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RESEARCH ARTICLE

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Conserved domains control heterochromatin localization and silencing properties of SU(VAR)3–7

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Abstract The *Drosophila* protein SU(VAR)3–7 is essential for fly viability, chromosome structure, and heterochromatin formation. We report that searches in silico and in vitro for homologues of SU(VAR)3–7 were successful within, but not outside, the *Drosophila* genus. Protein sequence homology between the distant sibling species *Drosophila melanogaster* and *Drosophila virilis* is low, except for the general organization of the protein and three conserved motives: seven widely spaced zinc fingers in the N-terminal half and the BESS and BoxA motives in the C-terminal half of the protein. We have undertaken a fine functional dissection of SU(VAR)3–7 in vivo using transgenes encoding truncations of the protein. BESS mediates interaction of SU(VAR)3–7 with itself, and BoxA is required for specific heterochromatin association. Both are necessary for the silencing properties of SU(VAR)3–7. The seven zinc fingers, widely spaced over the N-terminal half of SU(VAR)3–7, are required for binding to polytene chromosomes. One finger is necessary and sufficient to determine the appropriate chromatin association of the C-terminal half of the protein. Conferring a function to each of the conserved motives allows us to better understand the mode of action of SU(VAR)3–7 in triggering heterochromatin formation and subsequent genomic silencing.

Introduction

In eukaryotes, a large proportion of the genome is in the form of heterochromatin, the chromosomal fraction characterized by a dense cytological appearance throughout the cell cycle. Heterochromatic regions are late replicating, predominantly located near centromeres and telomeres, and consist mainly of repeated DNA sequences with a low density of unique genes. In contrast, euchromatin replicates relatively early in the cell cycle and contains the majority of single copy genes. Euchromatin and heterochromatin are also distinguished by specific histone posttranslational modifications (Turner et al. 1992; Jacobs et al. 2001; Li et al. 2002). Heterochromatin can silence the expression of euchromatic genes translocated in its vicinity, a phenomenon known as position-effect variegation (PEV; Weiler and Wakimoto 1995; Wallrath 1998). Repression typically occurs in a subset of cells and is heritable through mitosis, leading to mosaic patterns of gene expression. Genetic analysis in *Drosophila melanogaster* has identified mutations that enhance or suppress PEV (Reuter and Spierer 1992; Schotta et al. 2003). Some loci exhibit both a haplo-suppressor and a triplo-enhancer effect on PEV and, for this reason, are thought to encode structural components of heterochromatin. Among them, *Su(var)2–5* encodes HP1, a chromodomain protein (Eissenberg et al. 1992), and *Su(var)3–9*, a histone-methyl transferase (Schotta et al. 2002). A third haplo-suppressor, triplo-enhancer of PEV, *Su(var)3–7,* encodes a protein mainly associated with pericentric heterochromatin and telomeres, but also with a few sites on the euchromatic arms of polytene chromosomes (Cléard et al. 1997; Delattre et al. 2000). The loss of SU(VAR)3–7 is lethal (Seum et al. 2002), but reduced amounts allow survival and lead to bloating of the polytene male X chromosome and decondensation of the chromocenter (Spierer et al. 2005). Inversely, a lasting excess of SU(VAR)3–7 during fly development induces also lethality and results in a more condensed morphology of polytene chromosomes, most severely of the male X (Delattre et al. 2004). In this context, the hypercondensed male X is preferentially bound by the markers of heterochromatin: SU(VAR)3–7 itself, HP1, and histone H3 di-methylated on lysine 9 (H3-diMeK9), a modification that depends on Su(var)3–9 (Delattre et al. 2004). SU(VAR)3–7 plays therefore a dose-dependent regulatory role on chromosome morphol-

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ogy and heterochromatin formation. The 1,250-amino-acid-long SU(VAR)3–7 protein contains seven atypical and widely spread zinc finger motifs shown to bind DNA in vitro (Reuter et al. 1990; Cléard et al. 1995; Cléard and Spierer 2001), and the protein has indeed been shown to bind DNA in vivo (Perrini et al. 2004).

We have previously reported that self-interaction and specific binding to pericentric heterochromatin of polytene chromosomes are conferred to SU(VAR)3–7 by its C-terminal half, while the zinc fingers comprised in the N-terminal half are required for the binding to chromosomes arms (Jaquet et al. 2002). Here, to delineate more precisely functional domains of SU(VAR)3–7, we have searched for its homologues in other species. We found that *Su(var)3–7* is a fast evolving protein in the *Drosophila* genus which, beside the seven zinc fingers, contains two short conserved domains in its C-terminal tail, BESS, and BoxA. These domains drive the binding to pericentric heterochromatin and the silencing function, while the zinc fingers stabilize nonspecific interaction with chromatin. These findings lead us to propose a model for SU(VAR)3–7 action in heterochromatin, relying on cooperation among its conserved domains.

Materials and methods

Phylogenetical analysis

Su(var)3–7 polymerase chain reaction (PCR) amplification was performed in various species: DNA from *D. melanogaster*, *Drosophila virilis*, *Drosophila obscura*, *Musca domestica* and *Artemia franciscana* was extracted with the DNeasy Tissue Kit (Qiagen). Twelve oligonucleotides were synthesized degenerated from 64 to 256 times. Among them, a couple of oligos 5' TYTGGATGAARM GNTGGCCYTGG 3' and 5' RAGRTANACRAAYTCNA CGTTCAT 3' corresponding to positions 725 and 1,118 of the *D. melanogaster Su(var)3–7* cDNA were providing the best amplifications for *Drosophila* species. PCR was performed under unspecific conditions (50°C for annealing and 35 cycles), and products were analyzed on agarose gel stained with ethidium bromide. Amplified DNA fragments were cloned into a T-overhang-vector (pGEM-T easy vector, Promega) and sequenced. Cloning of *D. virilis Su (var)3–7* gene was executed as follows: A 0.4-kb PCR fragment from *D. virilis* SU(VAR)3–7 was cut out from the vector, purified, random labeled with P^{32}-α-CTP, and used as a probe to screen a *D. virilis* genomic lambda EMBL3 library (Hooper et al. 1992). DNA was isolated from positive clones, subcloned into the pBluescripts SK vector, and sequenced. The *D. virilis* SU(VAR)3–7 sequence has been submitted to GenBank. Searches for *Su(var)3–7* homologues were performed using the following: the BLASTP or TBLASTN program (Altschul et al. 1997) and databases at the National Center for Biotechnology Information (NCBI) web pages (http://www.ncbi.nlm.nih.gov/; last update April 2005). Multiple-sequence alignments were obtained using the default parameters of the T-COFFEE program (Notredame et al. 2000) on the EMBNET website (http://www.ch.embnet.org/software/TCoffee.html).

