Males of this allele (*R2a*) were crossed with females producing the I-Cre enzyme under the hsp70 promoter, giving rise to the deletion in 8 cases out of 9 tested. The homozygote mutant flies were then crossed to each other to confirm the recessive maternal effect observed by Seum et al. (Seum et al., 2002) and tested for a Su(var) effect on PEV by crossing them to the *Heidi* variegating line (Seum et al., 2000).

Cloning of the donor

The 5′ noncoding region of *Su(var)3-7* was amplified using oligos 5′-CTACTGCGGGAAAGCCATGTTAGAG-3′ (forward) and 5′-GTC-ATCCCGCATCGGGTACCTGTCCTC-3′ (reverse) on genomic DNA of wild-type adult flies. The 2.6 kb PCR product was digested with *Hin*dIII/*Kpn*I and cloned into pH7-30 (Seifert et al., 1986) (=pH7-30HK5′). The 3′ noncoding region of *Su(var)3-7* was amplified as two fragments using primers: 5′-GTGCGTTGTGTACCTCTTGAC-3′ (forward) and 5′-CGGCCAGCTGAAGCTTCATACGGGAC-3′ (reverse) for the most 5′ part (1.95 kb) and primers 5′-GTC-CCGTATGAAGCTTCAGCTGGCCG-3′ (forward) and 5′-ATCC-GATCCGAGCTCTTCGCATTTCG-3′ (reverse) for the 3′ portion (2.05 kb). Both PCR fragments were digested with *Sac*I/*Hin*dIII and inserted into pGemT easy (*Sac*I) (Promega). Two oligos containing the I-SceI recognition site and flanked with incomplete *Hin*dIII sites were generated (5′-AGCTGCTAGGGATAACAGGGTAAT-3′ and 5′-AGCTATTACCCTGTTATCCCTAGC-3′), annealed and inserted into the *Hin*dIII site of this vector (pGemT S I-SceI S3′). This construct was then digested with *Sac*I and the 4 kb fragment containing the whole 3′ untranslated region was cloned into pH7-30HK5′ (partial *Sac*I). The 5′ and 3′ noncoding regions of *Su(var)3-7* were finally joined into pTV2 (Rong and Golic, 2001) as an 8 kb *Not*I fragment, the 3′ part facing the I-CreI site [pTV2ΔSu(var)].

Targeting screen

We followed a procedure described previously (Rong and Golic, 2001). The pTV2ΔSu(var) plasmid was injected into a *w1118* strain and four donors on the second or X chromosomes were used for the recombination [rapid scheme (Rong and Golic, 2001)]. We carried out about 10 crosses, implicating 20 homozygous female donors and *yw*; *70FLP* (site-specific recombinase), *70I-SceI*, *Sco/CyO* males. Two heat shocks were done on first- and second-instar larvae for one hour at 37°C. We then made 267 crosses, each time using four mosaic females carrying the *70FLP*, *70 I-SceI* chromosome with *yw* homozygous males expressing *70FLP* constitutively. Screening was by selecting non-mosaic *w*$^{+}$ flies on the third chromosome. Homologous recombinants were obtained containing the deletion and a wild-type copy of the *Su(var)3-7* gene separated by the *w*^{Hs} marker (Fig. 7C).

Reduction

The reduction step was performed to eliminate the *Su(var)3-7* sequence (Cléard et al., 1997) and the *w*^{Hs} marker. The targeted allele used for the reduction was not intact. We chose a homologous recombinant bearing a 1033 bp deletion in *CG8449* (*R2a*, characterized with primers 1 and 2 and sequenced with primer 3 in order to increase the number of recombination events in the 5′ region of *Su(var)3-7*; Fig. 7D,F). *R2a/T(2;3)ap*^{Xa} males were crossed with *70I-Cre 1A*, *Sb1/TM6* females (Bloomington stock 6937). Heat shocks were made on first-instar larvae for one hour at 37°C and *Sb* variegated males were balanced with *w*^{1118}; *CyO; TM3/T(2;3)ap*^{Xa} females. *w*$^{–}$*/TM3* flies were crossed with each other and homozygotes for the deletion were analyzed by PCR using primers 4 and 5 but also crossed together to confirm the recessive lethal maternal effect of the mutation. Different couples of primers were used to confirm the deletion: we tested the absence of FRT (FLP recognition target) and *Su(var)3-7* using primers in FRT and all along the *Su(var)3-7* gene, and comparing homozygote to heterozygote single flies. Finally, the deletion was amplified using primers 4 and 5 and sequenced with primer 5 (Fig. 7F).

Staining and immunostaining of polytene chromosomes

Larvae were dissected, and salivary glands transferred and squashed in 45% acetic acid. Slides were dehydrated for at least 20 minutes in 100% ethanol and air-dried. A drop of staining solution (1% orcein

in a 1:1 mix of 60% acetic acid and lactic acid) was deposited on a coverslip and applied on the polytene chromosomes. Excess of staining solution was removed, and the coverslip sealed with nail polish. Procedures for immunostaining were as described previously (Platero et al., 1995). Briefly, salivary glands were dissected in Cohen's buffer, fixed for 2 minutes in 2% formaldehyde, 2% Triton X-100, and then squashed in 2% formaldehyde, 45% acetic acid. Primary antibodies were used at the following dilutions: 1:10 for anti-Su(var)3-7 antibody (Cléard et al., 1997), 1:400 for anti-HP1 antibody (a gift of L. Wallrath, University of Iowa, Iowa City, IA), and 1:200 for anti-MSL2 antibody (a gift of M. Kuroda, Harvard Medical School, Boston, MA) and for anti-H4AcK16 antibody (a gift of B. Turner, University of Birmingham, UK).

Immunostaining of neuroblasts mitotic chromosomes

Immunostaining of mitotic chromosomes was carried out according to the procedure of Roxane Blattes and Emmanuel Käs (personal communication). Larval brains were incubated for 15 minutes in 0.5% sodium citrate, then in HEN buffer [5 mM $MgCl_2$, 0.1% Triton X-100, 1× HEN (10 mM Hepes pH 7.4, 1 mM EDTA, 0.1 M NaCl)] for 2 hours. Brains were fixed in 2% paraformaldehyde in HEN buffer for 10 minutes and in 2% paraformaldehyde/45% acetic acid for 8 minutes after squashing. Rehydration was for 45 minutes in blocking solution (PMT-5% dried milk: 1× PBS, 0.1% Triton X-100, 1 mM $MgCl_2$, 5% dried milk). PMT-5% was removed and squashes were incubated with the primary antibodies overnight at 4°C in PMT with 0.5% dried milk (PMT-0.5%). After washing in PMT-0.5%, squashes were incubated with the secondary antibodies for 90 minutes in PMT-0.5%. Squashes were washed in PT (PMT without dried milk), stained with DAPI and mounted. Primary antibodies were used at the same dilutions as for polytenes.

