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A new gene in *Drosophila melanogaster*, *Ravus*, the phantom of the modifier of position-effect variegation *Su(var)3-7*

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ABSTRACT In a search for homologues of the dominant modifier of position-effect variegation *Su(var)3-7*, we have identified one ORF in *Drosophila melanogaster*. The 359 amino acid deduced protein is much shorter than the 1169 amino acid protein Su(var)3-7. Surprisingly, the two genes are very close to each other at 87E on the polytene chromosome map, and are transcribed divergently. The triplet coding for the N-terminus amino acid of the new gene lies only 368 base pairs from the start of transcription of *Su(var)3-7*. This opposite orientation of the homologue has led us to name it *Ravus*. The N-terminus of the Ravus protein contains only one of the seven unusual zinc fingers of Su(var)3-7. A second region of similarity encodes an acidic domain. Finally, there is a block of high similarity near the C-terminus of the two proteins. It corresponds to a new conserved protein domain, BESS, found also in the BEAF and Stonewall *Drosophila* proteins. We have constructed a tagged Ravus protein, and have expressed it as a heat-shock inducible transgene. Ravus associates *in vivo* with polytene chromosomes but, in contrast to the heterochromatin-associated protein Su(var)3-7, does not show specificity for the chromocenter. Ravus does not seem either to modify the genomic silencing of position-effect variegation, as over-expression of the transgene does not affect the variegated phenotype of a number of rearrangements tested.

KEY WORDS: *Drosophila, position-effect variegation, heterochromatin*

Introduction

Heterochromatin is defined cytologically as the regions of the genome condensed throughout the cell cycle. It replicates late during S phase, and is generally concentrated in centromeric and telomeric regions (reviewed in Elgin, 1996). Heterochromatin is involved in various nuclear functions including nuclear organisation, chromosome segregation and gene silencing (Henikoff, 2000, and references therein). Chromosomal rearrangements, or transgene insertions that juxtapose euchromatic genes to constitutive heterochromatin, frequently result in mosaic gene silencing, a phenomenon known as position effect variegation (PEV, Spofford, 1976; Weiler and Wakimoto, 1995; Wallrath, 1998). The extent of silencing depends both on the *cis*-DNA sequences and on *trans*-acting proteins.

Dominant genetic modifiers of PEV were isolated in Drosophila as mutations that can either increase or reduce the proportion of cells in which inactivation occurs. Interestingly, some loci exhibit both a haplo-supressor and a triplo-enhancer effect on variegation, making them good candidates for being structural components of constitutive heterochromatin (Reuter and Spierer, 1992). Among them figures the *Su(var)3-7* gene of *Drosophila melanogaster*. It encodes a large protein of 1169 amino acids containing seven widely spaced unusual zinc fingers, each preceded by a so-called tryptophan motif (Reuter *et al.*, 1990; Cléard *et al.*, 1995). The Su(var)3-7 protein is mainly associated with pericentromeric heterochromatin but also with some telomeres and euchromatic sites (Cléard *et al.*, 1997; Delattre *et al.*, 2000). Over-expression of the protein leads to a strong enhancement of variegation, and an increase in the number of sites bound by the protein on polytene chromosomes (Delattre *et al.*, 2000). We have previously shown by the two hybrid assay in yeast that Su(var)3-7 interacts physically with an other heterochromatic protein, HP1, and that both proteins co-localize *in vivo* at many sites (Delattre *et al.*, 2000). Very recently, two other HP1 proteins were found in *Drosophila melanogaster* (HP1b and c, Smothers and Henikoff 2001), and homologues of the HP1 proteins, HP1α, HP1β and HP1γ, have already been described in human and mouse (Singh *et al.*, 1991; Saunders *et al.*, 1993; Wreggett *et al.*, 1994). HP1β proteins associate with SUV39H1, the mammalian homologue of the *Drosophila* modifier of position-effect variegation Su(var)3-9, indicating the

Abbreviations used in this paper: EST, expressed sequence tag; HA, haemagglutinin; ORF, open reading frame; PEV, position-effect variegation

