SU(VAR)3-7, a *Drosophila* heterochromatin-associated protein and companion of HP1 in the genomic silencing of position-effect variegation

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An increase in the dose of the *Su(var)3-7* locus of *Drosophila melanogaster* enhances the genomic silencing of position-effect variegation caused by centromeric heterochromatin. Here we show that the product of *Su(var)3-7* is a nuclear protein which associates with pericentromeric heterochromatin at interphase, whether on diploid chromosomes from embryonic nuclei or on polytene chromosomes from larval salivary glands. The protein also associates with the partially heterochromatic chromosome 4. As these phenotypes and localizations resemble those described by others for the *Su(var)2-5* locus and its heterochromatin-associated protein HP1, the presumed co-operation of the two proteins was tested further. The effect of the dose of *Su(var)3-7* on silencing of a number of variegating rearrangements and insertions is strikingly similar to the effect of the dose of *Su(var)2-5* reported by others. In addition, the two loci interact genetically, and the two proteins co-immunoprecipitate from nuclear extracts. The results suggest that SU(VAR)3-7 and HP1 co-operate in building the genomic silencing associated with heterochromatin.

Keywords: *Drosophila*/genomic silencing/ heterochromatin/position-effect variegation

Introduction

Chromosomal rearrangements which break both in heterochromatin and euchromatin frequently produce mosaic expression of euchromatic genes relocated next to heterochromatin (reviewed by Spofford, 1976; Weiler and Wakimoto, 1995). Genetic and cytogenetic evidence indicate that the heterochromatic conformation expands in, or sequesters, neighbouring euchromatin and inactivates genes in some cells and not in others. Once established in a cell, silencing generally is maintained through cell divisions. This phenomenon, position-effect variegation (PEV), is found in a variety of organisms. It resembles other silencing mechanisms like telomeric position effects and mating type silencing in yeast (Laurenson and Rine, 1992), homeotic gene regulation (Peifer *et al.*, 1987; Moehrle and Paro, 1994), X chromosome inactivation in mammals (Gartler and Riggs, 1983) or genomic imprinting (Peterson and Sapienza, 1993). In some cases, components of one mechanism of silencing are also part of another, as described for modifiers of PEV that are also regulators

of homeotic gene complexes (i.e. Paro and Hogness, 1991; Dorn *et al.*, 1993; Fauvarque and Dura, 1993; Farkas *et al.*, 1994; Tschiersch *et al.*, 1994).

The genetic modifiers of PEV could identify components of the mechanisms of genomic silencing (Eissenberg, 1989; Reuter and Spierer, 1992). In *Drosophila melanogaster*, haplo-suppressor loci with a triplo-enhancer effect on PEV are candidates for being structural components of the heterochromatin conformation that spreads silencing on euchromatic genes. Among these, we have cloned the modifier locus *Su(var)3-7*, and shown that an increase in its dose enhances PEV (Reuter *et al.*, 1990; Cléard *et al.*, 1995). The deduced protein sequence predicts that it encodes a large protein with seven widely separated zinc fingers. Another candidate, HP1, initially isolated as a heterochromatin-associated protein (James and Elgin, 1986), later was found to be encoded by the haplo-suppressor triplo-enhancer of PEV *Su(var)2-5* (Eissenberg *et al.*, 1990, 1992). In polytene chromosomes, HP1 was found to associate with centromeric heterochromatin and chromosome 4, and to a lesser extent with a subset of telomeres and a few euchromatic bands (James *et al.*, 1989). In embryonic nuclei, HP1 co-localizes with centromeric heterochromatin in interphase chromosomes (Kellum *et al.*, 1995). The involvement of HP1 in the genomic silencing of PEV is also supported cytologically by its association with euchromatic loci inactivated by PEV (Belyaeva *et al.*, 1993).

It has been proposed that the heterochromatic conformation spreads silence by co-operative assembly of a variety of protein constituents (Zuckerkandl, 1974; Locke *et al.*, 1988, but see also other models in Karpen and Spradling, 1990; Dorer and Henikoff, 1994; Moehrle and Paro, 1994). The extent of expansion would then depend on the dose of each constituent by analogy with the chemical law of mass-action. Consequently, dose-dependent modifiers like *Su(var)3-7* and *Su(var)2-5* stand out as candidates for such constituents of heterochromatin.

Here we show that the product of *Su(var)3-7* is cytologically associated with centromeric heterochromatin and chromosome 4, and co-localizes there with the product of *Su(var)2-5*. Changes in the dose of each locus have a strikingly similar effect on the variegation of a number of chromosomal rearrangements and insertions, and the two loci interact genetically. This parallelism led us to test whether the products of the two suppressors of PEV are part of the same complex, as evidenced by co-immunoprecipitation from nuclear extracts. These results provide strong evidence for the co-operation of SU(VAR)3-7 and HP1 in the genomic silencing of PEV.