Results

Su(var)3-7 mutants

We have previously generated three *Su(var)3-7* mutants by homologous recombination: $Su(var)3\text{-}7^{14}$, $Su(var)3\text{-}7^{7.1A}$ and $Su(var)3\text{-}7^{9}$ (Seum et al., 2002). Although the mutants still contain *Su(var)3-7* sequences, one of them, $Su(var)3\text{-}7^{14}$, behaves genetically as a null mutation. This latter allele produces significant amounts of a chimeric protein made of Su(var)3-7 and Yellow (Seum et al., 2002). As we could not exclude an effect of this aberrant product, we report here the generation by homologous recombination of a new mutant, $Su(var)3\text{-}7^{R2a8}$. The strategy allowed us to delete precisely all the protein-coding sequence published by Cléard et al. (Cleard et al., 1995). Targeted mutagenesis in *Drosophila* is still far from trivial, and we recount in detail the design and construction of the mutant in the Materials and Methods section. In short, we used the 'ends-in' technology (Rong and Golic, 2001) and selected regions of homology 5′ and 3′ of the coding sequence to generate a null allele after homologous recombination (Xie and Golic, 2004). Unfortunately, a very recent correction of the *Su(var)3-7* gene sequence by the Berkeley *Drosophila* Genome Project has added a translation start in the *Su(var)3-7* transcript upstream of the one previously considered. This adds 81 amino acids to the previously reported 1169 amino acids, resulting in a new size of 1250 amino acids. These 81 amino acids out of 1250 do not contain nuclear localization signals, nor any known sequence motif, and were absent from transgene constructs rescuing the function (Jaquet et al., 2002). It is thus extremely unlikely that the fragment potentially produced by this mutant has any biological activity.

The new allele $Su(var)3\text{-}7^{R2a8}$ is a recessive maternal-effect lethal mutant. Progeny of homozygous mutant flies stop developing during the second larval stage. A zygotic wild-type dose rescues the maternal effect, but only half of the expected male progeny is recovered. This underlines the particular sensitivity of males to loss of Su(var)3-7. However, the maternal product alone allows about 90% viability of zygotic male and female null mutant progeny in equal proportion. These phenotypes are similar to those we observed with $Su(var)3\text{-}7^{14}$ (Seum et al., 2002).

In summary, we have four *Su(var)3-7* alleles: $Su(var)3\text{-}7^{R2a8}$, $Su(var)3\text{-}7^{14}$, $Su(var)3\text{-}7^{7.1A}$ and $Su(var)3\text{-}7^{9}$. $Su(var)3\text{-}7^{9}$ is the weakest and the only homozygous viable allele. $Su(var)3\text{-}7^{R2a8}$ and $Su(var)3\text{-}7^{14}$ have very similar phenotypes and behave genetically as null mutations.

The male X polytene chromosome and the chromocenter are affected by loss of either Su(var)3-7 or HP1

As Su(var)3-7 and HP1 are chromosome-associated proteins, we examined polytene chromosomes from salivary glands of mutant third-instar larvae. In order to reduce both maternal and zygotic doses of Su(var)3-7, we tested several homozygous and trans-heterozygous combinations of *Su(var)3-7* mutants allowing viability of third-instar larvae. One phenotype in particular is striking: the morphology of the X chromosome of affected male larvae is dramatically modified by severe loss of Su(var)3-7 (Fig. 1). The male X chromosome becomes shorter and bloated. The banding pattern is blurred and the whole

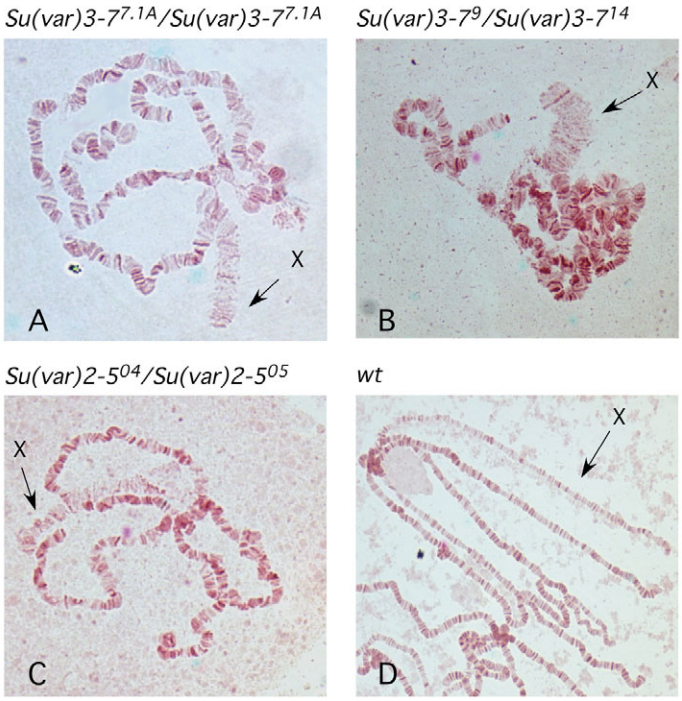

Fig. 1. Bloated X phenotype of salivary gland polytene chromosomes from *Su(var)3-7* or *Su(var)2-5* mutant males. (A) $Su(var)3\text{-}7^{7.1A}$ homozygote mutant. (B) $Su(var)3\text{-}7^{9}/Su(var)3\text{-}7^{14}$ mutant. (C) $Su(var)2\text{-}5^{04}/Su(var)2\text{-}5^{05}$ mutant. (D) Wild-type control male. Chromosomes are stained with orcein. X indicates the X chromosome.

chromosome is less dense. Morphology of the female X chromosomes does not differ from autosomes (not shown). All of the combinations tested show the bloated X phenotype in males, though the levels of male X bloating vary according to the amount of maternal and/or zygotic amount of Su(var)3-7 remaining. We also noted that X bloating varies according to the genetic background. Indeed, the yw^{67}, w^{1118}, or wild-type X chromosomes exhibit different degrees of sensitivity to *Su(var)3-7* mutation (not shown). Temperature also has an effect on this phenotype: raising *Su(var)3-7* mutant males at 18°C rather than at 25°C enhances the phenotype. All allelic combinations made with the newly designed mutant $Su(var)3\text{-}7^{R2a8}$ show less-severe phenotypes than those obtained with $Su(var)3\text{-}7^{14}$, $Su(var)3\text{-}7^{7.1A}$ and $Su(var)3\text{-}7^{9}$ (not shown). This result leads us to think that potential antimorphic effects of the first allelic series (Seum et al., 2002) could aggravate the male X bloating phenotype (see Discussion).

