Isolation of *Su(var)3-7* Mutations by Homologous Recombination in *Drosophila melanogaster*

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ABSTRACT

The *Su(var)3-7* gene, a haplo-suppressor and triplo-enhancer of position-effect variegation (PEV), encodes a zinc finger heterochromatin-associated protein. To understand the role of this protein in heterochromatin and genomic silencing, mutations were generated by homologous recombination. The donor fragment contained a *yellow*$^+$ gene and 7.6 kb of the *Su(var)3-7* gene inserted between two *FRT*s. The *Su(var)3-7* sequence contained three stop codons flanking an *I-Sce*I cut site located in the 5′ half of the gene. Using two different screening approaches, we obtained an allelic series composed of three mutant alleles. The three mutations are dominant suppressors of PEV. One behaves as a null mutation and results in a maternal-effect recessive lethal phenotype that can be rescued by a zygotic paternal wild-type gene. A P transposon zygotically expressing a *Su(var)3-7* full-length cDNA also rescues the mutant phenotype. One hypomorphic allele is viable and the pleiotropic phenotype showed by adult flies indicates that rapidly and late dividing cells seem the most affected by reduced amounts of Su(var)3-7 protein. All three mutants were characterized at the molecular level. Each expresses a portion of the Su(var)3-7 protein that is unable to enter the nucleus and bind chromatin.

POSITION-EFFECT variegation (PEV) results from the juxtaposition of euchromatin and heterochromatin by chromosome rearrangement or transposon insertion. It is characterized by the stochastic silencing of euchromatic loci placed in the vicinity of blocks of heterochromatin (Weiler and Wakimoto 1995). Many genes have been identified by mutations exhibiting a dominant suppressor effect on PEV (haplo-suppressors, *i.e.*, Reuter and Wolff 1981; Sinclair *et al.* 1983). Among them, *Su(var)3-7* encodes a heterochromatin-associated protein (Cleard *et al.* 1997). This gene is also a triplo-enhancer of PEV (Reuter *et al.* 1990). The N-terminal two-thirds of the Su(var)3-7 protein contains seven widely spaced zinc fingers. Several pair combinations of these zinc fingers bind DNA *in vitro*, with preference for some of the satellite DNAs tested (Cleard and Spierer 2001). Specific binding to pericentric heterochromatin is conferred to Su(var)3-7 by its C-terminal region (Jaquet *et al.* 2002). Overexpression of a C-terminal polypeptide suppresses PEV, probably by trapping endogenous Su(var)3-7 and depleting it from the chromocenter (Jaquet *et al.* 2002).

A number of links exist between *Su(var)3-7* and another modifier of PEV, *Su(var)2-5*, which encodes the heterochromatin-associated protein HP1 (reviewed in Eissenberg and Elgin 2000). First, the two genes show strong genetic interaction. The effect on PEV of decreasing the copy number of one of the genes is compensated by an increased dose of the other (Cleard *et al.* 1997). Second, the two proteins colocalize on polytene chromosomes not only at the chromocenter, but also at some telomeres and at several euchromatic sites (Delattre *et al.* 2000). Third, the two polypeptides co-immunoprecipitate, suggesting that they are components of the same complex (Cleard *et al.* 1997). And finally, a protein-protein interaction has been demonstrated in a yeast two-hybrid interaction-trap assay (Delattre *et al.* 2000). This interaction requires the chromoshadow domain of HP1 and any of three domains of Su(var)3-7 (Delattre *et al.* 2000), of which two contain a pentapeptide proposed as a consensus HP1-binding motif (Smothers and Henikoff 2000).

Until now, the only genetic tools available to study the role of the Su(var)3-7 protein were various transgenes allowing the overexpression of the protein and an ~25-kb deficiency, *Df(3R)Ace*^{HD1}, which uncovers at least five genes (Adams *et al.* 2000): *Ace*, *CG11686*, *CG15889* (now named *Ravus*; Delattre *et al.* 2000), *Su(var)3-7*, and *CG8449*. Because it uncovers several genes, including the two zygotic lethals, *Ace* and *l(3)G7* (Hilliker *et al.* 1980), *Df(3R)Ace*^{HD1} is not appropriate for the characterization of *Su(var)3-7* loss of function. The need for a mutation disrupting only *Su(var)3-7* has led to various unsuccessful mutagenesis attempts. For instance, screens for suppressors of PEV after EMS mutagenesis or *P*-element mobilization did not give hits within the locus (Reuter and Wolff 1981; Reuter *et al.* 1986; G. Reuter, per-

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sonal communication). In addition, local hopping of two *P* elements located in the 87 region and PCR screening using oligonucleotides 5′ of and into the *Su(var)3-7* gene were also unsuccessful (M. Delattre, unpublished results). Moreover, EMS mutagenesis saturating the 87E region for zygotic lethal mutations has not yielded *Su(var)3-7* mutations (Hilliker *et al.* 1980), suggesting that they would not be zygotic lethal.

Because of these past failures, we decided to try a completely different approach on the basis of homologous recombination. Gene targeting is widely used in both yeast and mouse embryonic stem cells, but has been developed only very recently in *Drosophila melanogaster* (Rong and Golic 2000, 2001). The gene knockout system is based on the generation of a linear donor DNA in the fly germline using two enzymatic activities, a recombinase (FLP) to excise a circular donor DNA and the I-SceI endonuclease to open the circle in the middle of the donor sequence. Until now, only a few successes have been reported with this method (Rong and Golic 2000, 2001), but a number of groups have failed to obtain mutants in their favorite gene despite extensive efforts.

We have successfully used two different strategies of screening for homologous recombinants and have obtained three mutant alleles for the *Su(var)3-7* gene, one of which behaves as a null. We describe here the molecular and genetic characterization of these mutations. Lethality is observed only when both the maternal and the zygotic contributions are completely removed, explaining why *Su(var)3-7* mutations have not been isolated in some other mutagenesis screens.

MATERIALS AND METHODS

DNA constructs: The *Car3yellowSu** plasmid (Figure 1) was constructed as follows: A 2083-bp *Bam*HI fragment containing most of the coding sequence of the *yellow* gene from *pC4yellow* (Sigrist and Pirrotta 1997) was introduced into the unique *Bam*HI site of a modified version of plasmid *pJ33R* (Struhl and Basler 1993) carrying two minimal *FRT*s separated by the polylinker *Nhe*I, *Cla*I, *Not*I, *Apa*I, *Bam*HI. The missing ATG as well as 2780 bp of the *yellow* regulatory sequences was added as a PCR fragment (amplified from *pC4yellow*) into the unique *Eco*RI site of the first construct, giving plasmid *pJ33RNCNyellow*. This cloning led to a duplication of 471 bp of *yellow* coding sequence 5′ of the *yellow* regulatory region. The whole *yellow* coding region was sequenced to ensure the fidelity of the PCR step.

