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Genetics

Molecular characterization of the *Drosophila* vermilion locus and its suppressible alleles

(suppressor of sable/transposon 412/tryptophan oxygenase)

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ABSTRACT **We have cloned vermilion (*v*), one of the genes required for brown eye pigment synthesis in *Drosophila*, using a mutant (v^{H2a}) "tagged" with the transposon *P* factor. Mutations that disrupt *v* gene expression are clustered within approximately 2 kilobases of DNA. A 1.4-kilobase transcript, homologous to this same region, is present in v^+ RNA but absent in RNA from several *v* mutants. The spontaneous *v* alleles that are suppressed by the suppressor of sable [*su(s)*] are apparently identical insertions of *412*, a *copia*-like transposable element. Preliminary evidence suggests that *su(s)*-suppressible alleles at other loci may also be *412* insertions.**

The wild-type eye color of the fruit fly *Drosophila melanogaster* is a composite of red and brown pigments. Elucidation of the pigment biosynthetic pathways has been accomplished primarily through studies of eye color mutants (reviewed in ref. 1). The bright red eye color of vermilion (*v*) mutants is the result of a defect at the initial step in the synthesis of the brown eye pigment. Tryptophan oxygenase (tryptophan 2,3-dioxygenase, EC 1.13.11.11, formerly EC 1.13.1.12), also known as tryptophan pyrrolase, catalyzes this step, the conversion of tryptophan to *N*-formylkynurenine. On the basis of several lines of evidence (see refs. 2–4), the *v* locus is presumed to be the structural gene for tryptophan oxygenase.

Certain spontaneous alleles at *v* (*v*, 1-33.0), sable (*s*, 1-43.0), speck (*sp*, 2-107.0), and purple (*pr*, 2-54.5) are suppressible by mutations at the suppressor of sable [*su(s)*, 1-0] locus. The response of *v* mutants to *su(s)* has been investigated in various genetic combinations of *v* and *su(s)* alleles by analysis of phenotypes (5–7), measurements of tryptophan oxygenase activity (2, 3, 7–9), and measurements of kynurenine autofluorescence in the larval fat body (10, 11). While the function of the *su(s)* locus remains unknown, these studies have demonstrated that a suppressible *v* mutation (v^s) results in a mutant phenotype only when the *su(s)* locus is expressed normally, i.e., in $su(s)^+$ v^s flies. On the other hand, recessive mutations at *su(s)* restore wild-type eye color, although the mutation at *v* persists, i.e., in $su(s)^-$ v^s flies. Suppression of the mutant phenotype is accompanied by partial restoration of tryptophan oxygenase activity.

Other recessive suppressors in *Drosophila*, notably suppressor of Hairy wing [*su(Hw)*] (12), suppressor of forked [*su(f)*] (13), and suppressor of white-apricot [$su(w^a)$] (14), have been shown to suppress mutations caused by insertion of specific transposable elements. *su(Hw)* suppresses mutations caused by insertion of the *gypsy* element at a number of different loci including Hairy wing, bithorax, cut, forked, scute, and lozenge (15). *su(f)* suppresses the same forked and lozenge alleles that are suppressible by *su(Hw)* (16, 17). The suppressible w^a mutation is a *copia* insertion (18). To initiate the investigation of allele-specific suppression by *su(s)* at the molecular level, we have cloned the *v* locus, analyzed DNA from *v* mutants, and initiated a preliminary examination of suppressible *pr*, *s*, and *sp* alleles.

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MATERIALS AND METHODS

Purification of Nucleic Acids. The *v* mutants used for nucleic acid isolation are described by Lindsley and Grell (16) or are referenced in the text. *Drosophila* DNA was isolated from adult flies as described by Bingham *et al.* (18). Procedures for isolation of bacteriophage λ DNA and plasmid DNA have been described (19). Total RNA was prepared from 2-day-old adults by using procedures described by Thireos *et al.* (20) except that 10 mM vanadyl–ribonucleoside complex was included in the homogenization buffer. Poly(A)$^+$ RNA was purified by chromatography on oligo(dT)-cellulose (21).