Results

SU(VAR)3-7 is a nuclear protein

Rabbit antisera Ab264.1 and Ab264.2 were raised against an 87 amino acid segment near the N-terminus of
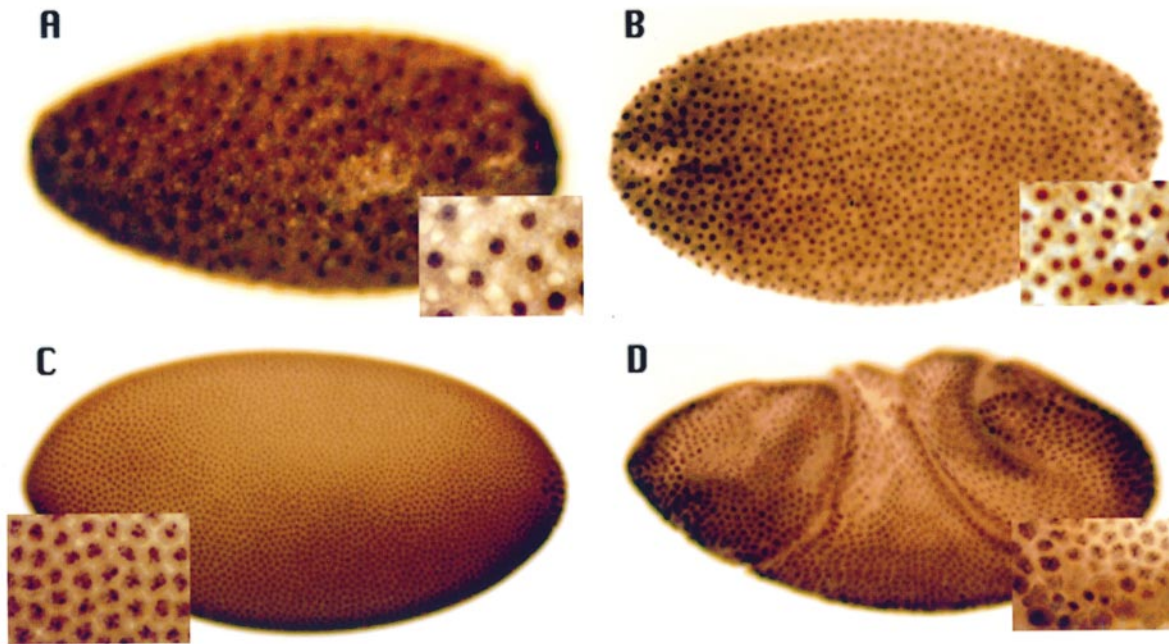
Fig. 1. Localization of SU(VAR)3-7 on whole mount embryos. Fixed embryos of various developmental stages were stained for SU(VAR)3-7 with crude Ab264 (1:1000). Staining was detected with HRP–anti-rabbit antibody and revealed with DAB and hydrogen peroxide. (**A**) Embryo at interphase 9; (**B**) syncytial blastoderm; (**C**) cellularized blastoderm; note the staining in pole cells; (**D**) embryo after gastrulation. The insets show a region of the same embryo at a higher magnification.

SU(VAR)3-7 fused to β-galactosidase. These antibodies and other preparations (Ab212.1 and Ab212.2) raised against a different part of the protein stain the same single 175 kDa band on Western blots of embryonic nuclear extracts (see Materials and methods for details). Figure 1 illustrates the distribution of SU(VAR)3-7, as revealed by immunostaining of embryos. In pre-blastoderm embryos, the protein, presumably of maternal origin, is ubiquitously distributed (not shown). This pattern is replaced rapidly by a nuclear localization clearly visible before the nuclei reach the egg periphery (Figure 1A). We cannot exclude that the protein becomes nuclear earlier, but the abundance of the cytoplasmic contribution makes this difficult to assess. At interphase 10, all nuclei are at the periphery and show a homogenous staining with anti-SU(VAR)3-7 (Figure 1B). When cellularization is completed, a distinct subnuclear pattern appears as dense spots within the nuclei (Figure 1C). The staining remains nuclear and spotty throughout further embryonic development, and until mitotic domains become visible. Then, in some regions, the signal becomes more diffuse (Figure 1D).

To examine the sub-nuclear localization of SU(VAR)3-7 in greater detail, embryos were stained with antibodies to the protein and examined by confocal microscopy (Figure 2). Early in embryogenesis, as soon as nuclei reach the surface of the embryo (mitotic cycle 11; Figure 2, upper lane), a fraction of SU(VAR)3-7 appears to co-localize with DNA whereas large amounts are detected in the cytoplasm. When cellularization proceeds (mitotic cycle 14; Figure 2, lower lane), the protein accumulates at the apical pole of the nuclei facing the external surface of the cell layer forming the blastoderm. This area is the region where DNA staining is also dense, and has been shown to correspond to centromeric heterochromatin (Foe and Alberts, 1983; Hiraoka *et al.*, 1990). This localization of

SU(VAR)3-7 in the nuclei has been confirmed by analysing fluorescence in a series of focal planes in a cycle 14 nucleus (Z-section; not shown).

SU(VAR)3-7 is redistributed during cell division
As illustrated in Figure 3, SU(VAR)3-7 is present throughout the nuclei at interphase 14, but concentrated at the apical pole. As soon as chromosomes start to condense to enter mitosis, the signal becomes diffuse and no longer correlates with DNA. Under the chromosome fixation conditions we use, the protein does not seem to be associated with chromosomes during all of the division process. On condensed chromosomes, neither of the two antibodies, raised against two different parts of the protein, were able to detect SU(VAR)3-7. This pattern resembles that of HP1, a heterochromatin-associated protein shown to correspond also to a haplo-suppressor, triplo-enhancer of PEV, *Su(var)2-5* (Eissenberg *et al.*, 1990, 1992; Kellum *et al.*, 1995). This observation should be viewed with caution as it might result from conditions of fixation of chromosomes. Indeed, studies with a mammalian homologue of HP1 have detected staining of constitutive heterochromatin on unfixed metaphase chromosomes (Wreggett *et al.*, 1994). At late telophase, when chromosomes decondense, staining again moves toward co-localization with DNA.