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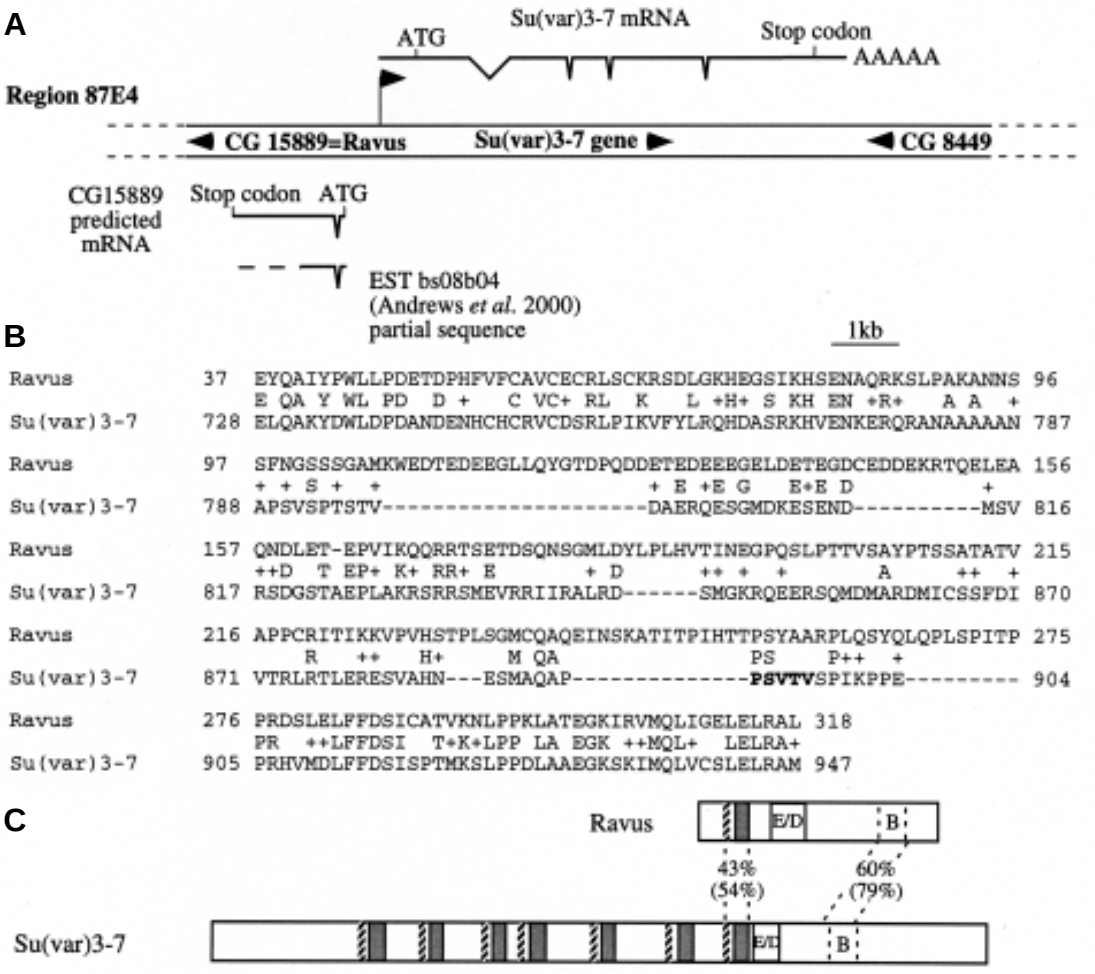

Fig. 1. Comparison of Su(var)3-7 and Ravus sequences. **(A)** *The* Ravus *and* Su(var)3-7 *genes, and their mRNA.* **(B)** *Alignment of the Su(var)3-7 and Ravus protein sequences. The consensus motive of proteins interacting with the chromoshadow domain of HP1 (Smoother and Henikoff (2000) is in bold.* **(C)** *Schematic representation of the Su(var)3-7 and Ravus proteins. Grey boxes represent the zinc fingers, black hatched bars represent the tryptophan boxes (Cléard et al., 1995), E/D boxes are aspartic and glutamic acid-rich regions, and B boxes represent the BESS motif. Amino acid identity and similarity (in brackets) are indicated for the regions of Su(var)3-7 and Ravus which exhibit the highest similarity.*

existence of a mammalian Su(var) protein complex (Tschiersch *et al.*, 1994; Aagaard *et al.*, 1999;).