Isolation of Genomic Clones. Genomic DNA libraries were prepared from v^{H2a}, v^+ (y^2w^{bf}), v^{36f}, v^k, and v^2 DNA as follows. *Drosophila* DNA (2.5 μg) was partially digested with various amounts of *Mbo* I (in the range of 0.009–0.075 units) for 1 hr at 37°C, and the reactions were terminated by heating to 65°C for 15 min. An aliquot (0.25 μg) of each sample was analyzed by agarose gel electrophoresis, and the digestion that yielded a majority of fragments greater than 15 kilobases (kb) was used to prepare the library. The sample was treated twice with 1 unit of calf intestinal phosphatase at 37°C for 30 min. The phosphatase was inactivated by heating to 65°C for 15 min; after the sample cooled, it was phenol extracted, chloroform extracted, and ethanol precipitated. The DNA sample was resuspended in 5 μl of 10 mM Tris·HCl (pH 8.0)/1 mM EDTA and ligated to 1 μg of *Bam*HI-digested λEMBL4 DNA in a 10-μl reaction mixture as described by Searles *et al.* (19). The ligated DNA was packaged as described by Hohn (22). Procedures for screening the libraries and purification of positive clones have been described (19). The λv^{H2a} library was probed with plasmid pπ25.1 (23), which contains a 2.9-kb P factor. The other libraries were probed with plasmid pv1.2, which contains the 1.9-kb *Eco*RI A fragment (coordinates 0 to +1.9, see Fig. 1). DNA fragments used in hybridization experiments were inserted into vector pUC8 as described (21).

Hybridization Procedures. DNA was analyzed by electrophoresis on 1% agarose gels. The conditions for DNA transfer and hybridization were described by Southern (24), except that 10% (wt/vol) dextran sulfate was included in the hybridization mixtures. RNA was analyzed by electrophoresis on agarose gels containing formaldehyde (25) and was transferred to nitrocellulose (26). The hybridization conditions were the same as described for Southern transfers. The hybridization probes were plasmid subclones that had been labeled with ^{32}P by nick-translation (27).

Abbreviations: kb, kilobase(s); bp, base pair(s).

To determine the identities of the transposable elements causing *v* mutations, DNA (1 μg) from 20 different plasmid clones, each containing a known transposable element, was spotted onto a nitrocellulose grid. After air drying, DNA on the filter was denatured and neutralized as described by Benton and Davis (28). The filter was hybridized separately with ^{32}P-labeled λv^k-7 and λv^{36f}-12 DNA under the same conditions described above. Subsequently, Southern transfers of λv^k, λv^2, and λv^{36f} DNA digested with *Eco*RI were incubated with labeled *412* and *roo* sequences to verify results.

For *in situ* hybridization experiments, tritium-labeled probes were prepared by nick-translation as described by Bingham *et al.* (18), and the procedures used for hybridization were described by Searles *et al.* (19). Alternatively, probes were labeled with biotinylated-UTP (29). Conditions for hybridization (Enzo Bio-Probe Labeling System; Enzo Biochemicals, New York) and detection using an avidin/biotin/peroxidase system (Vector Laboratories, Burlingame, CA) were as described by suppliers except as modified by Elizabeth Montgomery (personal communication).