### SU(VAR)3-7 and HP1 co-localize in embryos
The pattern of staining of SU(VAR)3-7 on embryos and during the cell cycle was found to be similar to that of HP1 (Figures 1, 2 and 3). To verify this potentially interesting co-localization, we have repeated all the analyses on embryos and through the cell cycle by double staining with antibodies against SU(VAR)3-7 and a monoclonal antibody to HP1 (provided by S.Elgin), either
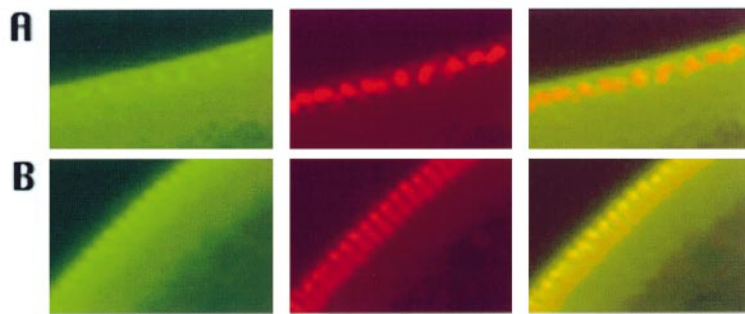
Fig. 2. Nuclear localization of SU(VAR)3-7 by confocal microscopy. Staining at the periphery of embryos before and after cellularization. Left: fluorescein-labeled SU(VAR)3-7 (green). Middle: propidium iodide staining of DNA (red). Right: merged image (regions of overlap are in yellow). (**A**) At syncytial blastoderm, when round-shaped nuclei reach the surface of the embryo (mitotic cycle 11), some SU(VAR)3-7 protein is already associated with DNA. (**B**) When cellularization is completed, SU(VAR)3-7 is concentrated at the apical pole of nuclei where the centromeric heterochromatin is located (Foe and Alberts, 1983; Hiraoka *et al.*, 1990).
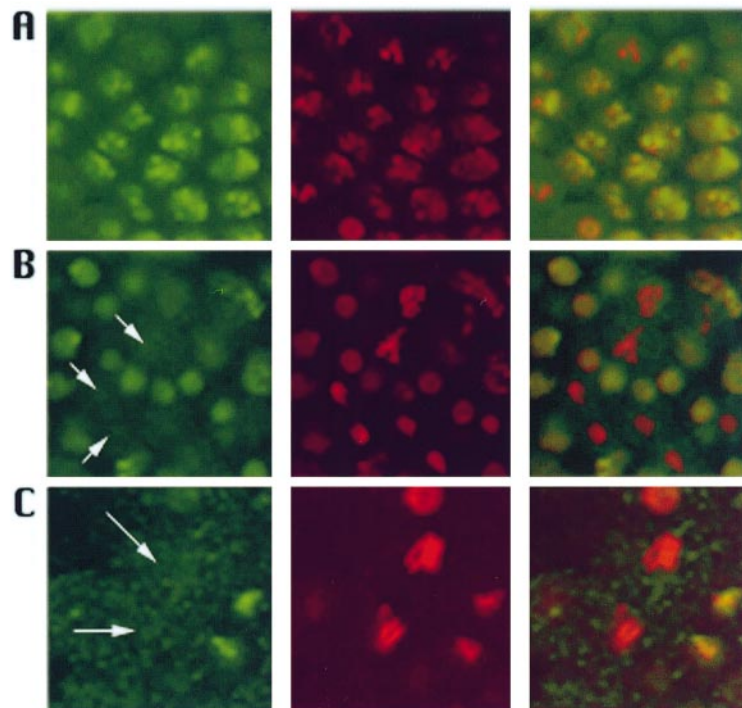
Fig. 3. Subnuclear localization of SU(VAR)3-7 in stage 14 embryos by confocal microscopy. (**A**) Interphase nuclei: scanning was done at the apical surface of nuclei. (**B**) Dividing and interphase nuclei: the position of some condensed nuclei is indicated by a white arrow. (**C**) Late anaphase and late telophase at a higher magnification. White arrows point to mitotic chromosomes.
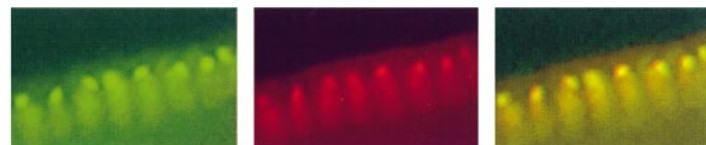
Fig. 4. Confocal analysis of HP1 and SU(VAR)3-7 double staining at the periphery of a cellularized wild-type embryo. Right panel, fluorescein-labelled SU(VAR)3-7 (green); middle panel, rhodamine-labelled HP1 (red); left panel, merged image (regions of overlap are in yellow).

independently or together. In all cases, the staining that we obtained was overlapping to the limits of the procedure. Figure 4 shows a subset of these data, namely the sub-localization observed in embryonic nuclei for SU(VAR)3-7 and the apparently identical distribution of HP1.

SU(VAR)3-7 associates with heterochromatin on polytene chromosomes
From the staining of embryos, we expected that the protein would localize to heterochromatin on salivary gland poly-

tene chromosomes, but we could not exclude that staining could also be found in euchromatin. Figure 5 shows that indeed staining of centromeric heterochromatin is strong, and that no other staining is detected, except for a banded pattern over the fourth chromosome. To confirm that the staining is linked to heterochromatin, we used the *brown*-variegating chromosomal inversion bw^{VDe2} which places a block of heterochromatin from the base of chromosome 2R near to the distal end of this chromosome. Figure 5 shows that, as revealed by immunostaining, SU(VAR)3-7
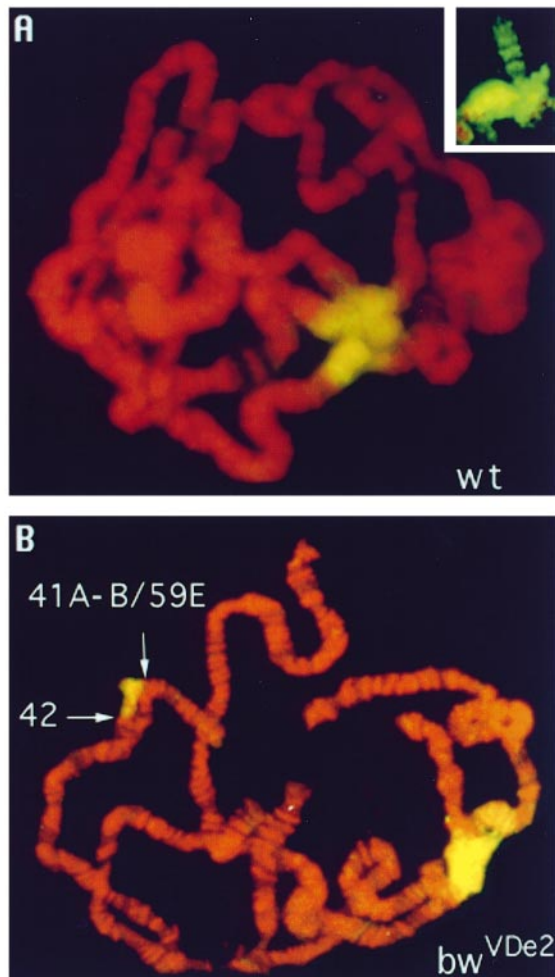
Fig. 5. Immunofluorescent staining of polytene chromosomes using anti-SU(VAR)3-7 antibodies. Green, fluorescein-labelled anti-SU(VAR)3-7; red, propidium iodide staining of DNA. (**A**) Chromosomes from wild-type larvae. Inset: enlargement of chromosome 4 from a different preparation. (**B**) Chromosomes from *bw*^{VDe2}/*CyO* larvae showing ectopic staining of the translocated heterochromatin and absence of staining of the unpaired homologue. The inversion *bw*^{VDe2} breaks at 41A-B and 59D6-E1 (Lindsley and Zimm, 1992).