It is therefore also of interest to look for relatives of Su(var)3-7 in *Drosophila* and other species. We report here that sequence similarity searches with the Su(var)3-7 protein have revealed the presence of one related protein. This *Drosophila melanogaster* protein is encoded by a gene identified as CG15889 in the Drosophila Genome Project (Adams *et al.*, 2000). It is very close and upstream of the *Su(var)3-7* gene. As it is transcribed in the opposite orientation, we have named it *Ravus*. The role of this homologue of Su(var)3-7 was examined by testing the effect of its over-expression on variegation and by visualising the localisation of the protein on polytene chromosomes.

Results and Discussion

Ravus, *a Gene Homologous to* Su(var)3-7 *in* Drosophila melanogaster

No protein homologous to *Drosophila melanogaster* Su(var)3-7 has yet been reported either from sequence similarity searches or by hybridisation. Our own efforts to retrieve a homologue outside of the genus *Drosophila* by hybridisation screens and PCR have not succeeded (C. Seum, C.H. Tonka and M.D., unpublished). We have performed sequence similarity searches (BLAST, Altschul *et al.*, 1997) using the Su(var)3-7 protein sequence (Cléard *et al.*, 1995; EMBL accession number X52187) against the protein sequences databases Genebank, PDB, SwissProt, PIR, and PRF, as well as against the EST databases (Boguski *et al.*, 1993). We find that a 359 amino acid open reading frame within the *Drosophila melanogaster* genome database (Berkeley Drosophila Genome Project, http://www.fruitfly.org) shares significant homology with Su(var)3-7. Interestingly, this gene, identified as CG15889, is localised shortly upstream of the *Su(var)3-7* gene. Only 368bp separate the start of transcription of *Su(var)3-7* (Cléard *et al.*, 1995) from the CG15889 predicted open reading frame which is transcribed in the reverse orientation. We have named CG15889 *Ravus,* as it is homologous to, and close neighbour of *Su(var)3-7,* but transcribed in the other orientation.

The predicted protein is small (359 amino acids) when compared to Su(var)3-7 (1169 amino acids). Ravus is homologous to the C-terminal part of Su(var)3-7. Figure 1 shows the alignment between Su(var)3-7 and Ravus, and schematises the regions of high similarity. The total percent of identity between the two proteins is 26%, and the similarity is 49%. This rather low level of similarity explains the failure to detect additional bands in whole genome Southern using *Su(var)3-7* as a probe (not shown). The Ravus protein comprises a number of protein sequence motives which correspond to landmarks of Su(var)3-7. First, Ravus contains a potential zinc finger of the C2H2 type. This finger is similar to the seven unusual zinc fingers present in the N-terminal moiety of Su(var)3-7, but it is most similar

to the seventh finger of Su(var)3-7. The identity is 43%, and the similarity 54%, including the 14 amino acids preceding the zinc finger. As in Su(var)3-7, the zinc finger is preceded by a tryptophan box (Cléard *et al.*, 1995), precisely localised at 13 amino acids before the first cystein doublet characteristic of the zinc finger. Second, the Ravus protein contains also a domain rich in acidic amino acids which is larger than the corresponding one in Su(var)3-7. There is a third region of homology. It lies toward the C-terminal end of Ravus and Su(var)3-7, and exhibits 60% of identity and 79% of similarity over 43 amino acids. Interestingly, this region is identified in CDD, the Conserved Domain Database (Altschul *et al.*, 1997), as the BESS motif. This motif is named after the proteins in which it is found, namely BEAF (Zhao *et al.,* 1995), Su(var)3-7, and Stonewall (Clark and McKearin, 1996). This motif is restricted until now to 19 proteins, all from *Drosophila melanogaster*.