RESULTS

Cloning the *v* Locus. We initially cloned the *v* locus using the strategy of transposon tagging with *P* element (18, 19), the transposable element that is mobilized in P–M hybrid dysgenic crosses (30). A *v* mutant (v^{hd81c}) was isolated by Margaret Kidwell during a hybrid dysgenic screen. We determined by *in situ* hybridization to polytene chromosomes that this mutant has a *P* element insertion on the X-chromosome at 10A, the cytogenetic location of *v*. Therefore, it seemed likely that the mutation was caused by insertion of a *P* element at the *v* locus. A genomic DNA library was made from a derivative, $v^{hd81c\text{-}H2a}$ (abbreviated v^{H2a}), of the original mutant by ligation of *Mbo* I partial restriction fragments into the *Bam*HI site of λEMBL4. Since the v^{H2a} line contained 30–50 copies of *P* element distributed throughout its genome we expected that it would be necessary to purify a large number of *P* element-containing clones in the λv^{H2a} library for analysis by *in situ* hybridization to polytene chromosomes to identify one clone containing *v* DNA. Among the first 26 clones that were purified, one clone (λv^{H2a}-1) hybridized to polytene band 10A in chromosomes lacking *P* elements. The cloned DNA contains an apparently complete 2.9-kb *P*-factor insertion flanked by approximately 10 kb of DNA from the *v* region (Fig. 1). A plasmid subclone of the 1.9-kb *Eco*RI fragment (coordinates 0 to +1.9), consisting of unique sequences, was used to screen a wild-type (y^2w^{bf}) library for λ phage clones homologous to this same region. Several clones were purified and a restriction map spanning approximately 26 kb of wild-type DNA was constructed. This entire region is represented in the combined restriction maps of two overlapping clones, λv^+-8 and λv^+-11 (Fig. 1).

Localization of *v* Mutations. Examination of DNA from *v* mutants revealed that several of these mutations result in detectable restriction pattern changes within the cloned region. Southern transfers containing *Eco*RI digests of wild-type and mutant genomic DNAs were probed with radioactively labeled plasmid subclones of *Eco*RI fragments spanning the entire 26-kb region shown in Fig. 1. This analysis demonstrated that all of the detectable mutations lie within two adjacent 1.9-kb *Eco*RI fragments (coordinates 0 to +1.9 and +1.9 to +3.8, Fig. 1). Because these two fragments are the same size, they will hereafter be referred to as the 1.9-kb *Eco*RI A fragment (coordinates 0 to +1.9) and the 1.9-kb *Eco*RI B fragment (coordinates +1.9 to +3.8). The spontaneous mutations v^1, v^2, and v^k, reported to be of independent origin, are apparently identical insertions of foreign DNA into the 1.9-kb *Eco*RI A fragment. By cloning the v^2 and v^k alleles and hybridizing the clones to 20 different *Drosophila* transposable elements, we identified the insertion as the 7.5-kb mobile element known as *412*, a member of the *copia*-like family of elements (31) (Fig. 2). Based on comparisons of v^1 restriction patterns to v^2 and v^k, we concluded that all three of these alleles are *412* insertions. Southern analysis of the allele v^{+37}, a partial revertant of v^1 (32), indicated that most of the *412* insertion remains in this mutant; however, a 100-base-pair (bp) deletion has occurred in the vicinity of the insertion (not illustrated). We have not yet determined whether the small deletion lies within the *412* insertion, within *v* DNA, or spans the insertion site. Genomic Southern analysis also demonstrated that the x-ray induced mutation v^{48a} is a 200-bp deletion in the 1.9-kb *Eco*RI A fragment. This deletion was further localized within the 0.9-kb *Xho* I/*Eco*RI fragment (coordinates +1 to +1.9) (Fig. 2). The spontaneous v^{36f} mutation is an insertion into the 1.9-kb *Eco*RI B fragment, the same fragment that contains the *P* element insertion. The v^{36f} allele was cloned and shown to be a different *copia*-like element, the 9-kb *roo* element, also known as *B104* (33, 34) (Fig. 2). A revertant of the v^{36f} mutation, v^{36f+} (provided by M. Green), appears wild type in genomic Southerns and thus has completely lost the *roo* insertion (not illustrated). The spontaneous v^{71P} mutation (35) and the x-ray induced v^{51c} mutation do not result in detectable restriction pattern changes over the 26 kb of DNA that were examined.