Knowing that the modifier of PEV *Su(var)2-5*, encoding HP1, interacts with *Su(var)3-7* (Cléard et al., 1997; Delattre et al., 2000), we also examined polytene chromosome of mutants of the following genotypes: $Su(var)2\text{-}5^{04}/Su(var)2\text{-}5^{05}$ or $Su(var)2\text{-}5^{02}/Su(var)2\text{-}5^{05}$ (Eissenberg et al., 1992). Larvae of these genotypes reach the third instar and then die. As a consequence of severe loss of HP1, the larval gonads are barely visible and determination of sex is difficult (Platero et al., 1995; Fanti et al., 1998). To determine whether a larva was a male or a female, one salivary gland of a pair was stained with orcein, whereas the other was immunostained with an antibody against MSL2, a subunit of the male-specific dosage compensation complex (DCC) (Lyman et al., 1997). In polytene chromosomes of *Su(var)2-5* mutants, we found the same bloating of the male X chromosome as in *Su(var)3-7* mutants (Fig. 1).

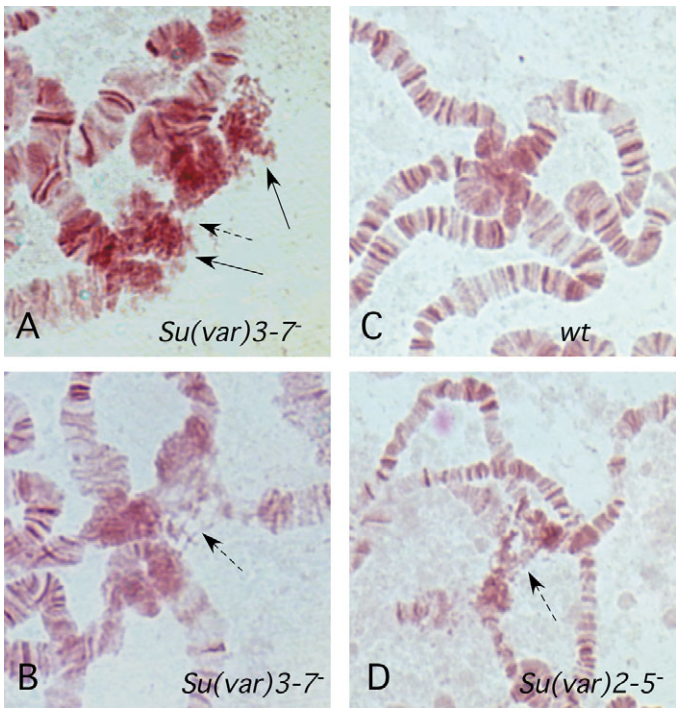

Fig. 2. Loose chromocenter morphology of salivary gland polytene chromosomes of *Su(var)3-7* or *Su(var)2-5* mutants. (A) Severe $Su(var)3\text{-}7^{14}/Su(var)3\text{-}7^{9}$ mutant, and (B) less-affected $Su(var)3\text{-}7^{14}$ homozygote. (C) Wild-type control. (D) *HP1* mutant: $Su(var)2\text{-}5^{04}/Su(var)2\text{-}5^{05}$. Chromosomes are stained with orcein. Arrows point at granules and dotted arrows at fibers.

A second phenotype seen in males and in females of all mutants concerns the morphology of the chromocenter. This region, where pericentric heterochromatin from all chromosomes coalesces, has a modified aspect in *Su(var)3-7* and *Su(var)2-5* mutants. It becomes larger than in wild type, and the chromosomes arms are not held together as tightly, but instead are attached by a loose net of thin fibers and aggregates of granules, occasionally forming small outgrowths (Fig. 2).

We conclude that severe loss of Su(var)3-7 or HP1 causes the same phenotypes on polytene chromosomes, suggesting strong similarity or cooperation in their chromatin-condensing function.

Su(var)3-7 requires HP1 for heterochromatin binding on interphase chromosomes

Immunostaining of polytene chromosomes has shown that Su(var)3-7 and HP1 colocalize on polytene chromosomes at the chromocenter, some telomeres and at several euchromatic sites (Cléard et al., 1997; Delattre et al., 2000). As mutations in *Su(var)3-7* and *Su(var)2-5* share common phenotypes, we wondered whether the loss of one protein influences the localization of the other. To answer this question, we examined the distribution of HP1 on polytene chromosomes of *Su(var)3-7* mutants. HP1 immunostaining is not perturbed on polytene chromosomes of $Su(var)3\text{-}7^{9}/Su(var)3\text{-}7^{14}$ larvae (Fig. 3) nor on $Su(var)3\text{-}7^{R2a8}$ and $Su(var)3\text{-}7^{14}$ homozygous mutant polytenes (not shown). The distribution of H3-diMe-K9, another marker of heterochromatin (Jacobs et al., 2001) that colocalizes in wild-type heterochromatin with HP1 and also with Su(var)3-7 (Delattre et al., 2004), is also not affected (not shown). We investigated then whether, conversely, Su(var)3-7 binding to polytene chromosomes was modified in a trans-heterozygous null mutant of *Su(var)2-5*, namely after loss of zygotic HP1 [as $Su(var)2\text{-}5^{04}/Su(var)2\text{-}5^{05}$]. Indeed, Su(var)3-7 loses its specific association with heterochromatin of polytene chromosomes and is found on euchromatin (Fig. 3). In addition, Fig. 3 illustrates, as a control, the situation in the *Su(var)2-5/+* heterozygous mutant, where one dose of HP1 suffices to keep Su(var)3-7 in heterochromatin. These experiments demonstrate that Su(var)3-7 relies on HP1 to associate specifically with pericentric heterochromatin on interphase polytene chromosomes. However, we still wondered how the loss of proteins mainly associated with heterochromatin in polytene chromosomes affects the morphology of a whole chromosome. We therefore examined mitotic chromosomes.