associates with the block of ectopic heterochromatin as well as with the chromocentre. This pattern again resembles that of HP1. We have therefore performed double immunostaining with our antibodies and the mono-clonal antibody to HP1 (data not shown). We see co-localization on the centromeric heterochromatin and on chromosome 4, the sites primarily stained by antibodies to HP1, but with our antibodies to SU(VAR)3-7 we do not detect the few euchromatic and telomeric sites decorated, though less intensively, by antibodies to HP1 (James *et al.*, 1989). We cannot exclude the possibility that SU(VAR)3-7 association with the euchromatic and telomeric sites was below the sensitivity of our antibodies. Genetic evidence discussed below suggests that this may well be the case in two instances.

Genetic tests of co-operation of Su(var)3-7 and Su(var)2-5

Previous work by others has determined that *Su(var)2-5*, the locus encoding HP1, is a dose-dependent modifier of centromeric heterochromatin-induced PEV (Eissenberg *et al.*, 1990, 1992). Furthermore, the dose of *Su(var)2-5* affects variegation of genes transposed in chromosome 4 (Wallrath and Elgin, 1995) or in the Y chromosome (Lu *et al.*, 1996), as well as variegation due to an ectopic block of heterochromatin (cited in Csink and Henikoff, 1996). PEV caused by a repeat array of a P-transposon also responds to the dose of *Su(var)2-5* (Dorer and Henikoff, 1994). In contrast, the phenotype of variegating insertions at telomeres of chromosome 2L, 2R and 3R is not affected by a change in the dose of *Su(var)2-5* (Wallrath and Elgin, 1995).

We have determined the effect of the dose of the *Su(var)3-7* locus on these same variegating lines to test the co-operation further. The results are summarized in Table I together with the published results obtained for the *Su(var)2-5* locus. In addition to *white-mottled 4* variegation, we find that the dose of *Su(var)3-7* affects the *white* variegation of genes transposed at three locations in chromosome 4 or in the Y chromosome. Moreover, the dose of *Su(var)3-7* also affects *brown* variegation in two rearrangements: *bw*^{D}, an insertion of a block of heterochromatin, and *bw*^{VDe2}, an inversion involving a large block of centromeric heterochromatin (references in Lindsley and Zimm, 1992). Finally, PEV caused by an array of four repeats of a P-transposon (Dorer and Henikoff, 1994) also responds to the dose of *Su(var)3-7*. In contrast, we find that variegation of *white* due to a transposon insertion at the telomere of chromosome 2L (Wallrath and Elgin, 1995) is not affected by changes in dose of *Su(var)3-7*.

In each case, the change of dose of either *Su(var)3-7* or *Su(var)2-5* has the same effect on a specific variegating line. An additional finding is the haplo-suppressor, triplo-enhancer effect of the dose of *Su(var)3-7* on *yellow* variegation of the minichromosome *Dp(1;f)8-23* (Karpen and Spradling, 1990; Tower *et al.*, 1993), which has yet to be tested with *Su(var)2-5*.

We then examined genetic interaction of the two loci. The initial published observations confirm that in the presence of two doses of one locus, a progression from one to three doses of the other results in a stepwise enhancement of silencing on the *white-mottled 4* rearrange-ment (Reuter *et al.*, 1990; Eissenberg *et al.*, 1992). We have combined one dose of *Su(var)2-5* with three or four doses of *Su(var)3-7* (see genotypes and crosses in Materials and methods). As illustrated in Figure 6, it results in an intermediate phenotype on the *white-mottled 4* variegating rearrangement. This means that the suppression of silenc-ing due to the loss of a dose of one locus is partially compensated by an excess dose of the other. The same partially compensated phenotype is obtained when one dose of *Su(var)3-7* is combined with three doses of *Su(var)2-5* (not shown). This result is strongly suggestive, but by no means definite proof, of co-operation of the two loci in silencing. Two other combinations have been tested: the phenotype of a combination of an excess dose of both loci, and the phenotype of a combination of a loss of a dose of both loci (see genotypes and crosses in Materials and methods). The combination of three doses of each locus results in a stronger enhancement of variega-tion than do three doses of either one or the other locus together with two doses of the putative partner. The

Table I. The effects of the dose of *Su(var)3-7* and *Su(var)2-5* on different variegating chromosomal rearrangements and insertions

Cause of variegation	Stock[a]	Dose[b]	*Su(var)3-7*[c]		*Su(var)2-5*[d]	
Centromeric heterochromatin (CH)	*w*^{m4}	1	<	Reuter *et al.* (1990)	<	Eissenberg *et al.* (1992)
		2	–	Reuter *et al.* (1990)	–	Eissenberg *et al.* (1992)
		3	>	Reuter *et al.* (1990)	>	Eissenberg *et al.* (1992)
	bw^{VDe2}	1	<	86.2 ± 2.7	ND	
		2	–	12.0 ± 0.4	ND	
	bw^D	1	<	73.0 ± 2.4	<	Csink and Henikoff (1996)
		2	–	9.1 ± 1.5	–	Csink and Henikoff (1996)
CH (minichromosome)	*Dp(1;f)8-23*	1	<	98.8 ± 2.2	ND	
		2	–	76.8 ± 10.9	ND	
		3	>	30.7 ± 9.5	ND	
Telomeric insertion on 2L	39C-5	1	–	0.5 ± 0.1	–	Wallrath and Elgin (1995)
		2	–	1.4 ± 0.6	–	Wallrath and Elgin (1995)
Insertion in 4, near centromere	118E-10	1	<	50.7 ± 2.4	<	Wallrath and Elgin (1995)
		2	–	1.7 ± 0.2	–	Wallrath and Elgin (1995)
Insertion in 4, medial	39C-12	1	<	80.3 ± 4.1	<	Wallrath and Elgin (1995)
		2	–	37.4 ± 2.6	–	Wallrath and Elgin (1995)
		3	>	2.4 ± 0.6	ND	
Insertion in 4, near telomere	118E-15	1	<	63.8 ± 1.9	<	Wallrath and Elgin (1995)
		2	–	2.5 ± 1.1	–	Wallrath and Elgin (1995)
Insertion in Y	*Tp(3;Y)BL2*	1	<	31.7 ± 1.0	ND	
		2	–	22.7 ± 1.5	–	Lu *et al.* (1996)
		3	>	0.7 ± 0.0	>	Lu *et al.* (1996)
Array of four P-repeats	1A-46	1	<	56.8 ± 0.2	<	Dorer and Henikoff (1994)
		2	–	1.1 ± 0.0	–	Dorer and Henikoff (1994)