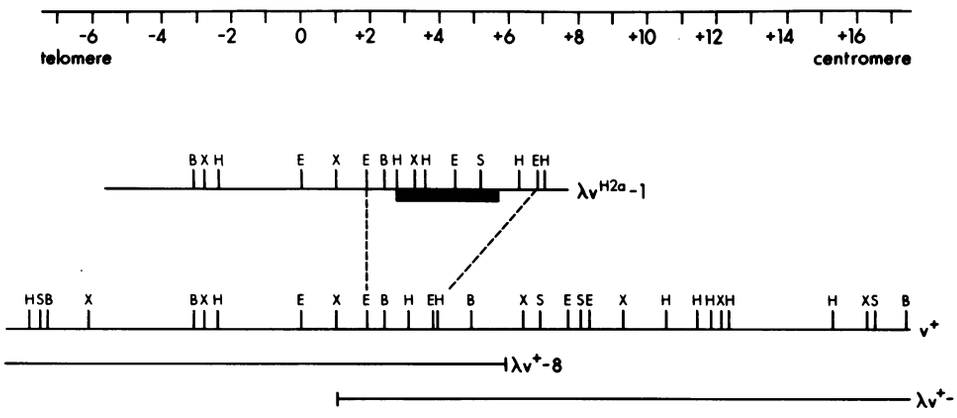

FIG. 1. Restriction map of DNA from the *v* region. *P* factor sequences within the λv^{H2a} clone are indicated by the solid bar (▭). The wild-type restriction map (v^+) was obtained by combining restriction data from clones λv^+-8 and λv^+-11, both of which were isolated from a wild-type (y^2w^{bf}) library. At the ends of the cloned fragments are *Eco*RI sites derived from the λEMBL4 polylinker. The coordinates on the scale above the restriction map are distances in kb and pertain to the v^+ map. The restriction enzyme abbreviations are: E, *Eco*RI; B, *Bam*HI; H, *Hin*dIII; S, *Sal* I; X, *Xho* I.

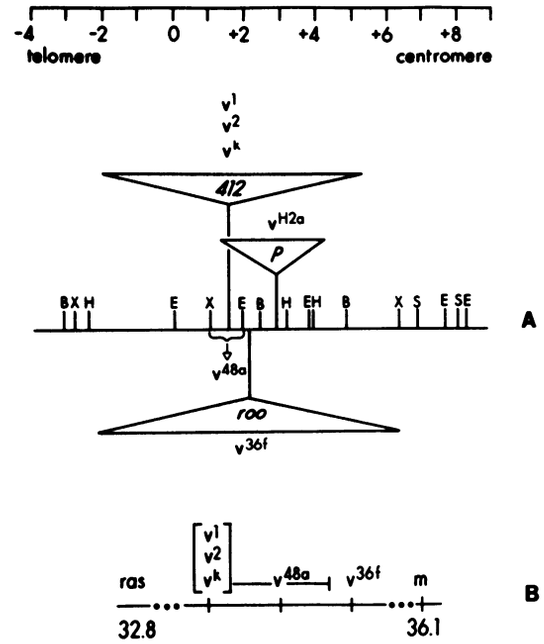

FIG. 2. Summary map of insertion and deletion mutations at the *v* locus. (*A*) The position of v^1, v^2, v^k, and v^{36f} insertions (▽) and the approximate location of the v^{48a} deletion (▽) are shown. (*B*) The genetic map of the *v* locus based on fine structure recombination studies [after Baillie and Chovnick (4)]. The numbers below the horizontal line indicate the map positions of the two visible markers, *ras* and *m*, in the *v* region.