The donor *Su(var)3-7* fragment was assembled and modified as follows: A 3022-bp fragment corresponding to 2656 bp of the regulatory region and the first 366 transcribed nucleotides of the *Su(var)3-7* gene was amplified by PCR with the following oligonucleotides designed to destroy the *Hin*dIII site located at the distal end of the regulatory region and to introduce an in-frame stop codon at the *Bam*HI site (753). Numbers in parentheses correspond to the cDNA sequence given in FlyBase (accession no. X52187) and sequences of the oligonucleotides used for the PCR are the following: *Hin*dIII modified, 5′ GGTATCGATATGCTTTTTAAGGGG, and *Bam*HI modified, 5′ ATCCCGGGAGGATCTAGGGCCAACG. This PCR fragment was cloned into the unique *Cla*I/*Sma*I sites of *pBluescript KSII* (Stratagene, La Jolla, CA; *KSII Suvar 5′stop*). An *I-Sce*I site was added by the annealing and cloning of the complementary oligonucleotides 5′ CTAGAGCTAGGGATAACAGGGTAATT and 5′ CTAGAATTACCCTGTTATCCCTAGCT into the *Spe*I site of *KSII Suvar 5′stop*. Integrity of the site was verified by digestion with I-SceI (New England Biolabs, Beverly, MA). The 3′ portion of *Su(var)3-7* was cloned from a genomic *Xba*I (830)/*Hin*dIII (2240) PCR fragment of 1962 bp DNA ligated to a cDNA *Hin*dIII (2240)/*Xba*I (3887) fragment of 1652 bp and inserted into the *Xba*I site of *KSII Suvar 5′stop*. The *Xba*I (830) and *Hin*dIII (2240) oligonucleotides used for the PCR were both modified to introduce in-frame stop codons (*Xba*I, 5′ GCTCTAGACACATCCAATAGCACAACGTG; *Hin*dIII, 5′ AAAAGCTTATTGTCCGCTCAAGCAGTC). The modified *Su(var)3-7* gene was then introduced into the *Cla*I/*Not*I sites of *pJ33RNCNyellow* (*pJ33R Suvar yellow*). Finally, the *pJ33R Suvar yellow* was partially digested with *Kpn*I and the 12.5-kb fragment was introduced into the *Kpn*I site of *Carnegie 3* (*Car3yellowSu**; see Figure 1).

Fly stocks and genetics: The stocks *y w (v); P[ry^{+}, 70FLP]4 P[v^{+}, 70I-SceI]2B Sco/S^{2} CyO* and *w^{1118}; P[ry^{+}, 70FLP]10* (homozygous on the second chromosome, expresses FLP constitutively) were provided by Yikang Rong and Kent Golic. Description of other stocks can be found at http://flybase.bio.indiana.edu.

Targeting was done from two different donors on the X chromosome for the normal scheme (donors 2 and 7.1) and three different donors on chromosome 2 for the rapid scheme (donors 1, 4.1, and 5). We crossed 732 G1 females for the normal scheme and 828 G1 females for the rapid scheme, using 3 females per vial. To maximize the efficiency of donor excision, two heat shocks (37°, 60 min) were performed on G1 first and second instar larvae. Mapping of chromosome linkage of the recombination events was done by mating to *y w^{67}; CyO/If; MKRS/TM6B* flies.

Molecular characterization of the targeted events: Southern and Northern blots were performed as in Gerard *et al.* (1996). The following specific primers were used for the precise mapping of the homozygous recombinant flies by PCR: (1) 5′ TGGTCGCTTGAGATTCGAC located just 3′ of the *yellow* coding sequence and (2) 5′ ATCCCGGGAGGATCTAGGGCCA ACG located at *Bam*HI (753) of *Su(var)3-7* in the reverse orientation (oligonucleotides 1 and 2 in Figure 1). Sequences were performed with a set of *Su(var)3-7* internal and external primers.

One-step reverse transcription (RT)-PCR was performed with a QIAGEN (Valencia, CA) kit and oligonucleotides at positions 520 and 2350 (reverse) on the *Su(var)3-7* coding region. RNA was prepared with TRIzol (no. 15596026; GIBCO BRL, Gaithersburg, MD) using 10 adult females as starting material.

Immunostaining: Immunostaining of whole mounts: Salivary glands were dissected in Cohen buffer (10 mm $MgCl_2$, 25 mm $Na_2GlycerolPO_4$, 3 mm $CaCl_2$, 10 mm KH_2PO_4, 0.5% NP40, 30 mm KCl, 160 mm sucrose) and fixed for 20 min in formaldehyde fixative buffer (0.1 m NaCl, 2 mm KCl, 2% Triton X-100, 2% formaldehyde, 10 mm NaH_2PO_4). The rest of the protocol was as in Cleard *et al.* (1997), using antibody 264 at a dilution of 1/20 and an anti-rabbit DTAF (Jackson Laboratories, West Grove, PA) at a dilution of 1/200. For immunostaining of polytene chromosomes (Platero *et al.* 1995), third instar salivary glands were dissected in Cohen buffer. They were fixed 3 min in formaldehyde fixative buffer, incubated 2 min in a 45% acetic acid/2% formaldehyde solution, and squashed. The rest of the protocol was done as described above. Immunostaining on whole-mount embryos was done as in Cleard *et al.* (1997) with antibody 264 at a dilution of 1/100 and an anti-rabbit Cy3 (Jackson Laboratories) at a dilution of 1/400.

RESULTS

Choice and construction of the donor: Modifiers of PEV are usually tested for their effect on a *white* gene relocated near centromeric heterochromatin, such as w^{m4h} (Reuter and Wolff 1981) or *Heidi* (Seum *et al.* 2000). For this reason, we could not use the targeting vector of Rong and Golic (2001), which carries a *white* marker gene whose expression would obscure the effect on the variegating *white* gene. Therefore, we constructed a new vector, *Car3yellowSu**, derived from *Carnegie 3* and carrying the *yellow* marker.