We have shown by the two hybrid assay in yeast that Su(var)3-7 interacts physically with HP1 (Delattre *et al.*, 2000), and with itself (our unpublished work). A domain of 126 amino acids downstream of the seventh finger promotes interaction between Su(var)3-7 and itself, and between Su(var)3-7 and HP1. Only the second half of this domain, corresponding to the BESS motif, is conserved in Ravus. The consensus motive found by Smoother and Henikoff (2000) in proteins interacting with the chromoshadow domain of HP1 is found there in the domain of Su(var)3-7 defined as interacting with HP1 (PSVTV), but is not conserved in Ravus. The three regions of Ravus with good homology to Su(var)3-7 (zinc finger, acidic domain, and part BESS motif) are colinear with the respective motives in the comparison of the two sequences depicted in Fig. 1.

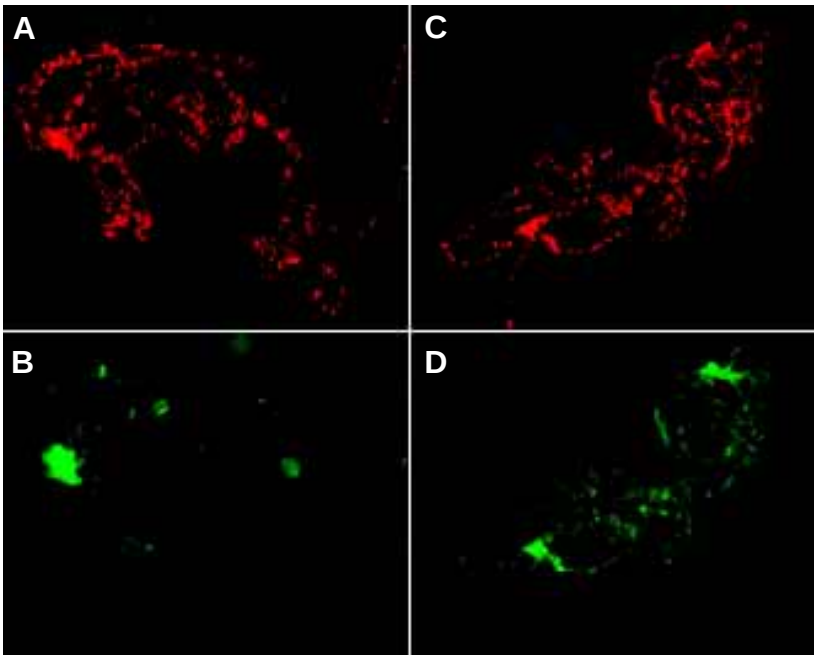

Fig. 2. Su(var)3-7 and HA:Ravus immunolocalisation on polytene chromosomes. *Polytene chromosomes were prepared from two different transgenic* P[HsHA:Ravus] *homozygous lines after heat shock.* **(A)** *Polytene chromosomes from* P[HsHA:Ravus] *12A homozygous line stained with an anti-HA antibody detected by a Cy3-conjugated secondary antibody (red).* **(B)** *The same nucleus as in A but stained with an anti-Su(var)3-7 antibody detected by a DTAF-conjugated secondary antibody (green).* **(C)** *and* **(D)** *same as in (A) and (B) but with polytene chromosomes from a* P[HsHA:Ravus] *13 homozygous line.*

The Genome project has identified one small intron in *Ravus*, after the triplet coding for the 11th amino-acid, in a region showing no similarity to Su(var)3-7. As expected hence, there is no intron in the *Su(var)3-7* gene at this position. We have not studied transcription of *Ravus,* but there is a report of an EST corresponding to the *Ravus* gene. This putative cDNA is present in a *Drosophila melanogaster* adult testis cDNA library (bs08b04 EST; Andrews *et al.*, 2000), and is evidence that *Ravus* is transcribed. This EST does not correspond to the complete *Ravus* cDNA as its 5' sequence starts with the 9th amino acid. Nevertheless, in the 380 bp available, the predicted intron is spliced, thus showing that the RNA is processed. Interestingly, we have not found ESTs corresponding to *Ravus* in other tissues. In contrast, we have not detected ESTs correponding to *Su(var)3-7* in the adult testis cDNA library. The two genes could share transcriptional regulation and, from the limited evidence presented here, one of the two genes only could be expressed at any time.