HA:Su(var)3–7 constructs

Fragments of the *Su(var)3–7* cDNA (Cléard et al. 1995) were cloned in frame downstream of the hemagglutinin tag (HA) of the Puc-HA vector (Delattre et al. 2002). HA:Su(var)3–7 fragments were inserted in a modified version of the C4-*yellow* transformation vector (Sigrist and Pirrotta 1997), downstream of the hsp70 promoter, to allow heat shock induction of tagged fragments. Constructs were made with the following fragments: a 2.24-kb fragment (amino acids 118–863) for HA:FL(ΔCT), a 1-kb fragment (amino acids 909–1,250) for HA:CT, a 0.86-kb fragment (amino acids 962–1,250) for HA:CT(ΔBoxA), and a 0.6-kb fragment (amino acids 1,052–1,250) for HA:CT(ΔBoxA-BESS). The HA:FL(ΔBESS) construct is issued from the HA:FL (Jaquet et al. 2002) with a deletion of amino acids 987–1,025: the corresponding base pairs have been replaced by a *Nhe*I restriction site during the cloning procedure. The HA:FL(ΔBoxA) construct is issued from the HA:FL construct with a deletion of amino acids 909–961: the corresponding base pairs have been replaced by a *Nhe*I restriction site during the cloning procedure. The HA:6CT(Δ7) construct is issued from the HA:6-Ct (Jaquet et al. 2002) construct with point mutations in the seventh zinc finger (C827F and C829T). The HA:Su(var)3–7 constructs were then inserted into the genome of *D. melanogaster* yw^{67} line by P element germline fly transformation.

Western blots and polytene chromosomes analysis

Ten males and ten females from each transgenic line were heat-shocked for 30 min. After 1 h of recovery, flies were homogenized in 200 µl of protein homogenization buffer (8 M urea, 100 mM NaH_2PO_4, 10 mM Tris–HCl pH 6.4). Sample buffer of 200 µl (4% SDS, 17.5% glycerol, 120 mM Tri–HCl pH 6.8, and 0.01% bromophenol blue) was added, and the samples were boiled for 5 min before Western blot analysis. Proteins were detected with an anti-HA monoclonal antibody (diluted 1:100) and stained with an anti-mouse IgG-alkaline phosphatase-conjugated antibody (diluted 1:2,000). Immunostaining on third instar larvae salivary gland polytene chromosomes was performed as described by Jaquet et al. (2002). For each construct, at least two independent insertions have been compared to avoid strong position effect on the expression of the construct. Localizations for most constructs [HA:FL (ΔBoxA), HA:FL(ΔBESS), HA:FL(ΔCT), HA:6CT(Δ7), HA:7-Ct, HA:7-B, HA:Δ7-B, and HA:Δ7-B] were tested in both mutant and wild-type backgrounds. There was no difference in most cases. Results of experiments in mutant background are reported only when relevant and/or different compared to wild type.

Effect of constructs on position-effect variegation

To test the effect of the heat shock induction of HA:SU(VAR) 3–7 proteins on the w^{m4h} (Tartof et al. 1989) and *Heidi* (Seum et al. 2000) lines, females bearing the variegated alleles were crossed with males homozygous for the *HA:Su (var)3–7* transgene, and with yw^{67} males as control. Heat shock was carried out by incubating embryos at 30°C until the beginning of the third larval stage, and then by administrating three 15-min heat shocks at 37°C per day in a water bath until adult emergence. Eye pigments measurements of males were made according to Sun et al. (2000): Heads of ten hemizygous adult male flies (4–5 days after emergence) were homogenized in 1 ml of homogenization buffer (0.01 M HCl in 30% ethanol). The homogenate was placed at 37°C overnight. The supernatant from a 10-min spin at 12,000×*g* was recovered, and the OD at 480 nm measured. The average value from female

Fig. 1 T-COFFEE alignment of deduced amino acids sequences from *Su(var)3–7* genes from *Drosophila melanogaster* (Dm), *Drosophila pseudoobscura* (Dpse), and *Drosophila virilis* (Dv). Color shading is used to highlight the presence of identical or biochemically related amino acids in a given position. Gap in sequence alignment are indicated by *dashes*. The conserved regions (Zinc fingers 1–7 and their upstream tryptophan box, BoxA, and BESS) are indicated above the alignment

yw[67]*;Heidi/+* flies was used as control. Three independent samples were analyzed for each transgenic line; means and standard deviations were normalized to the control.

Results

Su(var)3–7 is a fast evolving gene

Protein domains with a specific function are often conserved in the course of evolution. We screened databases and searched for *Su(var)3–7* orthologues by PCR with degenerated oligonucleotides in several *Drosophila* species as well as in *M. domestica* and in *A. franciscana*. These strategies did not allow us to find orthologues outside the *Drosophila* genus. The most distant species in which we found a *Su(var)3–7* orthologue is *D. virilis*, which has split from *D. melanogaster* 40–60 million years ago (Schmid and Tautz 1997), suggesting that *Su(var)3–7* varies rapidly during evolution. Comparison of *D. melanogaster*, *Drosophila pseudoobscura*, and *D. virilis* SU(VAR)3–7 deduced protein sequences (Fig. 1; DmSU(VAR)3–7, DpseSU(VAR)3–7, and DvSU(VAR)3–7, respectively) shows, indeed, only 34% identity and 57% similarity. Alignment between *D. melanogaster* and *D. virilis* shows 46% identity and 69% similarity. For comparison, *D. melanogaster* and *D. virilis* HP1 are 76% identical and 91% similar in protein sequence. Despite this relatively low level of conservation for a short phylogenetical distance, immunolocalization of *D. virilis* SU(VAR)3–7 with antibodies for the *D. melanogaster* protein shows that both orthologues associate likewise with pericentric heterochromatin, telomeres, and a few euchromatic sites on salivary gland polytene chromosomes (Fig. 2). In addition, the three SU(VAR)3–7 orthologues are similar in overall organization: They contain seven conserved zinc fingers preceded by tryptophan and box (Cléard et al. 1995), with similarity ranging from 82% for the fifth zinc finger to 100% for the seventh. Interestingly, two other domains are well conserved in the C-terminal half of the proteins. One of these two domains shows 95% similarity and is known as BESS, for BEAF-32, SU(VAR)3–7 and Stonewall, the first three proteins in which it was identified (PFAM accession number PF02944). The BESS domain is about 40 amino acids long (amino acids 987–1,026 in *D. melanogaster* SU(VAR)3–7), and its sequence predicts two alpha helices. This domain is mainly found in *Drosophila* but also exists in *Anopheles gambiae* and *Xenopus tropicalis*. Its function is unknown. The second domain, with 93% similarity between *D. melanogaster* and *D. virilis*, is 75 amino acids long (amino acids 887–961) and encodes three deduced alpha helices. We called it BoxA (Jaquet et al. 2002). We did not find this domain outside of the *Drosophila* genus, and it is not present in databases of conserved protein motifs. The conservation of the zinc fingers, BoxA, and BESS domains suggests that they play important functional roles.