Su(var)3-7 and HP1 do not overlap on mitotic chromosomes

We stained mitotic chromosomes from wild-type larval neuroblasts with antibodies against Su(var)3-7. Surprisingly, the pattern of Su(var)3-7 is different from the characteristic chromocentric staining of interphase polytene chromosomes. Fig. 4 shows an association of Su(var)3-7 throughout euchromatic chromosome arms, although with discontinuities.

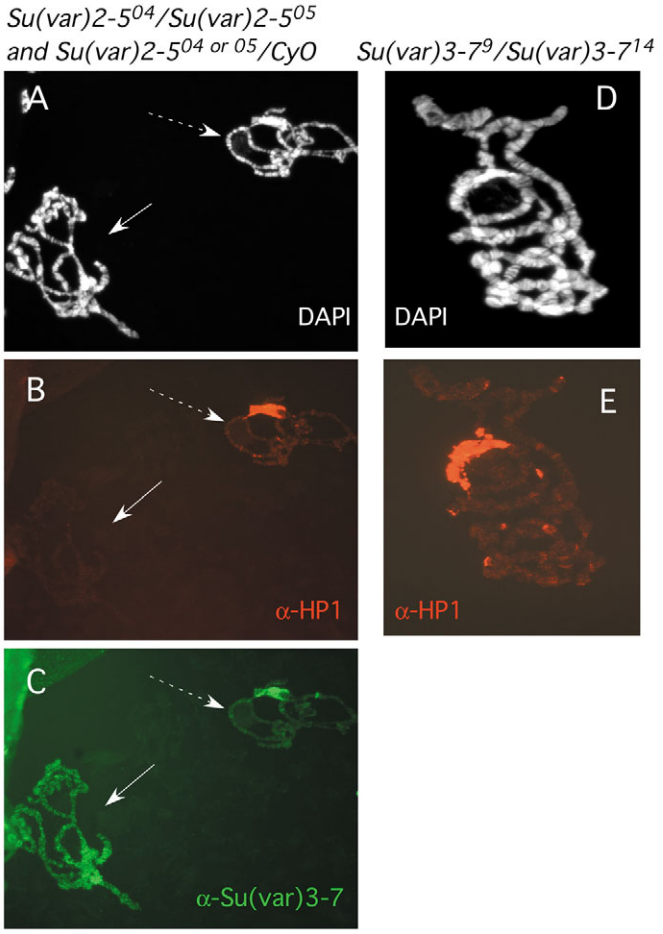

Fig. 3. Immunolocalization of Su(var)3-7 and HP1 on polytene chromosomes. Gray, DAPI; red, HP1; green, Su(var)3-7. (A-C) Detection of Su(var)3-7 protein on *Su(var)2-5*^{04}*/Su(var)2-5*^{05} mutant (arrow) with heterozygote control *Su(var)2-5*$^{04\ or\ 05}$*/CyO* on the same slide (dotted arrow). (D,E) Detection of HP1 protein on *Su(var)3-7*^{9}*/Su(var)3-7*^{14} mutant.

Within the limit of the method, the Su(var)3-7 protein was neither detected on the fourth chromosome, nor on heterochromatin of the third, second and X chromosomes. The Y chromosome is partially stained (Fig. 4B). By contrast, using the same fixation conditions, HP1 is seen on heterochromatin of all mitotic chromosomes including the fourth and the Y chromosome (Fig. 4C). Anti-H3-diMe-K9, another heterochromatin marker, shows the same staining pattern as HP1 (not shown). Thus, in contrast to polytene chromosomes, Su(var)3-7 and HP1 form an almost complementary pattern on larval brain mitotic chromosomes. HP1 staining appears unchanged in *Su(var)3-7* mutant brain neuroblasts (as illustrated for the *Su(var)3-7*^{9}*/Su(var)3-7*^{14} trans-heterozygotes in Fig. 4I). And staining of mitotic chromosomes from the *Su(var)2-5* mutant with the anti-Su(var)3-7 antibody shows that euchromatic localization of Su(var)3-7 is not modified (Fig. 4E), indicating that Su(var)3-7 association to mitotic euchromatin is not dependent on HP1.

We conclude that, within the limits of detection of the method, and in contrast to interphase polytene chromosomes (where both proteins associate mainly with pericentric heterochromatin), HP1 and Su(var)3-7 do not colocalize on larval mitotic chromosomes, where HP1 is still mainly seen on heterochromatin and Su(var)3-7 on euchromatic arms. This is a new lead towards the understanding of the whole X-chromosome phenotype of mutants, which will be explored in the Discussion.

Severe loss of either Su(var)3-7 or HP1 causes similar developmental defects

In our search for causes and consequences of chromosome defects in mutants, we wondered whether loss of Su(var)3-7 and HP1 results in developmental abnormalities. Indeed, we detected several interesting and unexpected phenotypes shared by larvae affected by a severe decrease in amounts of Su(var)3-7 or HP1. The most spectacular and penetrant phenotype concerns the ring gland. The ring gland is a composite endocrine organ containing the cells of the prothoracic gland (source of the moulting hormone ecdysone), the corpus allatum (source of the juvenile hormone) and the corpus cardiacum (source of peptide hormones). In wild-type larvae, the ring gland represents a small flat ring anterior to the brain hemispheres. *Su(var)3-7* and *Su(var)2-5* mutant larvae exhibit an extreme enlargement of their ring gland owing to an increase in size of the ecdysone-secreting prothoracic gland cells (Fig. 5A,B). These cells exhibit highly endoreplicated polytene chromosomes (Fig. 5D). In wild-type larvae, polytene chromosomes of prothoracic gland cells can reach a degree of polyteny of 256C, whereas salivary gland chromosomes reach 1024C or even 2048C (Hochtrasser and Sedat, 1987). Here, we show that *Su(var)3-7* and *Su(var)2-5* mutant larvae exhibit prothoracic gland cell nuclei that have polytene chromosomes similar in size to those of the salivary gland. As salivary glands and their polytene chromosomes are not larger in mutants than in wild type, we conclude that reduced levels of Su(var)3-7 or HP1 affect endoreplication specifically in certain polytene tissues. Other phenotypes are observed in both mutants. The gastric ceca are abnormal. Gastric ceca consist of four appendages growing out of the proventriculus (the larva stomach), at the junction between the foregut and the midgut. In larvae of *Su(var)3-7* and *Su(var)2-5* mutants, the ceca are shorter and wider, and pairs of appendages are often fused (Fig. 5F). Moreover, the proventriculus and the anterior midgut are swollen in mutants (Fig. 5F). These parallel phenotypes provide additional evidence of a functional link between Su(var)3-7 and HP1, with an impact on development.