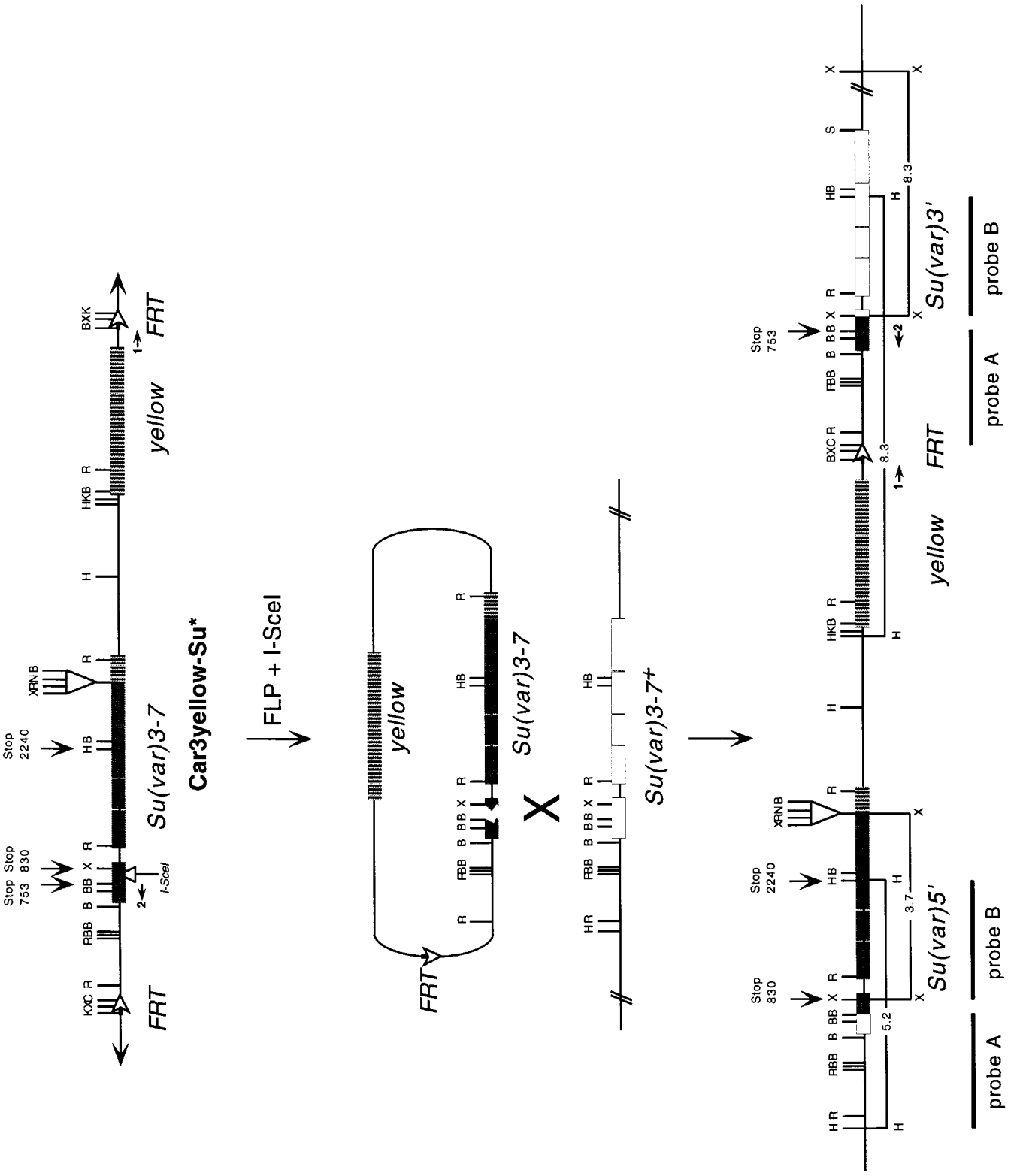
We estimated that a donor resembling the one used for the *pugilist* gene targeting (Rong and Golic 2001) might not be appropriate. In their experiment, the authors used a small donor creating a 3′ truncated gene and a 5′ truncated gene upon homologous recombination. Indeed, many truncated forms of Su(var)3-7 can be perfectly well expressed, and overexpression of some of them results in distinct effects on PEV. For example, a protein devoid of the zinc finger region has a strong dominant negative effect on PEV (Jaquet *et al.* 2002). We therefore decided not to create a 5′ truncated gene, as it might potentially be expressed from a cryptic promoter.

The most important feature in homologous recombination seems to be the length of homology. In mouse gene targeting, for example, a number of groups have described a relationship between the length of homology and the targeting frequency (Thomas and Capecchi 1987; Hasty *et al.* 1991). A recent review also discusses the importance of the length of homology in Drosophila homologous recombination (Gloor 2001). We decided to use a 7.6-kb fragment containing 2.7 kb of 5′ regulatory sequences and most of the *Su(var)3-7* gene, except for the last and very short intron and the 3′ untranslated trailer.

To make it an appropriate donor for targeted mutagenesis, we introduced several modifications in the *Su(var)3-7* sequence. A site for the I-SceI yeast endonuclease was inserted in place of the 77-bp sequence from 753 to 830 encoding part of the first zinc finger. Since the frequency of homologous recombination is affected by mismatches (see, for example, Te Riele *et al.* 1992), we chose to keep modifications of the gene to a minimum. Stop codons were introduced by single-point mutations at three different positions. Two of them were placed near the N terminus of the protein, one before and one within the first predicted zinc finger (the *Bam*HI 753 stop codon was located 5′ of and the *Xba*I 830 stop codon, 3′ of the *I-Sce*I site). The third stop codon was introduced at position 2240 between the fifth and sixth zinc fingers. Integration of this fragment by homologous recombination would lead to a duplication of *Su(var)3-7* as well as a duplication of the *Ravus* gene lying in opposite orientation 368 bp 5′ of the start of transcription of *Su(var)3-7*. *Ravus* has some sequence similarities with the C-terminal part of Su(var)3-7, but its duplication was not a concern since its overexpression does not affect PEV or fly viability (Delattre *et al.* 2002). The predicted Su(var)3-7 truncated proteins, if expressed, should not bind to centromeric heterochromatin and should not be functional, since similar polypeptides do not behave as enhancers of PEV when they are overexpressed (Jaquet *et al.* 2002). We introduced the 7.6-kb modified donor fragment in a vector carrying the *yellow* marker gene. Because of the cloning procedure, the modified *Su(var)3-7* gene does not have its normal stop codon and polyadenylation sites, but is fused in frame with the first 471 nucleotides of the *yellow* coding region. We named the final construct *Car3yellowSu** as the transformation vector derives from *Carnegie 3* (Figure 1).

Recombination: We injected the *Car3yellowSu** plasmid in $y\ w^{67}$ flies and recovered transformants on the X, second, and third chromosomes. To maximize the chance of recovering homologous recombinants, we started with donors at five different locations and used two different screening protocols. Two donors on the X chromosome were used for a "normal" targeting experiment (Figure 2A), which involved 732 G1 females for 244 crosses, and three donors on the second chromosome were used for the "rapid scheme" experiment (Rong and Golic 2001; Figure 2B), which involved 828 G1 females for 276 crosses. The advantage of the second approach is that the progeny are more abundant (~300 progeny per cross in the rapid scheme *vs.* ~100 progeny per cross in the normal screen) because the female parents have less balancer chromosomes and hence are more fertile. In addition, all the progeny could be safely screened even if the excision of the donor was not 100%. We induced targeting in females, as both the frequencies of total recombination and of homologous recombination are much higher in the female than in the male germline (Rong and Golic 2000). After screening ~105,000 progeny, we isolated 18 fertile potential recombinants (Table 1).

We tested all the potential recombinant lines by Southern blot analysis. Genomic DNA was prepared from heterozygous recombinant flies and digested with the restriction enzymes *Hin*dIII or *Xba*I. The *Hin*dIII blots were probed with a PCR fragment comprising the 2.7 kb of regulatory sequences of *Su(var)3-7* and the transcribed nucleotides up to the *Bam*HI site at 753 (Figure 1, probe A). The *Xba*I blots were probed with a PCR fragment from position 830 to 2240 (probe B). These Southern blots allowed us to determine whether the recombination was targeted and how many copies of the donor were inserted into the locus. We obtained different types of events: insertion of the donor into the *Su(var)3-7* locus or outside of the locus, insertion accompanied by a deletion, and duplication of the donor followed by an insertion into the *Su(var)3-7* locus or outside of the locus (see some examples in Figure 3).