The BoxA and BESS domains of SU(VAR)3–7 are required for specific binding to heterochromatin and silencing

To examine in vivo the function of the BoxA and BESS domains, we established transgenic lines expressing truncations of SU(VAR)3–7 under the control of the inducible *hsp70* promoter. In addition, the truncations were tagged with the hemagglutinin epitope. The truncated proteins contain a deletion of either BoxA, BESS, or the whole C-terminal domain (Fig. 3a). We verified by Western blot analysis that these proteins are expressed upon heat shock (Fig. 3b). We then compared the pattern of association of the truncated proteins with that of the full-length construct (Jaquet et al. 2002), by immunostaining of polytene chromosomes prepared from heat-shocked larvae. In contrast to the full-length protein, which has preferential affinity for heterochromatin, the three truncated proteins bind throughout chromosomes with no particular specificity for pericentric heterochromatin (Fig. 4). At a lower level of expression resulting from a shorter heat

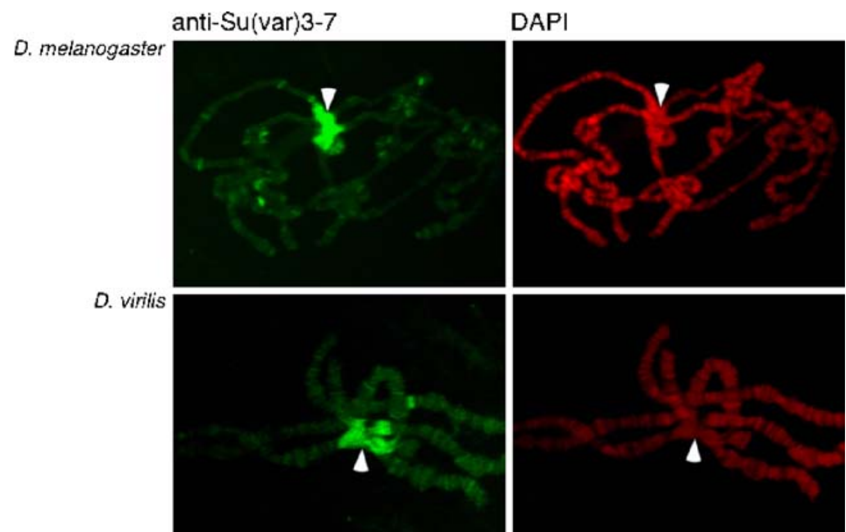

Fig. 2 The antibodies against *D. melanogaster* SU(VAR)3–7 recognize a heterochromatic protein on *D. virilis* salivary glands polytene chromosomes. *Arrowheads* indicate pericentric heterochromatin