The chromosome phenotype of *Su(var)3-7* mutants is suppressed by mutation of dosage compensation

We have been puzzled by two unexpected observations of *Su(var)3-7* mutant phenotypes. First, the greater sensitivity of males to lack of Su(var)3-7 (Seum et al., 2002) (M.D., unpublished observations), and second the bloated male X chromosome, a phenotype shared by mutants of *Su(var)2-5*. What could explain the specific sensitivity of the male X chromosome to low doses of Su(var)3-7 or HP1? A specific feature of the male X chromosome in *Drosophila* is dosage compensation, a mechanism by which the single male X chromosome is hyperactivated in order to reach the transcription level of the two female X chromosomes. This

activation is driven by a complex of at least five main proteins (MSL1, MSL2, MSL3, MLE and MOF) and two noncoding RNAs (*roX-1* and *roX-2*) that assemble specifically on the male X chromosome (for reviews, see Akhtar, 2003; Kelley, 2004). Hyperactivation results from acetylation of lysine 16 of histone H4, leading to an increase in transcription of X-linked genes (Smith et al., 2001).

We first tested by immunostaining whether the absence of Su(var)3-7 prevents the DCC from forming on the male X chromosome. The DCC is present, as the bloated male X of *Su(var)3-7* mutants is entirely stained by antibodies against MSL2 or histone H4 acetylated on lysine 16 (Fig. 6A,B). To examine the possible link between Su(var)3-7 and dosage compensation further, we looked at polytene chromosomes of flies mutant in *mle*, a gene coding for the RNA helicase component of the DCC (Kuroda et al., 1991; Lee et al., 1997). Mutants of this gene do not compensate for dose and die at the third-instar larval stage, allowing examination of polytene chromosomes (Kuroda et al., 1991). When we combined the mle^{1} null mutation (Rastelli and Kuroda, 1998) and the $Su(var)3\text{-}7^{14}$ mutation, both as homozygotes, the bloated X phenotype expected from the loss of Su(var)3-7 was rescued. The male X has an mle^{1}/mle^{1} configuration (Fig. 6). To ascertain that the effect was due to mutation of *mle*, we repeated this experiment with the mle^{9} allele, a mutation produced with another mutagen in another genetic background (Kernan et al., 1991). We obtained the same result (not shown). These experiments suggest that the presence of the DCC is necessary for the bloated X phenotype in *Su(var)3-7* mutant larvae. The fact that the loss of a component of dosage compensation counteracts the loss of Su(var)3-7 implies that the *mle* and *Su(var)3-7* genes interact, and reveals a possible participation of Su(var)3-7 in the chromatin structure of the male X chromosome.

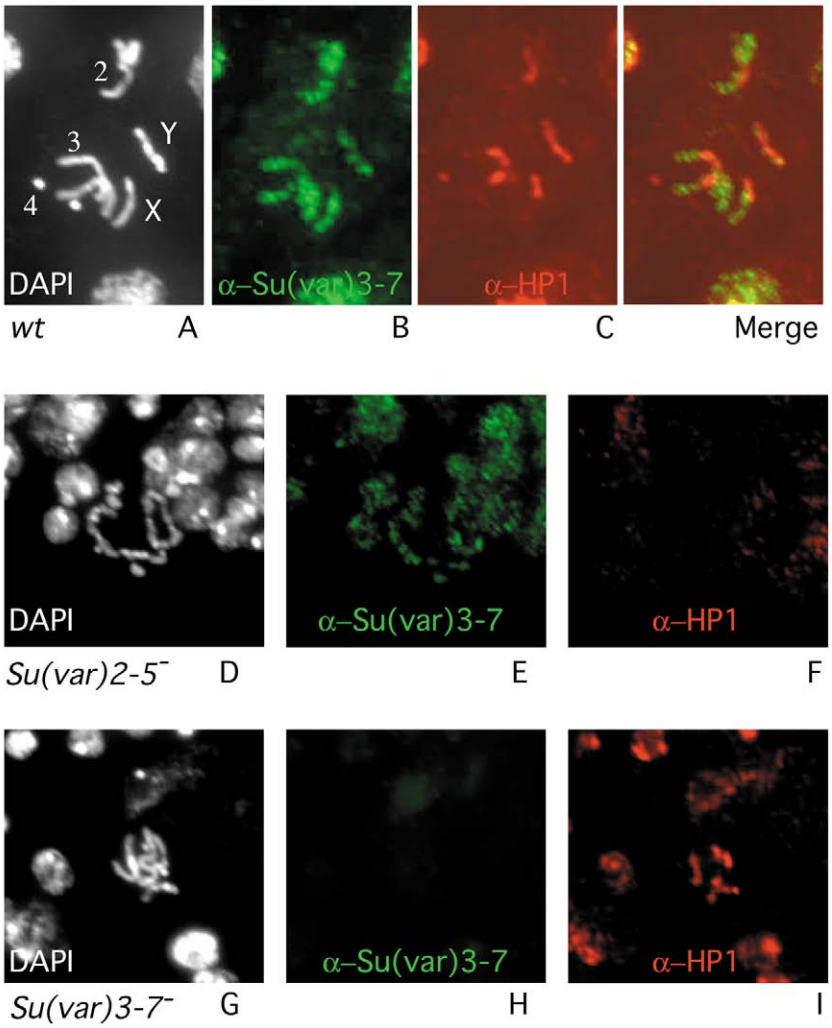

Fig. 4. Immunolocalization of Su(var)3-7 and HP1 on mitotic chromosomes from larval brains. Gray, DAPI; green, Su(var)3-7; red, HP1. (A-C) Wild-type mitotic chromosomes. (D-F) $Su(var)2\text{-}5^{04}/Su(var)2\text{-}5^{05}$ mitotic chromosomes. (G-I) $Su(var)3\text{-}7^{7.1A}$ homozygote mitotic chromosomes.