[a]References to stocks are in other columns of the Table.
[b]Number of gene doses of the suppressor of PEV. For *Su(var)3-7*, the loss of a dose is due to the small deficiency *Df(3R)Ace*^{HD1}, and the dose in excess to the insertion *T21A* (Reuter *et al.*, 1990). For *Su(var)2-5*, see references in the Table.
[c]The first column symbolizes the modifier effect on PEV: (<) suppression (less silencing); (–) level of variegation with wild-type dose of the modifier or level of variegation with other doses when effect is not different from the wild-type dose; (>) enhancement of silencing of PEV; (ND) not determined. In the second column, the results from one set of experiments is quantified by determining the percentage of red eye pigment relative to that of control flies. For the *Dp(1;f)8-23* stock, variegation of *yellow* was determined by scoring the colour phenotype of the middle bristles of the triple-row bristles at the anterior wing margin.
[d]Summary of effects of *Su(var)2-5* on variegating lines as published, for comparison.

evidence, besides that of the eyes being almost entirely white as expected for an excess dose of each locus, is that essentially all eyes show *roughest* clones (data not shown). *roughest* clones, seen as sectors of irregular facets in the eye, are evidence that inactivation has spread further than the *white* gene and up to the *roughest* gene. *roughest* is close to, but a few polytene chromosome bands downstream of, *white* relative to the block of heterochromatin (Demerec and Slizynska, 1937). We do not see this phenotype with three doses of either one or the other locus. Therefore, the silencing effect of an excess dose of each locus is additive. Finally, the combined suppressor effects of the loss of a dose of each locus in the same genetical background used for other combinations were difficult to assess as each locus is itself a strong haplo-suppressor locus. The eyes are essentially red, as seen with one dose of either one or the other locus, and no conclusion can be drawn.

Anti-SU(VAR)3-7 antibodies immunoprecipitate HP1

The parallels between SU(VAR)3-7 and HP1 in temporal pattern of expression and in cellular, nuclear and chromosomal localization, together with their common functional property of modifying the genomic silencing of PEV, prompted us to test further the 'brides to be'. The anti-SU(VAR)3-7 polyclonal antibody Ab212.2 was bound to protein A–Sepharose and mixed with embryonic nuclear extracts. Fractions retained by the anti-SU(VAR)3-7–protein A–Sepharose and the supernatants were analysed by Western blotting using the anti-SU(VAR)3-7 polyclonal antibody Ab212.1 and the monoclonal anti-HP1. Each of these two antibodies stains the complex specifically precipitated with anti-SU(VAR)3-7, meaning that SU(VAR)3-7 and HP1 are directly or indirectly associated (Figure 7, lane Ip/α-SUVAR). This co-operation is by no means evidence of a direct physical interaction. The specificity of the interaction was tested in a number of control experiments. When pre-immune serum is used instead of antibodies in the immunoprecipitation (Figure 7, lane Ip/PI), or in mock precipitations using phosphate-buffered saline (PBS) instead of serum (not shown), SU(VAR)3-7 and HP1 could not be detected in the pellet fraction. Significant depletion of SU(VAR)3-7 and HP1 was observed in the supernatant of the immunoprecipitated fractions obtained (not shown). Finally an antibody to an unrelated modifier of PEV (anti-RPD3, F.De Rubertis, unpublished) was not detected in the complex, though it is detected in other fractions, thus again indicating that the precipitation does not result from mere unspecific aggregation. There is a possibility that the co-immunoprecipitation does not result from the association of the proteins on chromosomes, but, for example, in a storage complex. There are two arguments making this unlikely. Staining of embryos after cellularization shows nuclear association in a pattern similar to heterochromatin, indicating that a majority of the protein lies there, and the genetic analysis shows a phenotypic response to small changes of dose (0.5 and 1.5) which might not be expected if large storage complexes exist.
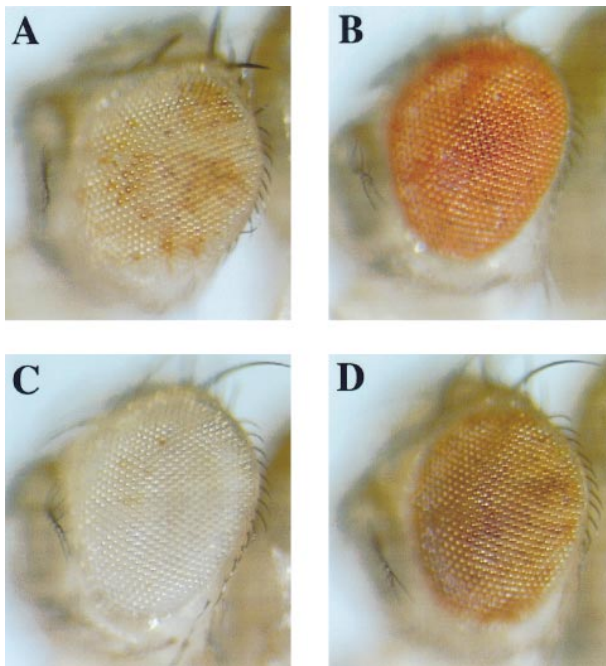
Fig. 6. Combined effect of the loss of a dose of *Su(var)2-5* with excess doses of *Su(var)3-7* on *white-mottled* variegation. Eyes of males produced from the cross between w^{m4};*Su(var)2-5^{05}/InCy* females and w^{m4}/Y;*T20A,T21A5/TM3* males. (**A**) w^{m4}/Y;*InCy/+*;*TM3/+* control male. (**B**) Strong suppression of *white* variegation by the loss of one dose of *Su(var)2-5* in w^{m4}/Y;*Su(var)2-5^{05}/+*;*TM3/+* males. (**C**) Strong enhancement of *white* variegation by the excess doses of *Su(var)3-7* in w^{m4}/Y;*InCy/+*;*T20A,T21A5/+* males. (**D**) Combination of one dose of *Su(var)2-5* with four doses of *Su(var)3-7*, resulting in an intermediate phenotype in w^{m4}/Y;*Su(var)2-5^{05}/+*;*T20A,T21A5/+* males. The relative red eye pigment content of the different genotypes compared with wild-type flies is (in per cent): 4.2 ± 0.9 (A), 80.5 ± 3.7 (B), 0.8 ± 0.1 (C) and 17.7 ± 2.3 (D).