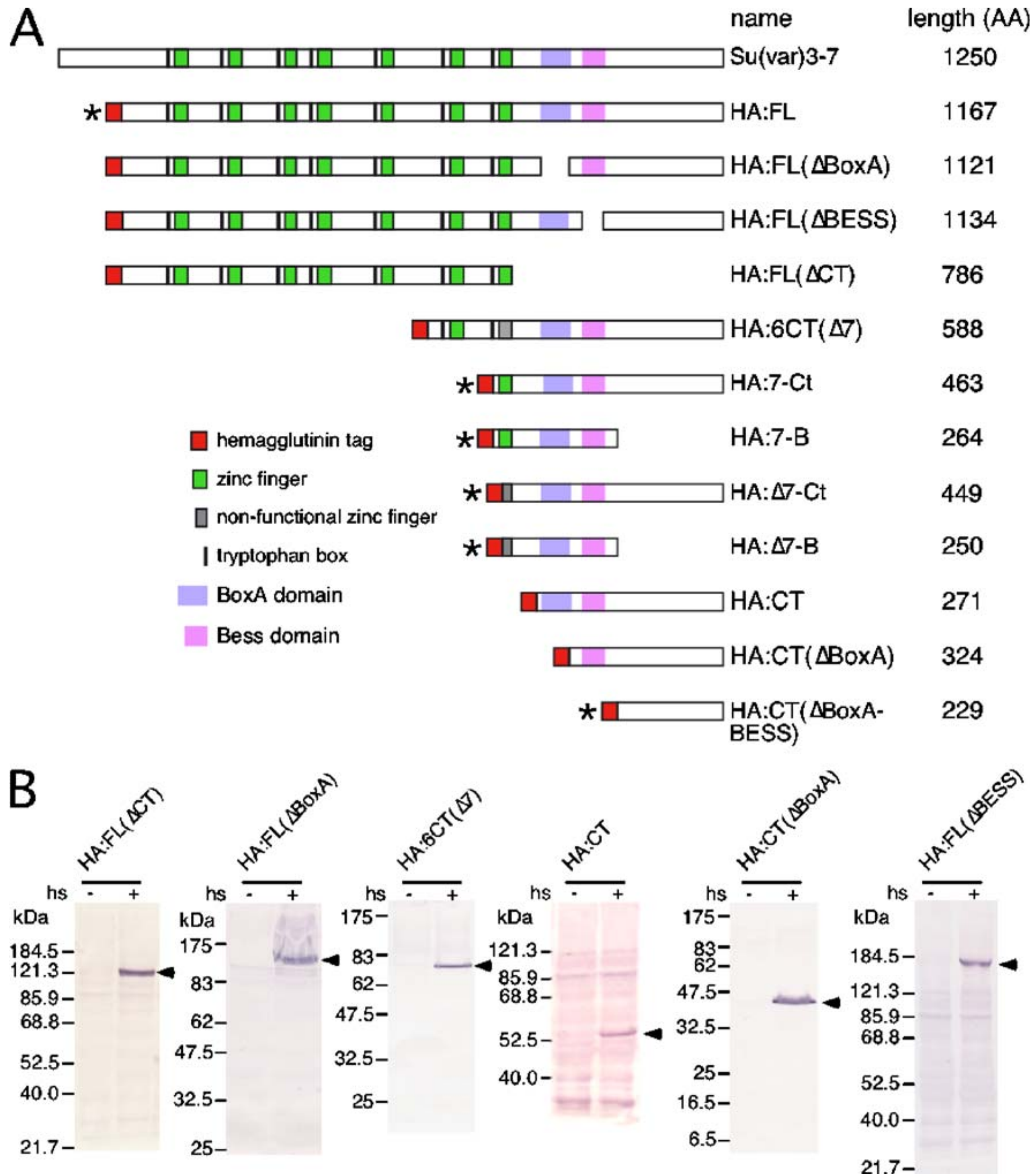

Fig. 3 Tagged SU(VAR)3–7 constructs. **a** Alignment of the tagged SU(VAR)3–7 constructs. Amino acids numbers include the tag. *Asterisk* shows constructs described by Jaquet et al. (2002). **b** Western blot of crude protein extracts from heat-shocked and nonheat-shocked adult flies expressing different tagged constructs. Proteins were detected with an anti-HA antibody. Positions of tagged proteins are indicated by *arrowheads*

shock, the result is the same (not shown). We conclude that deletion of the 53 amino acids of BoxA or of the 39 amino acids of BESS abolishes the preferential affinity of SU (VAR)3–7 for heterochromatin.

We showed previously that, when in large excess, the full-length tagged construct associates with the euchromatic arms of polytene chromosomes in a banding pattern different from DAPI DNA staining, resulting probably from affinity for specific DNA sequences or specific proteins (Jaquet et al. 2002). In contrast, we find here that constructs deleted for the C-terminal half or for BoxA exhibit a band–interband pattern along the chromosome arms identical to staining of DNA (Fig. 4). For the specificity of chromatin binding, deletion of the 53-amino-acid BoxA is equivalent to the deletion of the whole C-terminal 387 amino acids. BoxA plays a crucial role in the specificity of binding to heterochromatic and euchromatic sites. In contrast, the protein carrying a deletion of BESS binds to chromosome arms in a band–interband pattern neither identical nor complementary to DNA staining, but similar to the overexpressed tagged full-length protein. This implies that the C-terminal half of the protein, deleted

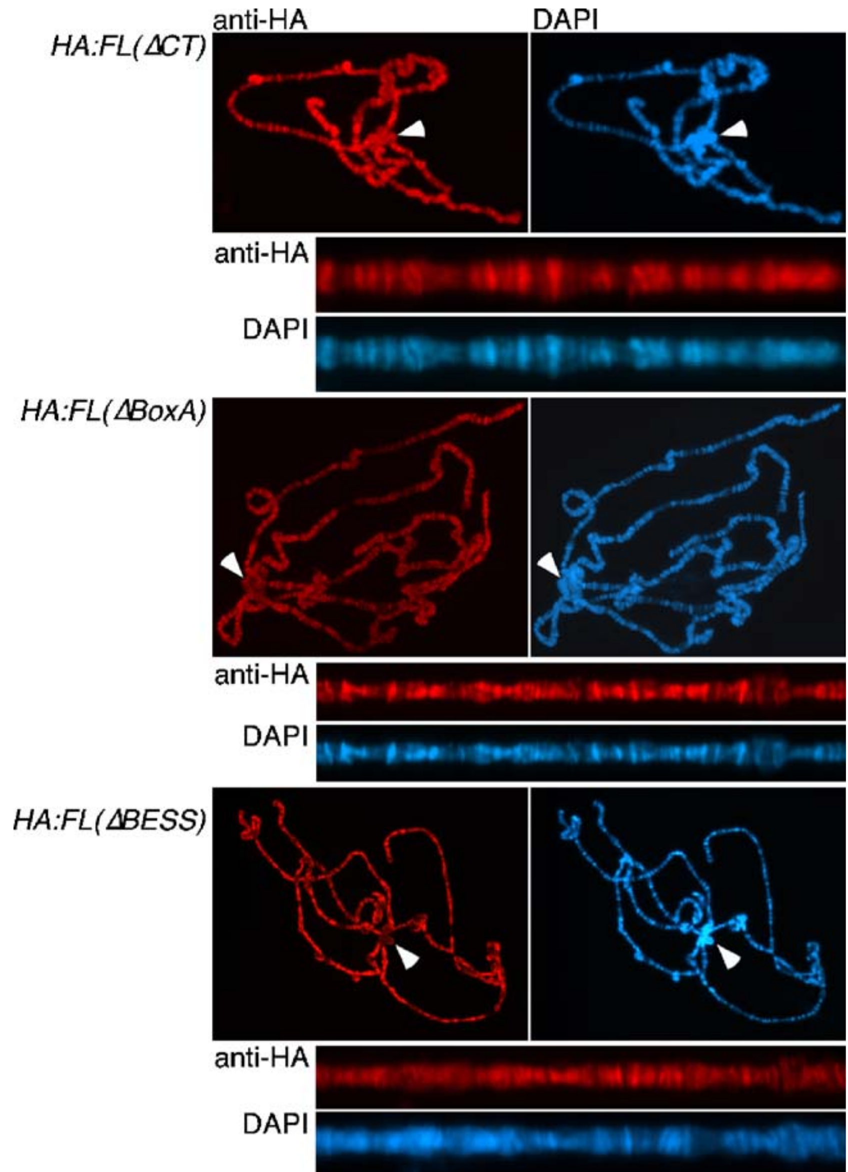

Fig. 4 HA:FL(ΔCT), HA:FL (ΔBoxA), and HA:FL(ΔBESS) bind the whole polytene chromosomes without specificity for heterochromatin. Polytene chromosomes from salivary glands of *HA:FL(ΔCT)*, *HA:FL (ΔBoxA)*, and *HA:FL(ΔBESS)* heat-shocked larvae. Staining with an anti-HA antibody (*red*) and DAPI (*blue*). *Arrowheads* indicate pericentric heterochromatin. For each construct, a higher magnification shows the staining of the protein on a chromosome arm segment. Chromosome section has been straighten using the Straighten Curvilinear Object tool (Barrett and de Carvalho 2003) of the Image SXM software (Barrett 2002)

for BESS, still contains the domain, providing chromatin affinity not specific for heterochromatin. This domain is not present in the proteins deleted for either the entire C-terminal part or BoxA, further supporting a crucial role for the 53-amino-acid BoxA in the specificity of SU(VAR)3–7 binding to chromatin.