Stop 753
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FRT
yellow
Su(var)3-7
Car3yellow-Su*
I-SceI
FLP + I-SceI
yellow
FRT
Su(var)3-7
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FRT
Su(var)3'
Su(var)5'
probe A
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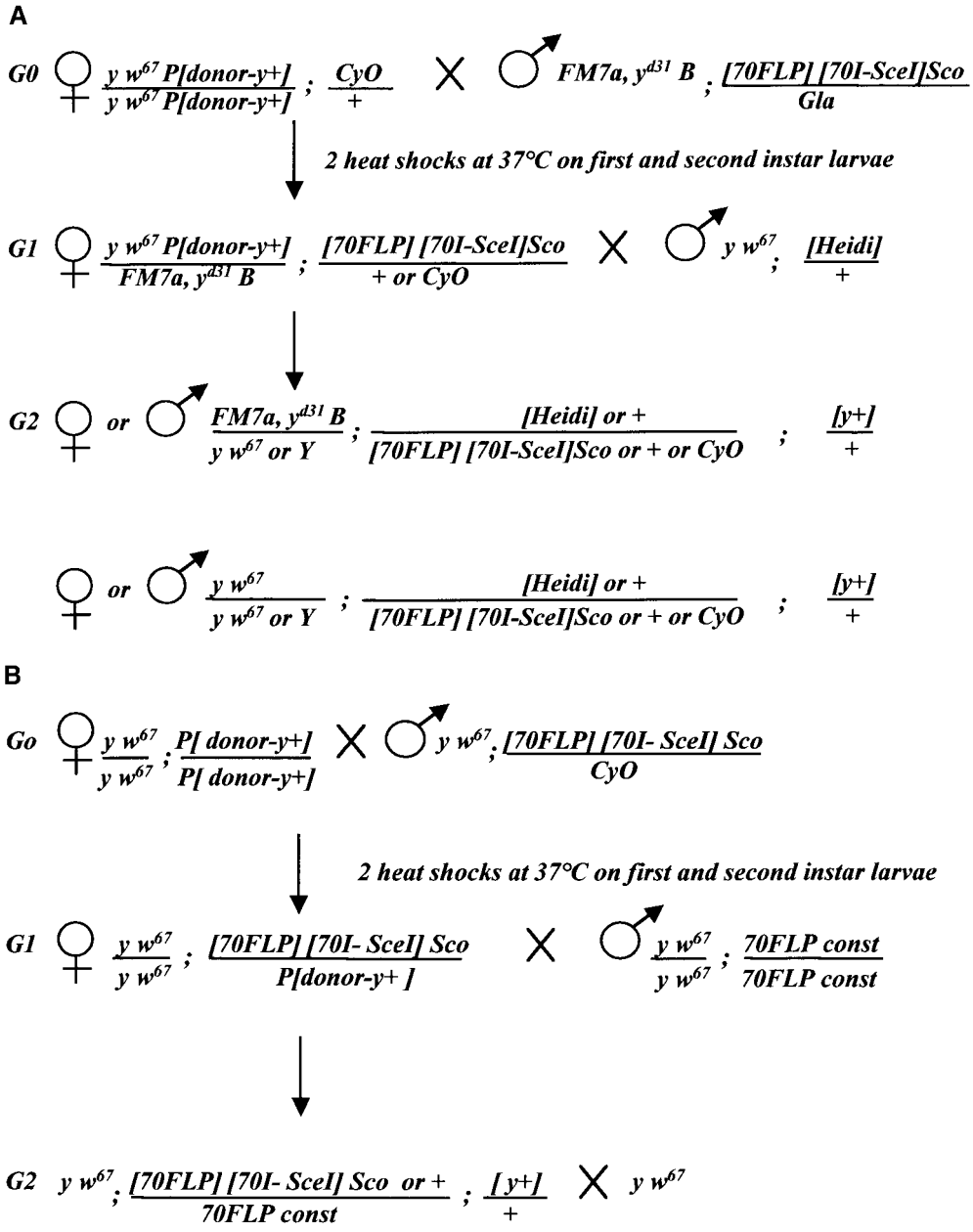

FIGURE 2.—Genetic crosses. (A) "Normal" targeting protocol: screen for y^+ and *Heidi*-suppressed flies or y^+ in non-*Heidi* flies. Two heat shocks were done on first and second instar G1 larvae to induce the FLP recombinase and the I-SceI enzyme. We selected y^+ G2 flies bearing the *FM7a, B* balancer, as well as flies in which variegation of the *white*$^+$ gene carried by *Heidi* (SEUM *et al.* 2000) was suppressed. Since the donor on the X chromosome was rather efficiently excised, we also kept flies that did not have the X chromosome balancer and had not received *Heidi*. False positives were identified by the linkage of y^+ to the X chromosome. (B) "Rapid scheme" approach (RONG and GOLIC 2001): screen for y^+ nonmosaic flies. The G1 females were crossed to a strain carrying a homozygous transposon that expresses the FLP recombinase constitutively. This ensures the somatic excision of the y^+ marker in the rare G2 flies coming from germ cells in which the donor had not been excised. Nonmosaic y^+ animals are true recombinants, because the unique *FRT* does not allow the excision of the marker.

We performed PCR amplifications to further characterize the insertions in the *Su(var)3-7* locus. Primers were designed to amplify the recombinant DNA only, but not the *Car3yellowSu** donor sequence (oligonucleotides 1 and 2 in Figure 1). Ten of the 18 recombinants were homologous insertions in the *Su(var)3-7* locus: 7 homologous events out of 8 total recombinants from donor 7.1; 1/2 from donor 2; 0/2 from donor 1; 1/3 from donor 4.1; and 1/3 from donor 5 (Table 1). On average, we estimated the frequency of recombination at 1 per 10,700 gametes. However, one donor gave no homologous recombinant, while another gave a frequency of homologous recombination of ~1 per 2300 gametes. These data suggest that donors located at different chromosomal positions may have very different targeting efficiencies and that it is safer to start with several donors.

Effect of the homologous recombinants on PEV: We

FIGURE 1.—"Ends-in" homologous recombination scheme. Constructs are described in MATERIALS AND METHODS, and genetic crosses are in the Figure 2 legend. The FLP recombinase expressed from a transgene located on the second chromosome induces excision of the donor as a circular molecule, which is then cut by the I-SceI endonuclease. Ends-in recombination of this linear product with the endogenous *Su(var)3-7* gene leads to a duplication of the locus flanking the *yellow* marker and a remaining *FRT* site. Integration of the donor DNA bearing mutations 5′ and 3′ of the *I-Sce*I site gives rise to two mutant copies of *Su(var)3-7*, named *Su(var)5′* and *Su(var)3′*.

TABLE 1

Summary of the recombination events

Name of donor (chromosome)	No. of G1 females (no. of crosses/ estimated no. of progeny per cross)	No. of recombinants (percentage per G1 female)	
		Homologous	Nonhomologous
7.1 (X)	480 (160/100)	7 (1.46)	1 (0.21)
2 (X)	252 (84/100)	1 (0.40)	1 (0.40)
1 (II), 4.1 (II), and 5 (II)	828 (276/300)	2 (0.24)	6 (0.72)

tested the 10 homologous recombinants for their effect on the *Heidi* variegating line (Seum *et al.* 2000). Seven of them showed no effect at all on expression of the *white* reporter gene, but 3 recombinants, all coming from donor 7.1 on the X chromosome, were strong dominant suppressors of PEV. These three lines, *Su(var)3-7*^{14}, *Su(var)3-7*^{9}, and *Su(var)3-7*$^{7.1A}$, were also tested on two other variegating lines, *w*^{m4h} and *bw*^{VDe2}. The effect was quantified by red eye pigment extraction (Table 2). Except for *Su(var)3-7*^{9} on *w*^{m4h}, the 3 recombinants were strong dominant suppressors on the variegating lines. *Su(var)3-7*^{14} showed pigment amounts similar to the *Df(3R)Ace*^{HD1} deficiency, suggesting that it is a genetically null mutation of *Su(var)3-7*. The two other recombinants appear to be hypomorphic alleles, with *Su(var)3-7*^{9} being the weakest.