The Ravus Protein Associates with Polytene Chomosomes

When discovering a homologue of a gene in the same genome, an obvious question to address is whether they both participate in the same endeavour. One avenue to this question is to examine the localisation of Ravus in the nucleus, as that of Su(var)3-7 is well known (Cléard *et al.*, 1997; Delattre *et al.*, 2000). To do this, we have cloned the full length protein, with the exception of the first six amino acids. The construct is also tagged by a HA-antigen and is placed under the control of a heat-shock promoter (see Material and Methods for details). We have introduced the construct in a P-element transformation vector, and have produced transgenic lines. Western blot analysis of crude extracts of transgenic adult flies using an antibody raised against the HA tag shows that the HA:Ravus protein is well produced after heat-shock, and that it has the expected size (data not shown). Double staining of polytene chromosomes from third instar larvae salivary glands were performed with antibodies against HA, and against the endogenous Su(var)3-7. In absence of heat-shock, we could not detect the HA:Ravus protein (not shown). After a moderate heat-shock of 5 minutes at 37°C, a strong staining appears on a large number of bands, and on the chromocenter (Fig. 2). A defined region of the chromocenter appears to be stained more intensely than the rest of heterochromatin (Fig. 2). Nevertheless, the tagged Ravus protein does not show preferential staining for the chromocenter in contrast to Su(var)3-7 (Cléard *et al.*, 1997). We did not either see preferential decoration of the HA:Ravus protein on the few euchromatic sites bound by Su(var)3-7 (Delattre *et al.*, 2000). If Ravus does not have by itself the specificity for the binding sites of Su(var)3-7 on polytene chromosomes, it could have acquired it by associating directly with its homologue or with HP1. This is not the case, thus suggesting that the Ravus sequence does not contain the domains necessary for interaction with Su(var)3-7 or HP1. We conclude that Ravus seems to have a general affinity for chromosomes, but not the specificity of its homologue Su(var)3-7.

Over-Expression of Ravus and Position-Effect Variegation

We have then made an attempt at finding a function for Ravus. From its deduced sequence, the obvious possibility is a role in position-effect variegation. There are no known mutants of the *Ravus* gene according to the database (flybase: http://flybase.bio.indiana.edu). The deficiency *Df(3R)Ace* ^{HD1} uncovers *Su(var)3-7*, *Ravus* and others gene in the 87E region. It has a strong suppressor effect on variegation (Reuter *et al.*, 1990; Cléard *et al.*, 1997). Two transgenes containing genomic DNA fragment from this locus enhance dramatically the variegation (the 10.4 kb SalI and the 6.5kb EcoRV fragments; Reuter *et al.*, 1990). Both contain the *Su(var)3-7* and the *Ravus* genes. However, over-expression of the *Su(var)3-7* gene alone is sufficient to enhance variegation, as an heat-shock inducible Su(var)3-7 transgene increases the level of w^{m4h} repression (Cléard *et al.*, 1995). As we do not dispose of an homozygous null mutant of Su(var)3-7, we can not distinguish between the two genes for the haplo-suppressor effect. Nevertheless we can over-express Ravus alone to test whether the homologue of Su(var)3-7 also participates in the enhancement of heterochromatin induced repression. The HA:Ravus transgene we have made to express a tagged version of the protein is under heat shock control, and allows us to express the full-length Ravus protein at the exception of the first six amino acids. We have combined the transgenic flies expressing the Ravus transgene with different variegating rearrangements, and have examined variegation of the lines in non heat-shock and heat-shock conditions. A panel of rearrangements were tested. They are listed in Table 1. We find that the over-expression of the Ravus protein has no effect on the level of heterochromatin induced repression of all the variegating rearrangements tested. Moreover, no modification of the level of w^{m4h} expression is seen when the over-expression of the Ravus protein is combined with the strong suppressor effect of the $Su(var)2\text{-}5^{05}$ allele of HP1, or the strong enhancer effect of *HS-Su(var)3-7* cDNA. The increased expression of the Ravus protein has no effect on variegation and does not genetically interact with classical modifiers of PEV.