To investigate the role of the C-terminal domain in the silencing properties of SU(VAR)3–7, we tested the effect of expression of the truncated proteins on PEV using the w^{m4h} and *Heidi* lines. w^{m4h} is an inversion within the X chromosome relocating the *white* (w^+) gene next to pericentric heterochromatin (Tartof et al. 1989), and *Heidi* contains a *P{*w^+*}* euchromatic transgene relocated in heterochromatin as a result of an x-ray-induced chromosomal inversion (Seum et al. 2000). Figure 5 shows that expression of proteins harboring deletion of BESS, BoxA, or of the whole C-terminal half does not enhance PEV as extradoses of the full-length protein do (Reuter et al. 1990; Cléard et al. 1997; Jaquet et al. 2002). Deletions of BESS or of the C-terminal tail have no effect on PEV, while deletion of BoxA has an antipodal suppressor effect, as seen by an increase in the eye pigment levels of *Heidi* (Fig. 5a) and w^{4h} lines (not shown). This result underlines the requirement for a complete C-terminal half of SU(VAR)3–7, containing intact BESS and BoxA, for its silencer effect on PEV. Moreover, the antipodal suppressor effect of the construct carrying a deletion of BoxA, but keeping intact the BESS domain, suggests that this construct has a dominant negative effect, probably by interacting with endogenous SU(VAR)3–7 and titrating it.

BESS mediates SU(VAR)3–7 self-interaction

In a previous study, we showed that ectopic expression of HA-tagged SU(VAR)3–7 fragments containing the C-terminal half and the C-terminal half truncated from its last 199 amino acids suppresses PEV (Jaquet et al. 2002).

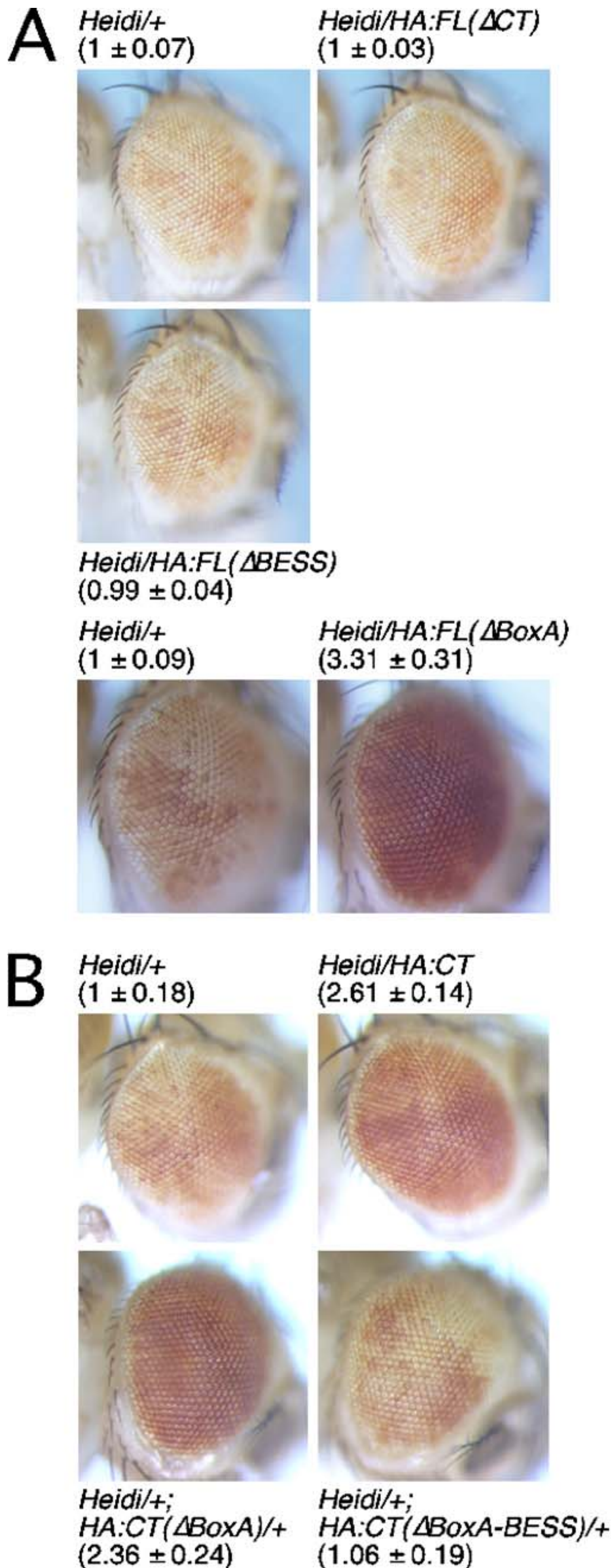

Fig. 5 Effect of SU(VAR)3–7 truncated proteins on PEV of the *Heidi* variegating line. Eyes of heat-shocked males coming from crosses between *Heidi/CyO* females and yw^{67}, yw^{67}*;HA:FL (ΔBESS)*, yw^{67}*;HA:FL(ΔCT)*, or yw^{67}*;HA:FL(ΔBoxA)* males (**a**) or yw^{67}, yw^{67}*;HA:CT*, yw^{67}*;HA:CT(ΔBoxA)*, or yw^{67}*;HA:CT (ΔBoxA-BESS)* males (**b**). Corresponding normalized eye pigment measurements are reported

We proposed that these fragments titrate endogenous SU (VAR)3–7, an interpretation supported first by interaction of the C-terminal half with itself in two-hybrid assays (Jaquet et al. 2002), and second by the delocalization in flies of endogenous SU(VAR)3–7 by these fragments on the polytene chromosomes (Jaquet et al. 2002). We have also noted above that BoxA is not involved in the proposed titration of endogenous SU(VAR)3–7. The best candidate domain for this self-interaction is therefore BESS itself. To test this hypothesis, we compared the effect on PEV of expression of three SU(VAR)3–7 tagged fragments: first, a construct containing the BoxA and the BESS domains in addition to the last 199 amino acids of the C-terminal half; second, a construct containing only the BESS domain and the last 199 amino acids of the C-terminal half; and third, a construct which corresponds only to these last 199 amino acids. These constructs are schematized in Fig. 3. We determined that the first two constructs have a suppressor effect on PEV, whereas the third has no effect (Fig. 5b). We conclude that BESS itself is responsible for the suppressor effect on PEV and thus should form the domain of self-interaction.