Discussion

We have shown that the protein encoded by *Su(var)3-7* is associated on polytene chromosomes with pericentromeric heterochromatin and chromosome 4, a chromosome described as being partially heterochromatic (Hochman, 1976), indicating that SU(VAR)3-7 is a component of, or at least is associated with, heterochromatin. We note that these are the major sites of association of HP1, the protein encoded by the *Su(var)2-5* locus. We also find that the two proteins co-immunoprecipitate from nuclear extracts. Co-localization on chromosomes and co-immunoprecipit-ation are the first evidence of direct or indirect co-operation between the two proteins. We have also determined that an increase in the dose of the *Su(var)3-7* locus enhances heterochromatin-induced silencing on a number of varie-gating lines previously found to respond to the dose of the *Su(var)2-5* locus. In addition to the enhancement of silencing of variegating lines by an increase in the dose of each locus, we have determined that the loss of a dose of one locus is partially compensated by an excess dose of the other.

There is a difference in the pattern of staining of polytene chromosomes between our results with SU(VAR)3-7 and the published work with HP1. James and co-workers (1989) report that anti-HP1 antibodies, which stain primarily centromeric heterochromatin and chromosome 4, also stain some telomeres and a few
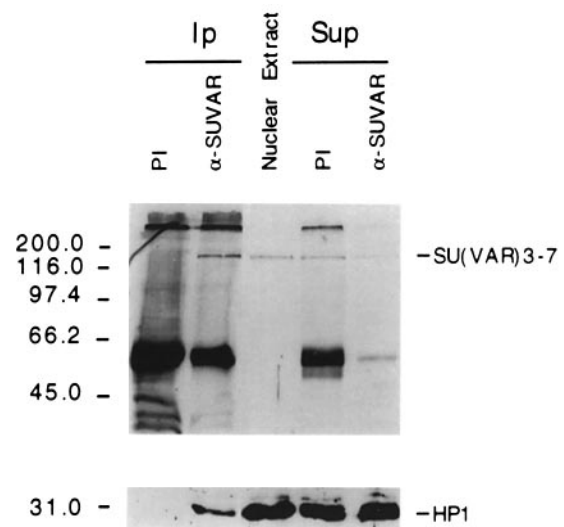
Fig. 7. Western blot analysis of immunoprecipitation of nuclear extracts with anti-SU(VAR)3-7 antibodies. Ip, proteins from nuclear extracts immunoprecipitated with pre-immune serum (PI) or Ab212.2 anti-SU(VAR)3-7 (α-SUVAR); nuclear extract, 10 µg of input proteins; Sup, proteins from supernatants of immunoprecipitations stained with pre-immune serum (PI) or anti-SU(VAR)3-7 (α-SUVAR); same protein amounts as in nuclear extract. Top: Western blot with Ab212.1 anti-SU(VAR)3-7 and alkaline phosphatase-conjugated anti-rabbit antibody, revealed with NBT/BCIP. Bottom: Western blot with C1A9 anti-HP1 and HRP-conjugated anti-mouse antibody, revealed by chemoluminescence.

euchromatic bands on polytene chromosomes. The inten-sity of staining of these sites is lower than that seen on pericentromeric heterochromatin. The difference between the pattern of SU(VAR)3-7 and HP1 could then either represent a true additional specificity of HP1, or a failure of our antibodies to detect lower amounts of SU(VAR)3-7 at these additional sites. We have noted that the staining of centromeric heterochromatin on polytene chromosomes with our antibodies provides a weaker signal than that of antibodies to HP1 kindly provided by S.Elgin. Therefore, our failure to detect the euchromatic and telomeric sites, should they indeed exist, may be due to the weaker signal produced by our antibodies. In at least two instances, our genetic data could support this explanation. The dose of *Su(var)3-7* affects variegation of the bw^D line, which has an insertion of a block of heterochromatin in a euchromatic arm. It also affects variegation due to an array of four repeated transposons (1A-46, Dorer and Henikoff, 1994). Under our conditions, we did not detect SU(VAR)3-7 at the sites of insertion. One could expect SU(VAR)3-7 at these sites where it acts, as occurs for HP1. On the other hand, if future work confirms that SU(VAR)3-7 does not associate stably and directly with sites affected by its dose, this would be rather interesting in view of mechanisms of heterochromatic silencing.