Lethal phenotype of two of the mutants: We crossed these heterozygous recombinants *inter se* or with *Df(3R)Ace*^{HD1} and obtained the expected Mendelian numbers of homozygous or hemizygous flies, showing that these *Su(var)3-7* mutations are not zygotic lethal. When crossed to wild-type flies, both the homozygous males and the homozygous females were fertile, although *Su(var)3-7*^{14} females produced a reduced amount of progeny. However, when we crossed homozygous *Su(var)3-7*^{14} males and females together, all the progeny died during the second larval stage. We observed the same result with *Su(var)3-7*$^{7.1A}$, except that the lethal phase was the third larval stage. When homozygous *Su(var)3-7*^{14} or *Su(var)3-7*$^{7.1A}$ females were crossed to heterozygous males, no homozygous progeny was recovered while a large number of fertile heterozygous progeny were obtained. Thus, elimination of both the maternal and the zygotic activity of *Su(var)3-7* is lethal. Furthermore, the maternal contribution is sufficient for normal viability even in the complete absence of zygotic activity and the absence of maternal contribution can be fully compensated by zygotic expression of a paternal wild-type gene. Hemizygous *Su(var)3-7*^{14}*/Df(3R)Ace*^{HD1} larvae from hemizygous female parents were also dying as second instar larvae, again indicating that this allele is amorphic. In contrast, homozygous *Su(var)3-7*$^{7.1A}$ larvae were found to die earlier when their mother was a hemizygote *Su(var)3-7*$^{7.1A}$*/Df(3R)Ace*^{HD1} rather than a homozygote *Su(var)3-7*$^{7.1A}$, further confirming the hypomorphic nature of this allele.

To verify that the lethality was due solely to *Su(var)3-7* inactivation, we tested the ability of *Su(var)3-7* transgenes to rescue the lethality of the *Su(var)3-7*^{14} mutation. Table 3 shows that partial rescue was obtained with a heat-inducible hemagglutinin-tagged full-length cDNA (Jaquet *et al.* 2002), even in absence of heat shock. Unexpectedly, we noted that the female progeny appeared to be rescued more easily than the male progeny. Trying to increase the expression of the transgene by three heat shocks at 32° every day did not improve the rescue (data not shown). Similar rescue of the *Su(var)3-7*^{14} mutation was also obtained with a transformant line carrying a 6.5-kb genomic fragment containing *Su(var)3-7* (T21A, Reuter *et al.* 1990; data not shown).

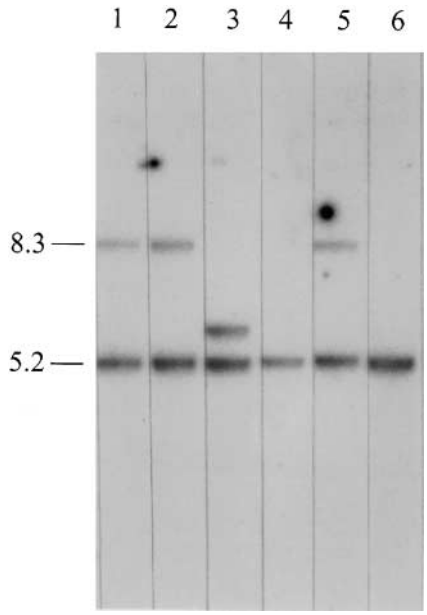

Figure 3.—Analysis of the recombinants by genomic Southern blot. Lanes 1–5, genomic DNA of five different heterozygous recombinants. Lane 6, *y w*^{67} control DNA. Samples were digested with *Hin*dIII, separated on a 1% agarose gel, blotted, and hybridized with probe A covering the 5′ end of the donor fragment (see Figure 1). The 5.2-kb band corresponds to nonrecombined *Su(var)3-7* and to *Su(var)5′* in homologous recombinants. The 8.3-kb band corresponds to *Su(var)3′* in homologous recombinants. Lanes 1, 2, and 5 show homologous integration into the *Su(var)3-7* locus. Lane 3 shows a nonhomologous recombination event (most probably into the *yellow* locus). Lane 4 shows a nonrecombinant line where the *yellow* mutation has been corrected from the *yellow*$^{+}$ of the donor molecule.

TABLE 2

Effect of *Su(var)3-7* mutations on three variegating genes

Rearrangement	+/+	*Su(var)3-7*^{9}/+	*Su(var)3-7*$^{7.1A}$/+	*Su(var)3-7*^{14}/+	*Df(3R)Ace*^{HD1}/+
Heidi	0.041 (±0.001)	0.171 (±0.012)	0.170 (±0.013)	0.220 (±0.017)	0.218 (±0.025)
w^{m4h}	0.018 (±0.000)	0.013 (±0.005)	0.074 (±0.014)	0.566 (±0.050)	0.455 (±0.023)
bw^{VDe2}	0.079 (±0.008)	0.697 (±0.048)	0.741 (±0.016)	0.815 (±0.008)	0.746 (±0.010)

Pigment measurements (Reuter and Wolff 1981) on F_1 3-day-old males from crosses of *Su(var)3-7*^{9,14} or *Su(var)3-7*$^{7.1A}$ males with *Heidi*, *w*^{m4h}, and *bw*^{VDe2} females. Data are the mean values of three measurements performed with 10 adult heads. The standard errors are given in parentheses.

Visible adult phenotype of a hypomorphic *Su(var)3-7* allele: In contrast to the two mutations described above, females homozygous for *Su(var)3-7*^{9} produced viable progeny even when the paternal allele was also mutated [*Su(var)3-7*^{9}, *Su(var)3-7*^{14}, or *Df(3R)Ace*^{HD1}]. Indeed, we have easily maintained a homozygous *Su(var)3-7*^{9} stock over >10 generations at 25°. However, the adult flies display many abnormalities. The most frequent and striking phenotype is an abnormal abdominal cuticle, where the anterior and posterior margins of the tergites are irregularly edged out. Also, many bristles are disorganized or missing (Figure 4). Other parts of the body also show abnormalities, though less frequently: thinner thoracic macrochaetes, missing humeral and sex comb bristles, spots of irregular ommatidia, and curled or ill-developed wings. Apart from these visible phenotypes, the flies look quite healthy although their development is slowed down. The hypomorphic nature of this mutation was confirmed by the finding that the progeny of hemizygous females [*Su(var)3-7*^{9}*/Df(3R)Ace*^{HD1}] crossed to hemizygous *Df(3R)Ace*^{HD1}*/TM3* males was only TM3. It is interesting to note that the most affected tissue, the abdominal cuticle, derives from cells, the histoblasts, which divide very rapidly and later during development than the imaginal cells (reviewed by Fristrom and Fristrom 1993). It is possible that the histoblasts are more affected because all the maternal contribution would be completely eliminated at the time of pupariation.

Structure of the three *Su(var)3-7* mutations: We characterized the molecular structure of the three mutant alleles in detail by PCR amplification followed by sequencing of the appropriate fragments. The results are schematized in Figure 5. Genomic Southern analysis of *Su(var)3-7*^{14} showed a 3.7-kb deletion of most of the *Su(var)3′* copy. We confirmed this finding by PCR using a primer at position 527 and a primer located 2.5 kb downstream of the *Su(var)3-7* coding sequence. The sequence of this PCR product was normal up to the *Bam*HI site (position 753). Then, one-half of the *I-Sce*I cut site was present followed by a 3′ 673-bp deletion starting from this point (position 830) and ending 14 nucleotides before the *Su(var)3-7* stop codon. The resulting potential polypeptide corresponds to the first 122 amino acids and is expected to be completely inactive since it misses all the zinc fingers as well as the C-terminal domain that is required for heterochromatin binding (Jaquet *et al.* 2002). We then analyzed the *Su(var)5′* copy. Sequencing of the whole coding region did not show any mutation compared to the wild-type *Su(var) 3-7*. In particular the stop codons at 830 and 2240 were found to be repaired, probably by the use of the sequence on the homologous chromosome. The only difference between this *Su(var)5′* copy and a wild-type *Su(var)3-7* gene is the fact that, due to the cloning procedure, the open reading frame of the former is fused at the C terminus in frame with 157 amino acids of the Yellow polypeptide.