The C-terminal half of SU(VAR)3–7 drives the specific association of the protein with heterochromatin (Jaquet et al. 2002). Furthermore, we have shown above that this C-terminal moiety of SU(VAR)3–7 contains a domain of self-association, the BESS domain. We surmised that the specific association of the C-terminal half with heterochromatin results from interaction of the truncated construct with wild-type endogenous SU(VAR)3–7. To test this hypothesis, we established fly lines homozygote for the *Su(var)3–7*^{14} mutation (Seum et al. 2002), and we combined them with *HA:Su(var)3–7* transgenes. The results show that in the absence of endogenous SU(VAR) 3–7, the C-terminal half construct does not bind polytene chromosomes (Fig. 6). We conclude that association of the C-terminal moiety with heterochromatin requires association with endogenous SU(VAR)3–7. Indeed, adding back one dose of endogenous SU(VAR)3–7 is sufficient to reestablish its binding to pericentric heterochromatin (Fig. 6).

One zinc finger suffices for heterochromatin binding of the C-terminal half of SU(VAR)3–7

The inability of the C-terminal half of SU(VAR)3–7 to associate with heterochromatin in the absence of endogenous SU(VAR)3–7 led us to explore the function of the zinc fingers distributed over the N-terminal half of the protein. A construct consisting of the N-terminal half, containing the seven zinc fingers, binds throughout polytene chromo-

anti-HA anti-Su(var)3-7 DAPI

HA:Δ7-Ct; Su(var)3-7^14/Su(var)3-7^14

HA:Δ7-Ct; Su(var)3-7^14/TM6B

HA:7-Ct; Su(var)3-7^14/Su(var)3-7^14

HA:7-Ct; Su(var)3-7^14/TM6B

HA:7-B; Su(var)3-7^14/Su(var)3-7^14

HA:7-B; Su(var)3-7^14/TM6B

Fig. 6 In the absence of endogenous SU(VAR)3–7, the binding of the C-terminal tail of SU(VAR)3–7 to heterochromatin requires a zinc finger. Polytene chromosomes from salivary glands of heat-shocked larvae carrying *HA:Δ(7)-CT*, *HA:7-CT*, or *HA:7-B* transgenes and the homozygous *Su(var)3–7*^{14} or heterozygous *Su (var)3–7*^{14}*/TM6B* mutation. Staining with an anti-HA antibody (*red*), an anti-SU(VAR)3–7 antibody (*green*), and DAPI (*blue*). *Arrowheads* indicate pericentric heterochromatin

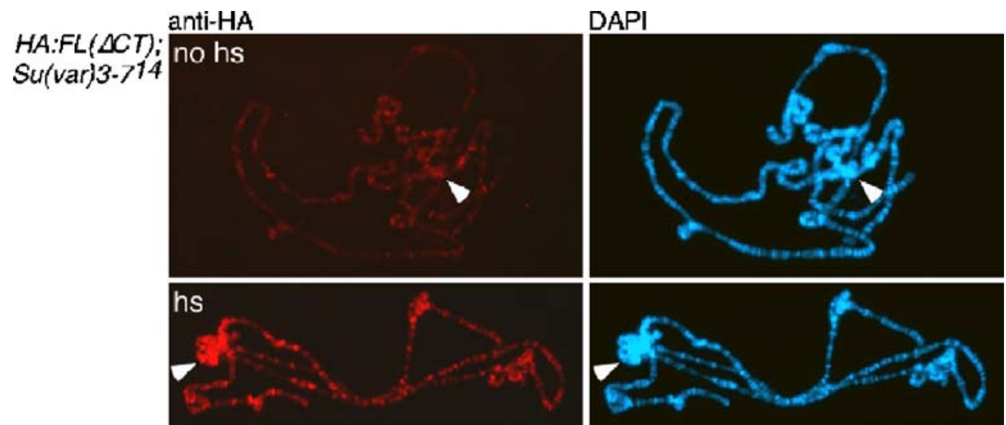

Fig. 7 In a *Su(var)3–7*^{14} mutant background, HA:FL(ΔCT) binds to polytene chromosomes without specificity for heterochromatin. Polytene chromosomes from salivary glands of nonheat-shocked and heat-shocked *HA:FL(ΔCT);Su(var) 3–7*^{14} larvae. Staining with an anti-HA antibody (*red*) and DAPI (*blue*). *Arrowheads* indicate pericentric heterochromatin

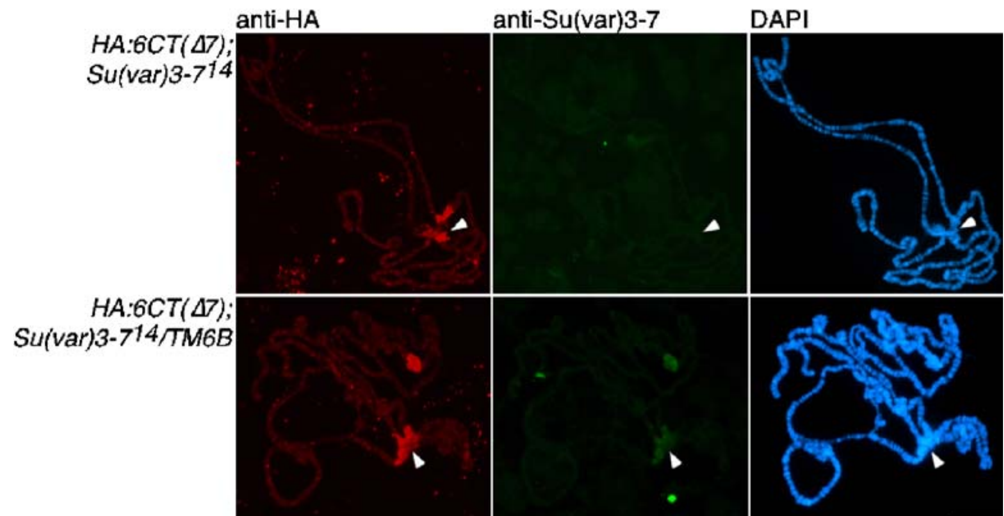

Fig. 8 The sixth zinc finger, like the seventh, is sufficient for the binding of the C-terminal tail of SU(VAR)3–7 to heterochromatin. Polytene chromosomes from salivary glands of heat-shocked *HA:6CT(Δ7);Su(var)3–7*^{14}, and *HA:6CT(Δ7); Su(var)3–7*^{14}*/TM6B* larvae. Staining with an anti-HA antibody (*red*), an anti-SU(VAR)3–7 antibody (*green*), and DAPI (*blue*). *Arrowheads* indicate pericentric heterochromatin