Zuckerkandl (1974) has proposed that the silencing of PEV results from expansion of heterochromatin due to assembly from diffusing factors he called 'locking mole-cules'. This model was reformulated by Locke *et al.* (1988). These authors proposed that the expansion results from co-operative assembly of heterochromatin as multi-meric complexes, and, by analogy with the chemical law of mass action, that the concentration of each component would affect the amounts of complex made, and hence

the extent of spreading and of variegation. The assembly proceeds from a pre-existing block of heterochromatin, split and relocated by a chromosomal rearrangement. This model makes two major testable predictions. First, there is a polar spreading of the silencing effect starting from the block of heterochromatin. Second, the genes encoding components of heterochromatin behave as haplo-suppressors, triplo-enhancers of PEV. Our data fulfil both predictions. First, the spreading effects were detected genetically long ago (i.e. Demerec and Slizynska, 1937) and also cytologically (Hartmann-Goldstein, 1967; Hayashi *et al.*, 1990; Belayeva and Zhimulev, 1991). In this report, evidence of spreading is provided by the *roughest* clones seen when variegation is strongly enhanced by a combination of an excess dose of both *Su(var)3-7* and *Su(var)2-5*. Second, haplo-suppressors, triplo-enhancers of PEV have been isolated in large mutagenesis screens, and are now being analysed further molecularly. Among these, *Su(var)2-5* was found to encode the HP1 protein, which, as predicted by the model, associates primarily with constitutive heterochromatin. The second, *Su(var)3-7*, encodes a protein which we also find to associate with constitutive heterochromatin. Moreover, the two proteins co-immunoprecipitate from nuclear extracts, and an extra dose of one locus can partially restore the effect of the loss of a dose of the other. These data for two haplo-suppressor, triplo-enhancer loci fit perfectly with the two major predictions of the expansion model.

The model is still challenged, however, by a number of observations, such as variegation of heterochromatic genes (Wakimoto and Hearn, 1990), discontinuous compaction (Belyaeva and Zhimulev, 1991; Belyaeva *et al.*, 1993), variegation of non-heterochromatic arrays of repeats (Dorer and Henikoff, 1994) and modifications of nuclear organization by variegating rearrangements (Csink and Henikoff, 1996; Dernburg *et al.*, 1996). Starting with the suggestion of Pontecorvo (1944) that heterochromatin could form from folding of DNA at any region comprising repetitive sequences, and by the fact that middle repetitive DNA is found both within satellite sequences of hetero-chromatin and within euchromatin, Dorer and Henikoff (1994) and Henikoff (1996) propose that heterochromatin nucleating from repeat arrays in euchromatin could fold back to centromeric heterochromatin. This 'sequestration' would drag nearby euchromatic genes and they would be silenced by their new environment. Using this line of thought, the concentration of factors like SU(VAR)3-7 and HP1 would contribute to the stability of the heterochro-matic complex. An interesting aspect of this possibility derives from analogies between modifiers of PEV and regulators of homeotic genes, and a similar model proposed for the latter (Pirrotta and Rastelli, 1994).

The finding by Ye and Worman (1996) that a human homologue of HP1 associates with the lamin B receptor, a component of the inner nuclear membrane, suggests that HP1 may also function in the subnuclear localization of heterochromatin. With its putative DNA-binding motifs, SU(VAR)3-7 is more likely to participate in the initial steps of compaction of DNA. In this respect, the dose-dependent assembly model of heterochromatin, for which we provide evidence here, and the cytological observation of sub-nuclear localization of heterochromatin could both be part of the process resulting in the silencing of PEV.

Materials and methods

Antibodies

Ab264 was raised against a β-galactosidase fusion protein in the pEX expression system (Stanley and Luzio, 1984) using a 260 bp *Bam*HI fragment starting 107 bp downstream of the first ATG. Crude antisera from two rabbits were tested on wild-type embryos and, as a control, on heat-shocked transgenic embryos carrying a copy of the *Su(var)3-7* cDNA under the control of the *hsp70* promoter (Cléard *et al.*, 1995). Dilution was 1:1000–1:2000. One of these antisera was affinity purified (Gu *et al.*, 1994) against another fusion protein where 258 amino acids including the *Bam*HI fragment were introduced in pQE-30 (Qiagen) to produce a 6×His-tagged protein. The affinity-purified fraction was the one used in the immunostaining experiments. Ab212 was made against a 639 bp *Xho*I–*Nsi*I fragment from amino acids 178 to 390, first cloned into *Sal*I and *Pst*I sites of an intermediate vector and taken out with *Bam*HI and *Hin*dIII to be introduced into the same sites of pQE32. Approximatively 2.4 mg of this 30 kDa protein were purified on an Ni-NTA column (Qiagen) and used for immunization of two rabbits. Crude antisera from both rabbits (Ab212.12 and Ab212.2) at a dilution of 1:1000 showed the same pattern on embryos as did Ab264. After affinity purification against the antigen, the eluted fraction was used at a dilution of 1:5000 on embryos and also in immunoprecipitation experiments. The two antibodies against different domains of the protein (Ab264 and Ab212) recognize one band at 175 kDa on Western blots (see the nuclear extract lane of Figure 7).

Embryo staining and analysis

Embryos were fixed and stained as described in Karch *et al.* (1990). Primary antibody was added at the appropriate dilution (1:1000 for crude Ab264, 1:25 for affinity-purified Ab264) and embryos incubated overnight at 4°C. For peroxidase staining, biotinylated anti-rabbit and subsequent reagents were from Vector. For immunofluorescence and confocal analysis, DTAF-conjugated anti-rabbit (1:400) antibodies were used for SU(VAR)3-7 detection, or FITC-conjugated anti-mouse anti-bodies (1:200) for HP1 detection. DNA staining was achieved after RNase A treatment (5 mg/ml, 2 h at 37°C with slow agitation) by adding propidium iodide (5 μg/ml) in the mounting medium. Double staining against SU(VAR)3-7 and HP1 was with a mixture of anti-SU(VAR)3-7 (Ab264) 1:25 and anti-HP1 (C1A9, kindly provided by S.Elgin) 1:400. The secondary antibodies were anti-rabbit–DTAF and anti-mouse–Cy3. Confocal microscopy was with a Zeiss LSM 410, and image processing with Adobe Photoshop 3.0.

Chromosome staining

Polytene chromosome squashes were prepared according to Zink and Paro (1989). For immunostaining, 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 h at room temperature, most of the blocking solution was removed and 5 μl of affinity-purified Ab264 were added. Slides were incubated overnight at 4°C in a humid chamber. After two 15 min washes in BBT (PBS + 100 mM NaCl, 0.1% BSA, 0.1% Tween-20), slides were incubated for 1 h at room temperature in a 1:200 dilution of DTAF-conjugated anti-rabbit antibody (Jackson) in PBS, 1% BSA, 0.1% non-fat dry milk. The slides were washed as above and mounted in 90% glycerol in PBS containing 1–2 μg/ml propidium iodide and 1 μg/ml paraphenylene diamine.