On Southern blots, *Su(var)3-7*^{9} appears to be a simple

TABLE 3

Rescue of the *Su(var)3-7*^{14} mutation by a *Su(var)3-7* cDNA transgene

Genotype of parents[a] (female × male)	Progeny				Relative viability of homozygous relative to heterozygous	
	Heterozygous *Su(var)3-7*^{14}		Homozygous *Su(var)3-7*^{14}			
	Females	Males	Females	Males	Females	Males
Su(var)3-7^{14} × *Su(var)3-7*^{14}*/TM6B*	251	131	0	0	0	0
Su(var)3-7^{14} × *FL1A;Su(var)3-7*^{14}*/TM6B*	276	183	128	34	0.46	0.19
FL1A;Su(var)3-7^{14} × *Su(var)3-7*^{14}*/TM6B*	192	110	32	6	0.17	0.05

[a] All the flies were *y w*. FL1A is a heat-inducible HA-tagged Su(var)3-7 cDNA, P[*y*$^{+}$, *hs-HA::Su(var)3-7*] (Jaquet *et al.* 2002).

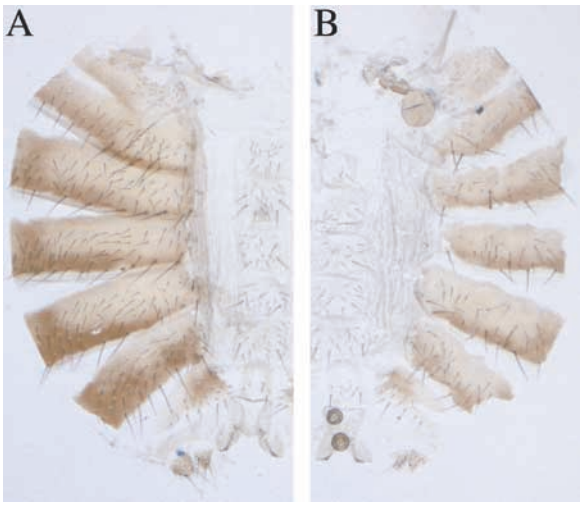

FIGURE 4.—Cuticle preparation of heterozygous (A) and homozygous (B) *Su(var)3-7*^{9} mutants. The procedure was described by MIHALY *et al.* (1997).

integration, as the *Hin*dIII genomic digest analyzed with probe A gave the expected two bands of 5.2 and 8.3 kb. Sequencing revealed that *Su(var)5′* retained the two predicted stop codons at positions 830 and 2240. However, the *Su(var)3′* copy is peculiar, because the stop codon at position 753 was repaired and an unexpected stop codon was found at position 830 (Figure 5). It is likely that during the integration process the 3′ copy was not repaired using the sister chromatid or the homologous chromosome as template but rather using the already integrated 5′ copy.

For the *Su(var)3-7*$^{7.1A}$ recombinant, a *Hin*dIII Southern blot hybridized with probe B spanning from positions 520 to 2350 gave three bands of 8.3, 5.5, and 5.2 kb, indicating the presence of three and not two copies of *Su(var)3-7* (Figure 5). Sequencing of each copy showed that *Su(var)5′* retained the stop codon at position 2240, but was repaired at position 830. *Su(var)3′* was repaired at position 753 but had an unexpected stop codon at position 2240. The middle *Su(var)* copy had a stop codon at position 830. This complexity is not compatible with the simple integration of a dimerized donor as found in some targeted events at the *yellow* locus (RONG and GOLIC 2000). As with recombinant *Su(var)3-7*^{9}, the 3′ copy was probably repaired with an already integrated 5′ copy.

Expression of the mutant genes: At this point of the analysis, the genetic and molecular data seemed conflicting. The hypomorphic *Su(var)3-7*^{9} allele contains stop codons in both gene copies and should make only a small N-terminal polypeptide, while the null allele, *Su(var)3-7*^{14}, should make a full-length Su(var)3-7 protein fused to an N-terminal portion of the Yellow polypeptide. To resolve this discrepancy, we analyzed the expression of the mutant genes at the RNA and protein levels.

Northern blot and RT-PCR analysis on total RNA from *Su(var)3-7*^{14} homozygous adult females showed that both *Su(var)* copies are efficiently transcribed (Figure 6) and spliced (not shown). From the sizes estimated on Northern blots, we deduced that the RNA from the *Su(var)5′* copy is probably polyadenylated at an A/T-rich region located in the regulatory sequence of *yellow* and that the RNA from the partially deleted *Su(var)3′* is surprisingly polyadenylated at the second consensus site, which is normally not used in adult females (CLEARD *et al.* 1995).

Immunostaining of blastoderm embryos with an antibody directed against the N-terminal portion of Su(var) 3-7 (CLEARD *et al.* 1997) showed that the potential fusion protein Su(var)3-7/Yellow and/or the deleted Su(var) 3′ polypeptide are produced, but appear to be unable to enter the nucleus and thus remain cytoplasmic (Figure 7a). Since this antibody does not discriminate between the predicted polypeptides, we do not know whether both are produced. Nevertheless, the absence

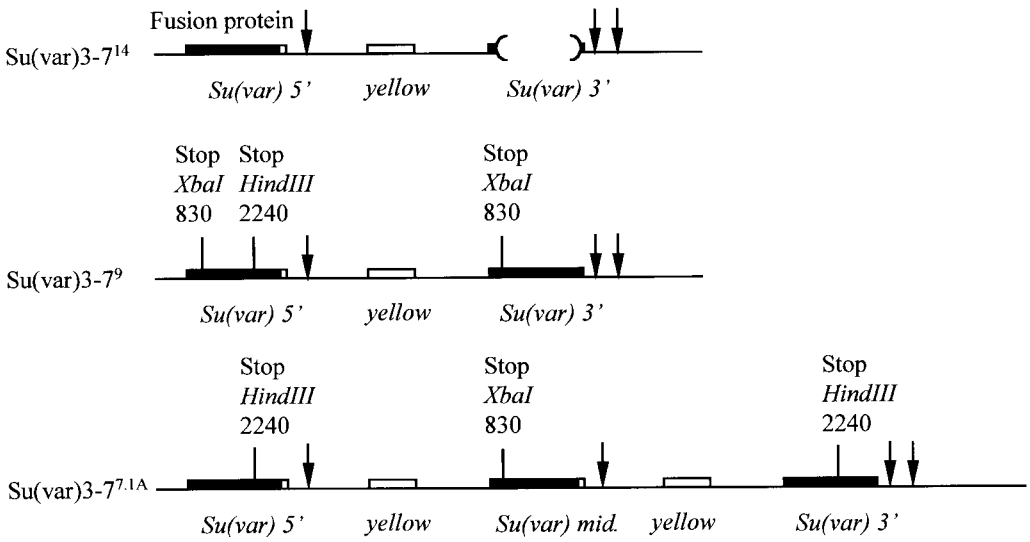

FIGURE 5.—Schematic representation of the *Su(var)3-7*^{14}, *Su(var)3-7*^{9}, and *Su(var)3-7*$^{7.1A}$ mutations. For a detailed description, refer to the text. The stop codons and the protein fusion are indicated above the genes. Arrows indicate the polyadenylation sites.