somes without specificity for heterochromatin. In the presence of one or two doses of endogenous SU(VAR)3–7, this construct stains the chromosomes as DAPI (not shown and Fig. 4). We speculated that the zinc fingers of the protein were needed for its association with heterochromatin, but that presence of endogenous SU(VAR)3–7 could interfere with the binding properties of the truncated protein. We therefore repeated the experiment in *Su(var)3–7* mutant background. Figure 7 shows that the chromosomes are decorated in a banded pattern identical to DAPI staining, as already described for the wild-type endogenous situation (Fig. 4). Thus, the zinc fingers are not sufficient for the specific binding of SU(VAR)3–7 to pericentric heterochromatin; the C-terminal half is necessary. A construct expressing the C-terminal half and the closest zinc finger binds to heterochromatin in the presence as well as in the absence of endogenous SU(VAR)3–7 (Fig. 6). This shows that the seventh zinc finger is necessary and sufficient for the binding of the C-terminal part of SU(VAR)3–7 to chromatin and more specifically to pericentric heterochromatin. In contrast, the last 199 amino acids of the C-terminal half are not required for this binding, since a construct containing the seventh zinc finger but devoid of this C-terminal end binds nevertheless to heterochromatin in the absence of endogenous SU(VAR)3–7 (Fig. 6).

We also wondered whether the zinc fingers are functionally equivalent, knowing the gradual loss of affinity for chromatin resulting from gradual deletion of the fingers (Jaquet et al. 2002). We made a construct starting from the sixth zinc finger and extending to the C-terminus but, in addition, containing a mutated seventh zinc finger [HA:6CT(Δ7); see "Material and methods"]. In the presence as well as in the absence of endogenous SU(VAR)3–7 protein, this construct binds specifically to pericentric heterochromatin (Fig. 8). This shows that the sixth zinc finger can also ensure the binding to heterochromatin. In this respect, it plays the same role as the seventh, suggesting that probably any zinc finger could play this role.

Discussion

Su(var)3–7 is a fast evolving gene

Whereas SU(VAR)3–7 interactors HP1 and SU(VAR)3–9 are conserved from yeast to human, all attempts to isolate homologues of *Su(var)3–7* outside of the *Drosophila* genus have failed. The most distant *Su(var)3–7* ortholog we found is in *D. virilis*, which has split from *D. melanogaster* between 40 and 60 million years ago (Schmid and Tautz 1997). Moreover, the 43% identity and 69% similarity between *D. melanogaster* and *D.virilis* SU(VAR)3–7 are low compared to what can be expected from their phylogenetical distance. For comparison, *D. melanogaster* and *D. virilis* HP1 are 76% identical and 91% similar in protein sequence. The numerous members of the C2H2 zinc finger family of proteins encountered in animal genomes originate mainly by gene duplications in a lineage specific manner followed by rapid and divergent evolution (Looman et al. 2002). It is thus difficult to find conserved orthologous genes across the animal kingdom. For example, one of the most conserved C2H2 zinc finger gene harbors about 50% identity between nematods and human (Liang et al. 2000). However, *Su(var)3–7* might represent an extreme case of divergent evolution. Indeed, no orthologous gene can be found outside Drosophilids, and clear paralogous genes are absent from *Drosophila* with the exception of the very short gene *Ravus* (Delattre et al. 2002). The lower conservation of *Su(var)3–7* means that it is a fast evolving gene. Poorly conserved genes between *D. virilis* and *D. melanogaster* are not exceptions, since one third of random cDNAs from a *D. melanogaster* library do not cross-hybridize with *D. virilis* DNA (Schmid and Tautz 1997). Furthermore, for some of the genes involved in early embryogenesis in *Drosophila*, it has been difficult or even impossible to isolate homologues outside of insects (Akam et al. 1994). Also, a few *Drosophila* genes diverge so fast that they cannot even be cloned from distantly related Drosophilids (Reuter et al. 1989; O'Neil and

Belote 1992). Rapid divergence of *Su(var)3–7* seems to be not related to the location at a junction between syntenic blocks. Preliminary examination of the synteny between *D. melanogaster* and *D. pseudoobscura* as defined in Flybase (http://flybase.bio.indiana.edu/) indicates that Su(var)3–7 is near but not at the end of a syntenic block, and that the two flanking genes are found in both species. On the other hand, it is known that conservation of folding structure does not necessarily require the conservation of the primary sequence (Sander and Schneider 1991). Thus, homologues of SU(VAR)3–7 in other species may exist and should be looked for according to their similarity in overall structure rather than in their primary sequence similarity. Nevertheless, three domains are well conserved along the SU (VAR)3–7 sequence: the zinc fingers and their associated tryptophan box, the BoxA, and the BESS domains, suggesting that they play an important role in the protein function.

The BESS domain

We have previously provided evidences that SU(VAR)3–7 interacts with itself and narrowed the domain of interaction to a 162-amino-acid-long domain in the C-terminal half of the protein (Jaquet et al. 2002). In this report, we add support to this interpretation by showing that the tagged C-terminal half of SU(VAR)3–7 binds to pericentric heterochromatin only in the presence of endogenous wild-type SU(VAR)3–7. Moreover, in a wild-type background, expression of a construct deleted for BoxA suppresses PEV, an effect most likely caused by titration of endogenous SU(VAR)3–7. Fine mapping of the self-interaction domain using a number of constructs shows that a stretch of 65 amino acids (amino acids 987 to 1,052) is responsible for the self-interaction, as measured by suppression of PEV. Within these 65 amino acids, the conserved 40-amino-acid-long BESS domain most likely forms the self-interaction domain of SU(VAR)3–7. In addition, expression of a SU (VAR)3–7 transgene deleted for BESS only has no effect on PEV, underlining that the domain suppresses silencing by interacting with endogenous SU(VAR)3–7. This conclusion on the role of BESS is supported by work of Bhaskar and Courey (2002), suggesting that the BESS domain mediates protein–protein interactions and, in particular, self-interaction in the MADF–BESS domain factor Dip3. Finally, the BEAF-32 protein oligomerizes through its C-terminal domain, a region that contains the BESS (Zhao et al. 1995; Hart et al. 1997). From these data, we speculate that SU(VAR)3–7 contributes to packaging chromatin into heterochromatin by self-interaction of monomers bound to chromatin at distance.