Discussion

Polytene chromosome phenotypes of Su(var)3-7 and HP1 mutants

Polytene chromosomes are affected similarly by severe loss of Su(var)3-7 or HP1. In both cases, the main mutant phenotype is a bloated X in males, and an expanded chromocenter in males and females. Why is chromosome morphology modified when HP1 or Su(var)3-7 amounts are strongly reduced? We see several possible explanations. First, Su(var)3-7 and HP1 are both required for stability of chromatid association, and reduction of dose could lead to dissociation. As discussed below, Umbetova and Zhimulev proposed this mechanism for similar phenotypes in other conditions (Umbetova and Zhimulev, 1987). This hypothesis could be tested by determining whether a phenomenon based on chromatid association, such as transvection, is affected in *Su(var)3-7* or *HP1* mutants. A second possibility is that Su(var)3-7 and HP1 are required for compaction of intercalary heterochromatin on euchromatic arms. The loss of this compaction, similar to what we have seen at the chromocenter, could lead to bloating and disruption of the banding pattern. If indeed Su(var)3-7 and HP1 are instrumental in chromosome compaction, then one could expect that excess amounts of the proteins lead in turn to an excess of compaction. This is actually the case for Su(var)3-7, as increasing amounts of Su(var)3-7 first affect the male X chromosome, which becomes strongly compacted (Delattre et al., 2004). Furthermore, we have shown that targeting HP1 to an ectopic site promotes chromosomal loops linking this ectopic site with sites of intercalary heterochromatin (Seum et al., 2001). The question remains of the particular sensitivity of the male X chromosome to loss and excess of Su(var)3-7 and to loss of HP1.

That the male X chromosome is affected first and most severely could result from association of this chromosome with

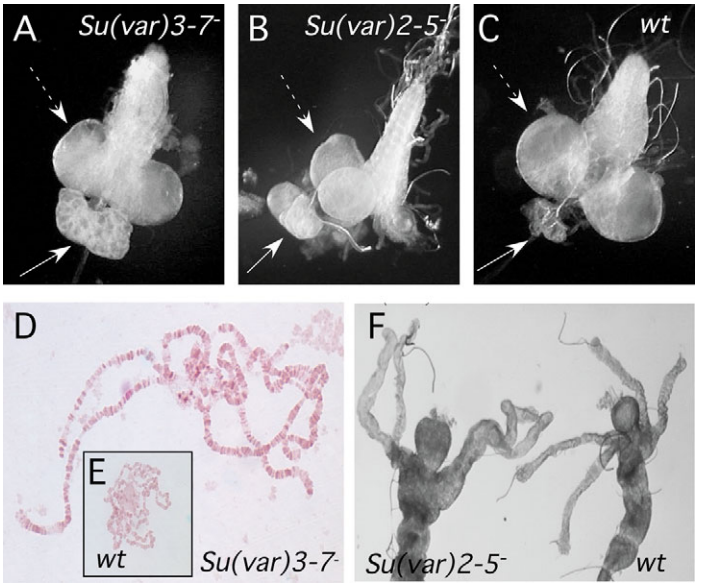

Fig. 5. Developmental defects shared by larvae mutant for either *Su(var)3-7* or *Su(var)2-5*. (A-C) Enlarged ring glands. (A) *Su(var)3-7*^{R2a8} mutant. (B) *Su(var)2-5*^{02}*/Su(var)2-5*^{05} mutant. (C) Wild-type control. Arrows point to the ring glands and dotted arrows show the larval brains. (D,E) Polytene chromosomes from the prothoracic gland of *Su(var)3-7*^{R2a8} mutant and wild-type control at the same magnification. Stained with orcein. (F) Abnormal gastric ceca, swollen proventriculus and anterior midgut of *Su(var)2-5*^{02}*/Su(var)2-5*^{05} mutant and wild-type control.

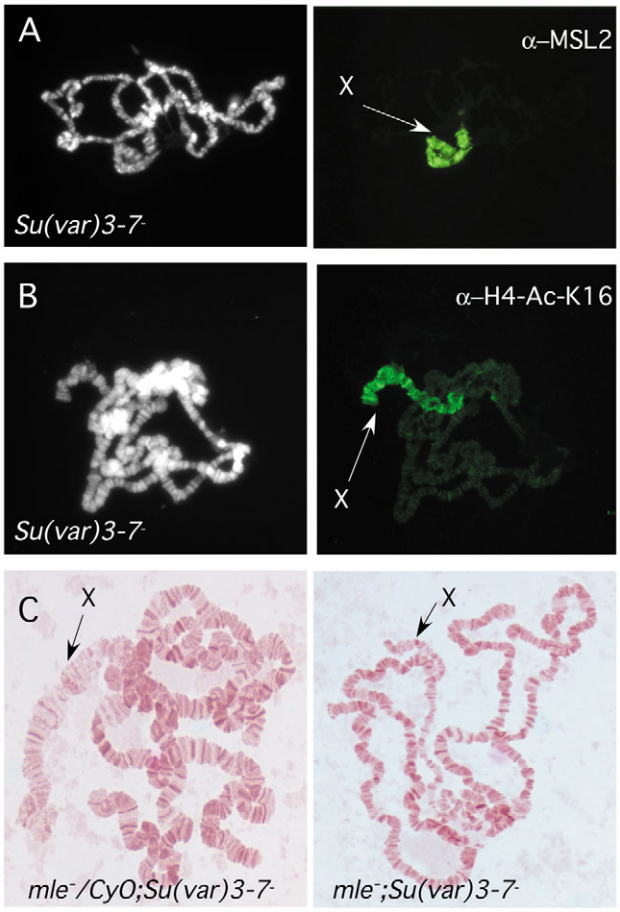

Fig. 6. Bloating of the male X chromosome in *Su(var)3-7* mutant requires a functional dosage compensation complex (DCC). (A,B) The DCC associates with the *Su(var)3-7* mutant bloated X. (A) DAPI and anti-MSL2 staining of a bloated X from a *Su(var)3-7*^{14} homozygous male. (B) DAPI and anti-H4-Ac-K16 staining of a bloated X from a *Su(var)3-7*^{9}*/Su(var)3-7*^{14} male. (C) Male X bloating occurs only with a functional DCC. Bloating of the X is lost in the *mle*^{1}*/mle*^{1}; *Su(var)3-7*^{14}*/Su(var)3-7*^{14} mutant, in contrast to its *mle*^{1}*/CyO*; *Su(var)3-7*^{14}*/Su(var)3-7*^{14} brother. Stained with orcein.

the DCC. Chromatin relaxation triggered by the DCC in the male X would render it more sensitive to variations of the amount of chromatin-associated proteins. Indeed, male X bloating and shortening has been observed in several conditions, and has been named the 'pompon' phenotype by Pavan and described as resulting from specific environmental aggressions or mutations (see Zhimulev, 1996). More recently, although less spectacularly, male X bloating was described as resulting from the loss of several chromatin-modifying factors such as Jil-1 (Wang et al., 2001) or the Nurf complex (Deuring et al., 2000; Badenhorst et al., 2002). The various environmental and genetic conditions in which bloating of the male X occurs underline the peculiar sensitivity of the phenotype, and could explain the differences of phenotype intensity we see using different X chromosomes.