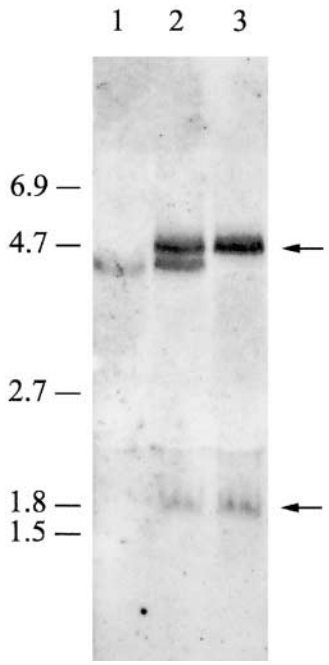

FIGURE 6.—RNA expression. Twenty micrograms of total RNA from adult females was separated on a 1% agarose-formaldehyde gel, blotted, and hybridized to a cDNA probe extending from position 520 to 2350. Lane 1, Canton-S control RNA; lane 2, heterozygous *Su(var)3-7*^{14}/+ RNA; lane 3, homozygous *Su(var)3-7*^{14} RNA. Lane 1 shows a single band corresponding to the *Su(var)3-7* transcript polyadenylated at the first site (CLEARD *et al.* 1995). The top arrow shows a 4.7-kb band corresponding to the transcript of *Su(var)5′*, using a cryptic polyadenylation site in the *yellow* regulatory region downstream of the *Eco*RI site (see Figure 1). The bottom arrow shows a 1.9-kb band corresponding to transcription of the partially deleted *Su(var)3′*, using the second *Su(var)3-7* polyadenylation site (CLEARD *et al.* 1995).

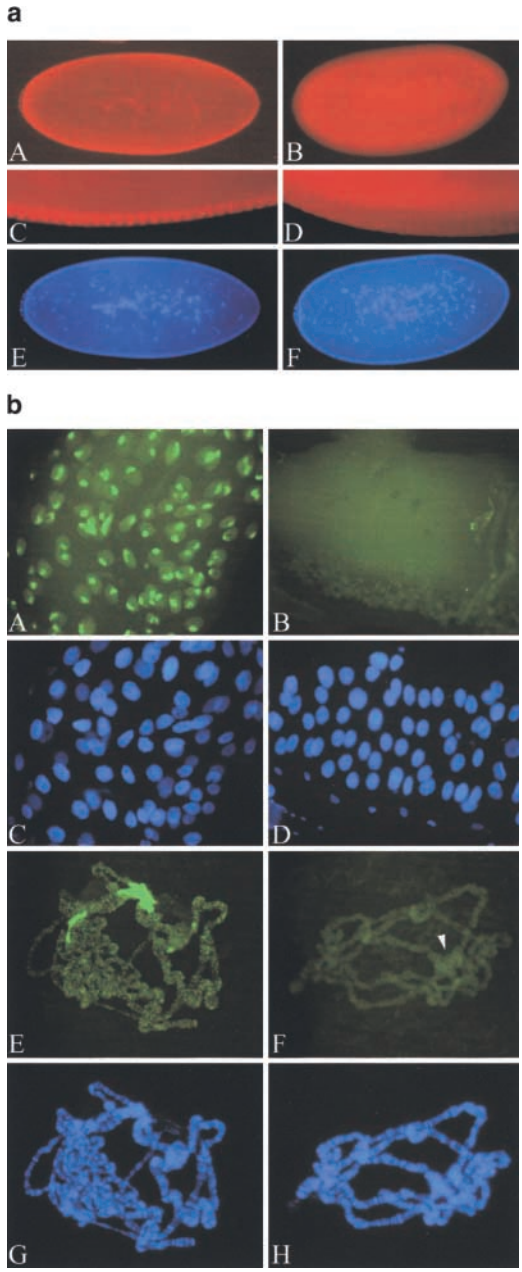

FIGURE 7.—Protein expression. (a) Immunostaining of cellular blastoderm embryos: A, C, and E, wild-type embryos; B, D, and F, homozygous *Su(var)3-7*^{14} embryos from homozygous mothers; A–D, immunostaining using anti-Su(var)3-7 antibody 264 (CLEARD *et al.* 1997) and an anti-rabbit Cy3 secondary antibody; C and D, magnifications of A and B showing the absence of the Su(var)3-7 protein in the apical region of the cellular blastoderm nuclei in *Su(var)3-7*^{14}; E and F, 4′,6-diamidino-2-phenylindole (DAPI) staining. (b) Immunostaining of whole-mount salivary glands and polytene chromosomes: A and C, wild-type third instar salivary glands; B and D, salivary glands of homozygous *Su(var)3-7*^{14} from heterozygous mothers; A and B, immunostaining with anti-Su(var)3-7 antibody 264 and an anti-rabbit DTAF secondary antibody. Note the absence of staining for *Su(var)3-7*^{14}. The maternal contribution has disappeared at this stage. C and D, DAPI staining; E and G, wild-type polytene chromosomes; F and H, homozygous *Su(var)3-7*^{14} polytene chromosomes. The arrowhead points at the chromocenter, which is not stained in *Su(var)3-7*^{14} chromosomes. G and H, DAPI staining.

of nuclear staining strongly suggests that the proteins produced are not functional. Immunostaining of polytene chromosomes of homozygous *Su(var)3-7*^{14} did not show the characteristic chromocenter staining (Figure 7b). On whole-mount salivary glands, no staining was observed either in the nucleus or in the cytoplasm (Figure 7b). Although the absence of cytoplasmic staining contrasts with the observation made in embryos, the lack of nuclear staining also supports the view that *Su(var)3-7*^{14} does not produce a functional Su(var)3-7 protein.

Further evidence that the Su(var)3-7/Yellow fusion protein is inactive was provided by the analysis of the seven homologous recombinants that did not behave as dominant suppressors of PEV. We found that three of them had repaired all the stop codons on both *Su(var)3-7* copies. If the Su(var)3-7/Yellow fusion protein was functional, we would expect these three recombinants to be enhancers of PEV (triplo-enhancement), which is clearly not the case.

We also performed immunostaining on *Su(var)3-7*^{9} and *Su(var)3-7*$^{7.1A}$ homozygous mutants. In both cases we found similar cytoplasmic staining in embryos and

absence of staining in whole-mount salivary glands, as in *Su(var)3-7*^{14}. However, on polytene chromosomes, *Su(var)3-7*^{9}, unlike *Su(var)3-7*^{14} and *Su(var)3-7*$^{7.1A}$, showed a faint, but highly reproducible, staining at the chromocenter (data not shown). This observation shows that *Su(var)3-7*^{9} does produce a small amount of probably full-length protein, which is compatible with the genetic data indicating that this mutation is not an amorph. An explanation of this case is developed in the DISCUSSION.