In respect to partners of SU(VAR)3–7, mammalian HP1 proteins have been shown to homo- and heteromerise in vivo and in vitro (Le Douarin et al. 1996; Ye et al. 1997; Nielsen et al. 2001). Self-association of HP1 bound on adjacent or nearby nucleosomes might render DNA inaccessible to transcription factors and/or prevent nucleosome sliding induced by chromatin remodeling machines (Li et al. 2003). By interacting with HP1 and itself, SU (VAR)3–7 could further stabilize the complex. Dimerization of SU(VAR)3–7 could also be necessary for interaction with partners like HP1 itself, required in vitro as a dimer for interaction with CAF1 and TIF1β in mice (Brasher et al. 2000), and with SUV39H1 (Yamamoto and Sonoda 2003).

The BoxA domain

Deletion of the 53 amino acids corresponding to the BoxA domain suffices to lose the pericentric heterochromatin specificity of SU(VAR)3–7 binding on polytene chromosomes and results in a pattern similar to DNA staining. We propose that BoxA mediates interactions of SU(VAR)3–7 with other partners, whether proteins or nucleic acids. These interactions could ensure specific binding of SU (VAR)3–7 to pericentric heterochromatin. When BoxA is deleted, and these specific interactions missing, the affinity of the zinc fingers for DNA drives the DAPI-like staining of chromosome arms by the protein. Proper localization of endogenous SU(VAR)3–7 depends on the presence of its interactor HP1. Indeed, in *Su(var)2–5* mutants larvae, SU (VAR)3–7 localization is not restricted to pericentric heterochromatin, and the level of Su(var)3–7 immunostaining at the chromocenter is lower, while staining appears on the chromosome arms (Spierer et al. 2005). Similarly, on polytene chromosomes from *Su(var)2–5* mutants larvae, SU(VAR)3–9 and H3-meK9 are not restricted to heterochromatin but also bind domains of euchromatic arms, showing that their restricted localization to pericentric heterochromatin requires HP1 (Schotta et al. 2002). In conclusion, we speculate that deletion of BoxA prevents interaction with heterochromatic partners.

The zinc fingers

The C-terminal half of SU(VAR)3–7 does not bind to polytene chromosomes in the absence of the endogenous wild-type protein. Binding to pericentric heterochromatin is restored by addition of the seventh zinc finger to the C-terminal half. The same phenotype is observed when we permutated the sixth zinc finger for the seventh in the constructs. Knowing that the seven fingers are very similar in sequence, this suggests that any of the seven zinc fingers could play this role. This assumption is supported by the fact that in vitro, any modules of two zinc fingers, even nonadjacent in the wild-type protein, are able to bind DNA (Cléard and Spierer 2001). We propose that the zinc fingers stabilize SU(VAR)3–7 at its binding sites by a direct contact with DNA. In vitro, a fragment of five of the seven fingers shows preference for AT-rich satellite DNA sequences (Cléard and Spierer 2001). This preference for satellite sequences concentrated in pericentric heterochromatin suggests that the zinc fingers confer the heterochromatin-specific affinity. Nevertheless, constructs containing the zinc finger domains are not sufficient for this specificity

in vivo. We conclude that in vivo, the heterochromatin specificity is not given by its zinc fingers alone but requires also the C-terminal tail of the protein.

In wild-type conditions, SU(VAR)3–7 binds also to telomeres and some sites on the chromosomes arms, in addition to pericentric heterochromatin. This binding to the arms depends on the zinc fingers because the tagged C-terminal half construct devoid of zinc fingers binds exclusively to pericentric heterochromatin, even when it is strongly overexpressed. Moreover, the capacity of SU(VAR)3–7 to expand on chromosome arms depends also on its zinc fingers: Indeed, addition of an increasing number of zinc fingers to the C-terminal tail leads to the expansion of the protein on the arms in a way that is proportional to the number of zinc fingers (Jaquet et al. 2002).

A model for SU(VAR)3–7 function

The capacity of SU(VAR)3–7 to extend on the chromosomes when overexpressed, suggests that in wild-type conditions, the expression of the protein must be narrowly controlled to constrain the protein mainly on pericentric heterochromatin. Its capacity to invade euchromatin might result from the general affinity of its zinc fingers for DNA. When in excess and without excess of its heterochromatic partners, such as HP1 and SU(VAR)3–9, SU(VAR)3–7 escapes the heterochromatin complex and invades the rest of the chromosome by its affinity for DNA. Thus, the precise pattern of SU(VAR)3–7 on the chromosome could result from a balance between the general affinity of its zinc fingers for DNA and the interactions with partners mediated by the C-terminal tail of the protein. The dose-dependent capacity of SU(VAR)3–7 to spread over chromosomes reminds the dose-dependent effect of SU(VAR)3–7 on PEV. The loss of a dose results in a strong suppression of variegation, whereas extra doses of the gene gradually enhance silencing of genes at vicinity of heterochromatin. Thus, in this respect also, a tight control of SU(VAR)3–7 expression is required to limit its silencing effect.

We have now several arguments to propose that SU(VAR)3–7 has powerful silencing effects. Increased expression of SU(VAR)3–7 enhances PEV and very efficiently condenses chromatin by recruiting both HP1 and SU(VAR)3–9 (Reuter et al. 1990; Delattre et al. 2004). To our knowledge, SU(VAR)3–7 is the only protein of *Drosophila* able to heterochromatinize complete chromosomes. So SU(VAR)3–7 is therefore very efficient in heterochromatin building. We propose that in the current model of heterochromatin formation, SU(VAR)3–9-dependent H3K9 methylation acts as a docking site for HP1 which in turn recruits SU(VAR)3–9 for H3K9 methylation of the adjacent nucleosomes, allowing the self-propagation of the system (Lachner et al. 2001). However, few arguments lead to think that HP1/H3methylK9 could not be so efficient to maintain silencing (Noma et al. 2004). Then we propose that a higher level of compaction of chromatin is reached by the further recruitment of SU(VAR)3–7.

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