In conclusion, it is very likely that the homology and position of *Su(var)3-7* and *Ravus* reflect a common ancestor. The likely duplication and divergence must be ancient, as the proteins have a rather low level of similarity. However, the strong conservation of some of the landmark sequence motives suggests nonetheless that Ravus has maintained a function, albeit perhaps not in the genomic silencing of position-effet variegation. The loss of most of the zinc fingers, and of parts of domains necessary to interaction with HP1, the companion of Su(var)3-7, is additional evidence for a divergence in function. But chromosomal association is maintained, and it is interesting to speculate on the parallel between the strong expression of *Su(var)3-7* in ovaries to provide a maternal contribution to the egg, and the predicted expression of *Ravus* in testis.

TABLE 1

VARIEGATING REARRANGEMENTS AND MUTATIONS COMBINED WITH THE *RAVUS* TRANSGENE

Strain	Genotype	Variegating gene	Reference
w^{m4h}	*In(1)3C1-2; 20A*	*white*	Lindsley and Zimm (1992)
bw^{VDe2}	*In(2R) 41A-B; 59E*	*brown*	Lindsley and Zimm (1992)
Heidi	*In(2L) P[w⁺WinkD]*	*miniwhite* transgene	Seum *et al.* (2000)
BL2	*Tp(3;Y) P[w⁺HS-lacZ]*	*miniwhite* transgene	Lu *et al.* (1996)
w^{m4h};*FLTX*	w^{m4h}; *P[HS-Su(var)3-7)]*	*white*	Cléard *et al.* (1995)
w^{m4h}; $Su(var)2\text{-}5^{05}$	w^{m4h}; $Su(var)2\text{-}5^{05}$/*Cy*	*white*	Wustmann *et al.* (1989), Eissenberg *et al.* (1992)

Flies homozygous for the heat-shock inducible *Ravus* transgene were crossed with the variegating rearrangements and mutant, and tested as described in Material and Methods. Over-expression of *Ravus* did not affect variegation.

Materials and Methods

The Heat Shock Inducible* HA:Ravus *Transgene

A genomic *Hpa*I DNA fragment from the *Ravus* gene locus was cloned in phase downstream of the HA tag into the *Sma*I site of the Puc-HA plasmid of N. Hulo and V. Pirrotta (unpublished). This fragment encodes the complete Ravus protein, except for the six first amino acids. Then, a *Xba*I/*Sal*I DNA fragment containing the HA-Ravus tagged protein was inserted into the *Xba*I/*Sal*I sites of a modified version of the C4-*yellow* transformation vector (Sigrist and Pirrotta 1997). Expression of the tagged Ravus protein was verified by Western blots as in Cléard *et al.* (1997), using the HA antibody at a dilution of 1:100.

Immunostaining of Polytene Chromosomes

Immunostaining of polytene chromosomes from salivary glands was done as described in Delattre *et al.* (2000). Primary antibodies were used at dilutions of 1:20 for the purified anti-Su(var)3-7 Ab212 (Cléard *et al.*, 1997), and 1:10 for the mouse monoclonal anti-HA. DTAF-conjugated anti-rabbit (1:400) and Cy3-conjugated anti-mouse (1:400) were used as secondary antibodies.

Induction of HA:Ravus for Phenotype Analysis

Heat shock was carried out either by keeping flies constantly at 30°C during the whole development, or by administrating three 37°C heat shocks per day from the first instar larvae until adult emergence from pupae.

Effect of HA:RAVUS on Variegation

Chromosomes and mutations are described in Flybase. To test the effect of the heat shock induction of HA:Ravus protein on w^{m4h}, Heidi (Seum *et al.*, 2000) and bw^{VDe2}, females bearing the variegated alleles were crossed with males homozygous for the *HA:Ravus* transgene, and with *yw* males as control for eye pigmentation without *HA:Ravus* expression. For the line containing a Y-linked variegating *P[w+]* transgene, males *yw/Tp(3;Y)BL2* (Lu *et al.*, 1996) were crossed to females homozygous for *P[HA:Ravus]* transgene or to control *yw* females. The combined effect on w^{m4h} variegation of over-expression of the *Ravus* gene with loss of dose of the *Su(var)2-5* gene or over-expression of the *Su(var)3-7* gene, were tested by crossing homozygous *P[HA:Ravus]* females with w^{m4h}; $Su(var)2\text{-}5^{05}$/*Cy* males or with w^{m4h}; *FLTX/FLTX* males (Cléard *et al.*, 1995; Delattre *et al.*, 2000).

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