DISCUSSION

Although the *Su(var)3-7* gene was identified >10 years ago as a strong haplo-suppressor and triplo-enhancer of PEV, the exact role of its protein product in heterochromatin structure and function is still poorly understood, in part because of the lack of appropriate mutant alleles. We have described here the generation by homologous recombination of three S*u(var)3-7* mutations. Gene targeting was realized with a 7.6-kb fragment of *Su(var)3-7* modified to contain three stop codons and the recognition sequence for the I-SceI endonuclease. Starting with donor DNA at five different chromosomal positions, two targeting approaches were used: a normal (two donors) and a rapid (three donors) scheme, as described by RONG and GOLIC (2001). In our hands, the normal scheme turned out to be more efficient (eight homologous recombinants for ~25,000 progeny screened *vs.* two homologous recombinants for ~80,000 progeny screened in the rapid scheme). This conclusion is, however, likely to be strongly biased by the fact that seven of the eight recombinants obtained in the normal screen came from an X-linked donor not used in the rapid scheme. Our results underline the importance of using donors at different chromosomal locations. A probable explanation for the better efficiency of some donors is likely to be the frequency of excision of the targeting DNA by the FLP recombinase. Indeed, one of the donors on the X chromosome was quite resistant to FLP recombinase, producing y^{+}/y^{-} mosaic animals with our heat-shock protocol. This donor produced only one homologous recombinant. The other donor was very efficiently excised (only nonmosaic y^{-} flies) and produced homologous recombinants with a fourfold higher frequency.

Out of 10 homologous integrations, only 3 turned out to be mutant alleles (dominant suppression of PEV and recessive lethal or visible phenotype). This low frequency is probably due to the fact that two of the stop codons were located very close to the *I-Sce*I cut site and were usually repaired during the integration process. Even the third stop codon located at ~1.5 kb was found to be corrected in 4 of the 10 recombinants. Unexpected events (triplication instead of duplication of the gene, partial deletion of the 3′ copy, or repair of the 3′ copy from the 5′ copy instead of the homologous chromatid or chromosome) were found in 4 of the homologous recombinants. Indeed all three mutant alleles had an unexpected modification. A conclusion from our experiment is that it is safer to introduce several stop codons and that they should be placed as far as possible from the *I-Sce*I site. Furthermore, the isolation of many recombinants increases the chance to obtain unexpected events, including some deletions, which could lead to the production of an allelic series from a single donor construct.

One of the recombinants, *Su(var)3-7*^{14}, behaves genetically as a null mutation in two different assays. First, its effect as dominant suppressor of PEV, tested on three chromosomal rearrangements, is undistinguishable from the effect of a complete deletion of the *Su(var)3-7* locus. Second, this allele is a recessive maternal effect lethal mutation whose phenotype appears to be identical in the homozygous and in the hemizygous state: The mutant progeny of mutant females die during the second larval stage. Molecularly, the *Su(var)3-7*^{14} allele is the result of a complex recombination event. Its 3′ copy is an almost complete deletion of the coding region with the remaining small peptide expected to be nonfunctional since it lacks all the zinc fingers, as well as the two C-terminal domains needed for heterochromatin and self-association of the protein (JAQUET *et al.* 2002). Surprisingly, the 5′ copy also appears to be nonfunctional despite the fact that it does not contain the possible stop codons or other unplanned mutations. Due to the cloning procedure, the 5′ copy is an in-frame fusion between the 3′ end of *Su(var)3-7* and a piece of the 5′ *yellow* coding sequence. We believe that the resulting fusion protein is not translated, unstable, or unable to enter the nucleus. Unfortunately, our antibody does not allow us to distinguish on whole-mount embryos between the two proteins potentially produced by the *Su(var)3-7*^{14} mutation. We therefore do not know whether they are both produced. An important point is that the fusion protein appears to be nonfunctional because three other recombinants, each potentially capable of making this protein together with two doses of wild-type Su(var)3-7 protein, do not behave as triplo-enhancers of PEV. In addition, the Su(var)3-7 protein can be made nonfunctional by the C-terminal fusion of unrelated polypeptides, since such a fusion with the GAL4 DNA binding domain is poorly expressed, does not bind to pericentric heterochromatin, and has no effect on PEV (C. SEUM, unpublished data). These observations suggest that a good strategy to improve the odds of getting a null mutation by homologous targeting might be to use donors in which one or both ends of the mutant protein are fused in frame with a sequence promoting its inactivation, such as a ubiquitination signal.

In contrast to *Su(var)3-7*^{14}, the two other mutant alleles are genetically hypomorphic. The *Su(var)3-7*^{9} allele clearly keeps substantial residual activity: It is not a suppressor of PEV on one of the three chromosomal re-

arrangements tested, and it does not show the recessive maternal-effect lethality. In view of its hypomorphic nature, the molecular structure of this allele was surprising: The 5′ copy bears two stop codons at positions 830 and 2240 and the 3′ copy a stop codon at position 830. Although this latter position indicates an unexpected gene conversion (the stop codon should be at position 753), we predicted that this allele should not make a functional polypeptide. Immunostaining on polytene chromosomes showed, however, a weak but significant staining of the chromocenter. Considering that a polypeptide truncated at amino acid 122 would either not be made or not be able to enter the nucleus, as was the case for *Su(var)3-7*^{14}, and would anyway not bind to the chromocenter (Jaquet *et al.* 2002), staining with an antibody directed against the N terminus of Su(var) 3-7 can be explained only if some full-length protein is produced. We believe that occasional translation through the stop codon at position 830 of the 3′ mRNA is a plausible molecular explanation for the production of a small amount of Su(var)3-7 protein. This could suffice for the survival of this allele as a homozygous stock. Translational readthrough has been described at least twice in Drosophila. In the case of the *headcase* gene, efficient translational readthrough is required to produce a functional protein and has been shown to depend on a stem loop sequence located downstream of the stop codon (Steneberg *et al.* 1998; Steneberg and Samakovlis 2001). In a second example, a sensitive electrophysiological test showed that some stop codons in the *opsin* gene can be read through, even in a genetic background without engineered nonsense suppressors (Washburn and O'tousa 1992).

Su(var)3-7$^{7.1A}$ is close to being a null allele: It is a strong suppressor of PEV and the lethal phase of the recessive maternal-effect lethality is only slightly delayed compared to *Su(var)3-7*^{14}. Molecularly, *Su(var)3-7*$^{7.1A}$ is a triplication of the locus and could produce two types of polypeptides. The longest, encoded by the 5′ and 3′ copies, would be truncated after amino acid 613 between the fifth and the sixth zinc finger. Jaquet *et al.* (2002) found that a slightly longer polypeptide does not enhance PEV and has lost the preferential heterochromatic binding of the wild-type protein. Our data suggest that a truncated polypeptide with only the first five zinc fingers does retain a very weak activity. The middle copy bears the same stop codon as *Su(var)3-7*^{9}, but the proposed translational readthrough of this stop codon would not produce the full-length protein but the Su(var)3-7/Yellow fusion.

In conclusion, the isolation of a large number of homologous recombinants allowed us to recover three mutant alleles of *Su(var)3-7*. These mutations, which were not simple integrations, form an allelic series that allows us to make the following conclusions: First, *Su(var)3-7*, like the gene encoding HP1, is essential for *D. melanogaster* viability. Second, complete removal of both the maternal and the zygotic contributions is necessary to kill the organism, explaining why *Su(var)3-7* mutations had not been isolated in an extensive screen for essential loci in region 87E (Hilliker *et al.* 1980). Third, our analysis suggests that a small amount (maybe only 5–10% of the wild-type amount) of Su(var)3-7 protein is sufficient for normal cellular function. Fourth, the observation of visible adult defects in the *Su(var)3-7*^{9} mutant indicates that, besides a role during embryogenesis and/or the early larval stages, the gene is also required later during development. Finally, the adult phenotype suggests that cells that divide very rapidly and late during development such as the histoblasts may be the most sensitive to reduced amounts of Su(var)3-7 protein.

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