Finally, the X-chromosome-specific phenotype might result from a direct interaction between the DCC and silencing factors. In this paper, we demonstrate indeed a genetic interaction between an essential gene of the dosage compensation machinery, *mle*, and *Su(var)3-7*. However, we have not detected, in the wild type, preferential association of Su(var)3-7 with the polytene male X chromosome using either a polyclonal antibody raised against Su(var)3-7 sequences, or a monoclonal antibody raised against the tag of HA-Su(var)3-7. However, we clearly see preferential association with the male X when Su(var)3-7 is over-expressed from a transgene (Delattre et al., 2004). We cannot at this point distinguish between two possibilities: either Su(var)3-7 modulates the transcription level of the X chromosome by counteracting the DCC relaxing effect, or it protects the X-linked genes that do not need to be dosage compensated. We are currently exploring further the interaction of Su(var)3-7 and the DCC. The role of HP1 also remains to be explored. We and others have not seen preferential association of HP1 with the male X polytene chromosome. Nevertheless, when Su(var)3-7 is over-expressed, HP1 is found associated preferentially with the male X (Delattre et al., 2004).

The 'null' mutant described in this paper, in which only weak and variable bloating of the male X polytene chromosome occurs, leads us to reconsider the phenotypes of previously obtained mutants where some altered Su(var)3-7 is still produced. For example, an hypothesis would be that the truncated portions of Su(var)3-7 synthesized in *Su(var)3-7*^{14} or *Su(var)3-7*$^{7.1A}$ have the ability to titrate away a component stabilizing or participating in the DCC/X chromatin interaction. Nonetheless, other truncations of Su(var)3-7 expressed from a transgene do not cause bloating of the X chromosome (Y. Jaquet, personal communication). We do not know whether this last observation disfavors the titration

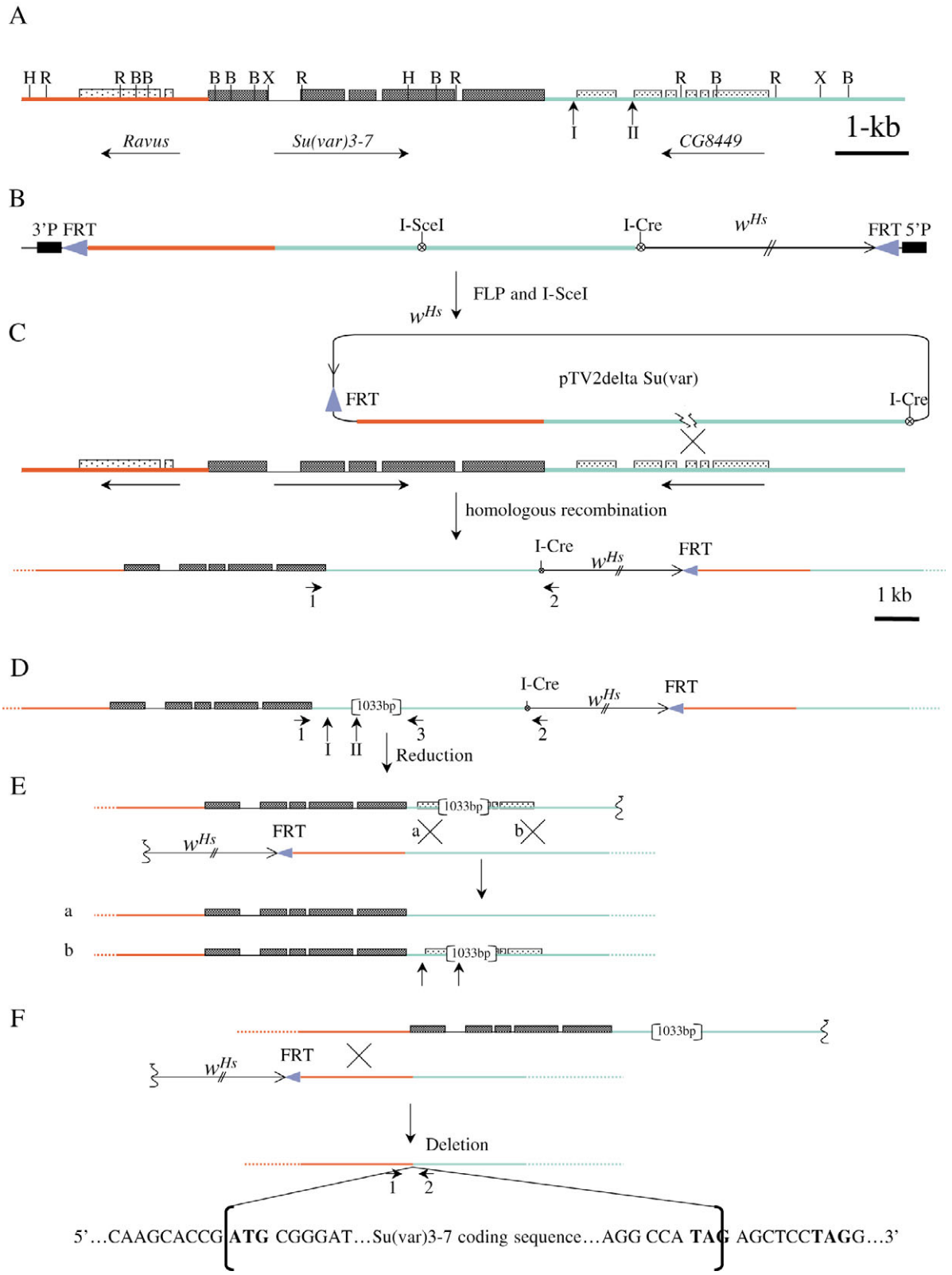

Fig. 7. Targeted mutagenesis of *Su(var)3-7*. (A) The *Su(var)3-7* locus and neighboring genes. (B) Donor construct using 2.6 kb of homology upstream of *Su(var)3-7* (red) and 4 kb downstream (blue). (C) Combination of the donor transgenic lines with an I-SceI and FLP-producing strain leads to a double-strand break in the middle of the downstream region (blue) and can give rise to homologous recombination. (D) Use of homologous recombinant bearing a 1033 bp deletion 5′ of the I-SceI site displaces the equilibrium towards a reduction in the 5′ region of *Su(var)3-7*. (E,F) Crosses of recombinants with a strain expressing the I-CreI restriction enzyme lead to a double-strand break permitting many different reduction events. (F) Repair of the break in the 5′ region of *Su(var)3-7* results in deletion of the gene. B, *Bam*HI; H, *Hin*dIII; R, *Eco*RI; X, *Xba*I.