Immunoprecipitation and Western blot analysis

Nuclear extracts were prepared from overnight collections of wild-type embryos according to Han *et al.* (1993). Antibodies to SU(VAR)3-7 were bound to protein A–Sepharose: 50 μl of beads were mixed with either affinity-purified anti-SU(VAR)3-7 (Ab212.2, ~8 mg/ml) or, as a control, the corresponding pre-immune serum (80 mg of protein per ml) in 150 μl final volume of PBS for 1 h at 4°C. Beads were then washed several times with 1 ml of PBS and once with 1 ml of the interaction buffer (120 mM KCl, 50 mM Tris–HCl pH 8, 3 mM MgCl$_2$, 1 mM EDTA, 0.1% NP-40). The nuclear extract (300 μg diluted in 300 μl of interaction buffer) was added to the beads and incubation carried out for 3 h at 4°C. The supernatant was separated, the beads washed five times in 1 ml of interaction buffer (brought to 420 mM final of KCl) and resuspended in 40 μl of SDS protein gel loading buffer. Ten μl were

analysed by SDS–PAGE in parallel with the unbound fractions and an equivalent amount of the input. After electrophoresis, the proteins were transferred to nitrocellulose. The filter was blocked in the same solution used for slides (see above) for 1 h at room temperature before addition of the primary antibody [anti-SU(VAR)3-7 Ab212.1, 1:1000 or anti-HP1, 1:400] and incubation overnight at 4°C. The secondary antibody was an alkaline phosphatase-conjugated anti-rabbit antibody to detect SU(VAR)3-7 and a horseradish peroxidase-conjugated anti-mouse antibody for HP1.

Genetic analysis

Chromosomes and mutations are described in Lindsley and Zimm (1992) and in other references cited. The haplo-suppressor effect of *Su(var)3-7* was tested on different variegating chromosomal rearrangements and insertions using the *Df(3R)Ace^{HD1}* deletion which uncovers the locus (Reuter *et al.*, 1990). The triplo-enhancer effect of *Su(var)3-7* was tested with the transformant line *T21A/CyO* which contains a 6.5 kb genomic fragment found to rescue the function of the modifier of variegation (Reuter *et al.,* 1990). To examine the effects of the dose of *Su(var)3-7* on *white* variegation due to an insertion in chromosome 4 or in a telomere, females homozygous for *y* and *w* and for one of the *P[w^+]* insertions 39C-5, 118E-10, 39C-12 or 118E-15 (Wallrath and Elgin, 1995) were crossed to *w/Y;Df(3R)Ace^{HD1}/TM2* males and, in the presence of high levels of *white* expression, with *w/Y;T21A/CyO* males. The percentage of red eye pigment in the male offspring with one or three wild-type copies of *Su(var)3-7* was determined (Reuter and Wolff, 1981) and compared with the controls bearing two wild-type copies of the gene. For the line containing a Y-linked variegated *P[w^+]* transgene, males yw/*Tp(3;Y)BL2* (Lu *et al.*, 1996) were crossed to *w;Df(3R)Ace^{HD1}/Ap^{Xa}* and *w;T21A/CyO* females. For the *white* variegation associated with the array of repeats of a P-transposon (Dorer and Henikoff, 1994), females *w;T(2;3)V21^e 1A-46/TM6B*, containing a four-copy repeat of *P[lacW]* at 92E, were crossed to *w/Y;Df(3R)Ace^{HD1}/Ap^{Xa}* males. The effect on *bw* variegating alleles was studied by crossing females *bw^{VDe2}/CyO* or *bw^D sp;st* to *w/Y;Df(3R)Ace^{HD1}/TM2* males. The modifier effects of *Su(var)3-7* on *yellow* variegation in the *Dp(1;f)8-23* minichromosome (Tower *et al.*, 1993) were tested by scoring the colour phenotype of the middle bristles of the triple-row bristles at the anterior wing margin (Karpen and Spradling, 1990). Females *y;ry^{506};Dp(1;f)8-23 (y^+, ry^+)* were crossed to males containing the *Df(3R)Ace^{HD1}* deficiency balanced either by *TM2* or *Ap^{Xa}* and with *w/Y;T21A/CyO* males. The percentage of yellow bristles in the male offspring with one or three wild-type copies of *Su(var)3-7* was compared with the controls bearing two wild-type copies of the gene.

Loss of or excess doses of *Su(var)3-7* were combined with loss of or excess doses of the *Su(var)2-5* locus, and the effect of these different combinations were studied on *white-mottled* variegation. The *Su(var)2-5^{05}* allele was used for the loss of dose of the *Su(var)2-5* locus (Eissenberg *et al.*, 1992) and DpP24, which corresponds to a duplication of a fragment of the second chromosome comprising the *Su(var)2-5* locus, as an extra dose of the gene (Wustmann *et al.*, 1989). The combined suppressor effect of the loss of a dose of each locus was tested by mating *w^{m4};Su(var)2-5^{05}/InCy* females with *w/Y;Df(3R)Ace^{HD1}/Ap^{Xa}* males. The combined effect of an excess dose of both loci was studied by crossing *w^{m4};DpP24/InCy* females with *w^{m4}/Y;T21A/CyO* males. The combination of one dose of the *Su(var)2-5* locus with three and four doses of *Su(var)3-7* was tested by mating *w^{m4};Su(var)2-5^{05}/InCy* females with *w^{m4}/Y;T21A/CyO* males and with *w^{m4}/Y;T20A,T21A5/TM3* males. *T20A* and *T21A* are transgenes of *Su(var)3-7* (Reuter *et al.*, 1990). The reciprocal combination was also tested by a cross between *w^{m4};DpP24/InCy* females [three doses of *Su(var)2-5*] and *w/Y;Df(3R)-Ace^{HD1}/Ap^{Xa}* males [one dose of *Su(var)3-7*].

Acknowledgements

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