hypothesis, or whether the explanation resides in the different times, amounts and distributions resulting from expression of a transgene.

The second observation made on polytene chromosomes of *Su(var)3-7* and *Su(var)2-5* mutants is the decondensed aspect of the chromocenter, where pericentric heterochromatin from the different chromosomes coalesces. As heterochromatin is under-replicated in polytene chromosomes, a possible explanation is that heterochromatin, freed from the 'condensing' constraint of Su(var)3-7 or HP1 in mutants, reaches higher degrees of polytenization and expands. The phenotype we describe for *Su(var)3-7* and *Su(var)2-5* mutants (large amount of granules and loose reticulum of fibers) is however very different from the phenotype seen in *Suppressor of Underreplication* mutants where blocks of heterochromatin are more replicated, become polytenized and acquire a banding pattern (Belyaeva et al., 1998). We rather believe that heterochromatin of *Su(var)3-7* and *Su(var)2-5* mutants adopts a less tightly packed conformation. Moreover, the loosened and expanded chromocenter phenotype makes sense as Su(var)3-7 and HP1 are both known to associate primarily with pericentric heterochromatin and to promote genomic silencing of PEV (Reuter et al., 1990; Cléard et al., 1997; James et al., 1989; Eissenberg et al., 1992). The silencing of PEV is relieved by loss of a dose of either factor, and it is therefore not surprising that the characteristic condensed state of heterochromatin is affected. This phenotype strongly suggests a direct role of Su(var)3-7 and HP1 in compacting heterochromatin.

We must also consider that the phenotypes described in this study could be indirect, and result from mis-regulation of *Su(var)3-7* and *HP1* target genes. Amounts of HP1 do affect expression of genes embedded in heterochromatin (Lu et al., 2000), but also seem to regulate euchromatic genes (Hwang et al., 2001; Nielsen et al., 2001; Greil et al., 2003; Piacentini et al., 2003). Microarray experiments suggest that *Su(var)3-7* also regulates euchromatic genes (Y. Jaquet, Functional dissection of Su(var)3-7, a heterochromatic protein from *Drosophila melanogaster*, PhD thesis, University of Geneva, 2004). There is therefore a significant body of data arguing for a role of Su(var)3-7 and HP1 on gene regulation not only in heterochromatic context, but also in euchromatin.

Su(var)3-7 and HP1 do not overlap on mitotic chromosomes

We find Su(var)3-7 associated with diploid chromosomes undergoing mitosis in wild-type larval brains. Whereas Su(var)3-7 is associated with euchromatin arms, it is not detected on heterochromatin, except for partial staining of the Y chromosome. This is in contrast to HP1, which is primarily associated with heterochromatin. Detection of Su(var)3-7 on euchromatin is not unexpected. On polytene chromosomes, several sites have indeed been detected on euchromatin for both Su(var)3-7 and HP1 (Delattre et al., 2000; Fanti et al., 2003; Piacentini et al., 2003). Moreover, in excess amounts of Su(var)3-7, association is seen all over the euchromatic arms (Delattre et al., 2000; Jaquet et al., 2002), suggesting general affinity of the protein for euchromatin. Finally, Su(var)3-7 expansion on polytene chromosome euchromatic arms in a *Su(var)2-5* mutant background further confirms its capacity to bind euchromatin. This might result from the affinity of Su(var)3-7 for DNA in vitro (Cléard and Spierer, 2001) and in vivo (Perrini et al., 2004). It is interesting to note that Su(var)3-9 localization in heterochromatin depends also on HP1 and that, in the absence of HP1, Su(var)3-9 also expands in euchromatin (Schotta et al., 2002).

We propose several possible explanations for the different pattern of Su(var)3-7, namely on heterochromatin of polytene chromosomes and euchromatin of mitotic chromosomes. First, on mitotic chromosomes, interaction between HP1 and Su(var)3-7 could be prevented by a competitor recruited by HP1 such as, for example, the cohesin complex that is highly enriched in heterochromatin regions during mitosis and not in interphase (Nonaka et al., 2002). Second, a specific modification of HP1 (Eissenberg et al., 1994; Huang et al., 1998) in mitotic chromosomes could abolish its interaction with Su(var)3-7. In this case, Su(var)3-7 would associate with other partners yet to be determined. The Su(var)3-7 pattern in the *Su(var)2-5* mutant supports this model as we showed that it does not move away from mitotic euchromatic arms. Other chromatin-associated proteins, such as for example GAGA or PROD (Platero et al., 1998; Torok et al., 1997), are known to exhibit a cycling pattern, although in the opposite way, as these proteins are on heterochromatin during mitosis and on euchromatin on interphase chromosomes. Another hypothesis is that Su(var)3-7 has a specific affinity for condensed chromatin, and consequently is associated with condensed heterochromatin of polytene chromosomes and with condensed euchromatic arms of neuroblast mitotic chromosomes.

In conclusion, we have shown that Su(var)3-7 and HP1 participate in chromocenter and male X polytene chromosome integrity. The similarity of the phenotypes seen in mutations of either one, the partial compensation of the loss of dose of one by an increase of dose of the other in PEV (Cléard et al., 1997), and the physical interaction seen in vitro and in vivo (Cléard et al., 1997; Delattre et al., 2000) all point to the same conclusion. These two structurally very different proteins cooperate closely in chromosome organization. We have also discovered an interaction between Su(var)3-7 and compensation of dose. This interaction between the genomic silencing of PEV dependent on Su(var)3-7 association, and hyperactivation dependent on association of the DCC, need to be unravelled.

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