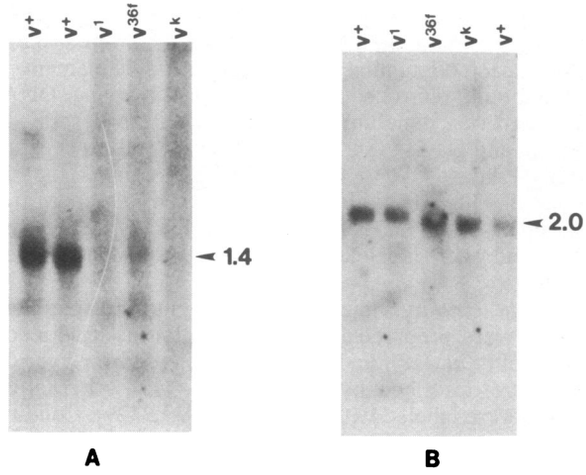

FIG. 3. RNA blot hybridization analysis of transcripts homologous to the cloned DNA. Poly(A)$^+$ RNA from v^+ (y^2w^{bf} and Oregon R) and *v* mutant adults was size fractionated on formaldehyde/agarose gels and transferred to nitrocellulose. The sizes (kb) of the transcripts, indicated on the right of the autoradiograms, were determined relative to known sizes of alcohol dehydrogenase, actin, and white transcripts. (*A*) RNA (10 μg) from each strain was probed with either the subcloned 1.9-kb *Eco*RI A fragment (coordinates 0 to +1.9) or the *Eco*RI B fragment (coordinates +1.9 to +3.8). (*B*) RNA (3 μg) from each strain was probed with the subcloned 4.0-kb *Eco*RI fragment (coordinates +3.8 to +7.8).

The mutants v^1, v^2, and v^k are suppressed by *su(s)* (5). The v^{36f} allele is unsuppressible with respect to the eye color phenotype (5); however, fluorescence measurements of the enzyme reaction product kynurenine in the larval fat body (10, 11) and enzyme activity measurements (3) indicate that the amount of tryptophan oxygenase activity is slightly elevated in *su(s)* v^{36f} flies, although the small increase in activity is apparently insufficient to restore wild-type eye color. Thus, *su(s)* suppresses mutations caused by *412* insertion and slightly affects the expression of a *roo* insertion mutation at *v*.

Transcription Mapping in the *v* Region. To define the location of the *v* structural gene, RNA transfers (26) containing polyadenylylated RNA from wild-type and *v* mutant adults were probed with plasmid subclones of *Eco*RI fragments spanning the entire region cloned. Two different transcripts were detected in the 26 kb of DNA shown in Fig. 1. When filters were probed with either the 1.9-kb *Eco*RI A fragment or the 1.9-kb *Eco*RI B fragment, a 1.4-kb transcript was observed in wild-type RNA but not in *v* mutant RNA (Fig. 3*A*). This transcript, the v RNA, is homologous to the region where *v* mutations are located. A 2.0-kb transcript was detected when RNA blots were probed with the 4.0-kb *Eco*RI fragment (coordinates +3.8 to +7.8). Since this transcript is present at the same level irrespective of the *v* genotype of RNA (Fig. 3*B*), it is apparently the product of a gene that lies immediately to the right of *v*.

***In Situ* Hybridization of *412* and *roo* Sequences to *s*, *pr*, and *sp* Mutant Chromosomes.** Several *Drosophila* suppressors have been shown to affect the expression of mutations caused by insertion of a specific transposable element at a number of different loci. If this is true of *su(s)*, then one would predict that, like the suppressible *v* alleles, the *s*, *sp*, and *pr* alleles, which are also suppressible by *su(s)*, might be *412* or possibly *roo* insertions. To investigate this possibility polytene chromosome squashes from relevant *s*, *pr*, and *sp* mutants were probed separately with *412* and *roo* sequences to ask if either of these elements is associated with the cytological intervals that contain the mutations. The results of this analysis are summarized in Table 1. When chromosomes from four suppressible mutants, *pr*, pr^{bw}, *sp*, and *s*, were probed with *412*, labeling was observed at *pr* (38B4-6) with both *pr* mutants and at *sp* (60B13-C5). However, *412* sequences were not found at *s* (11F1,2-12A1,2) in chromosomes carrying the suppressible *s* mutation. Labeling with *roo* was observed only in the *sp* region. Although the parental chromosomes were not available for examination, no labeling was observed at these sites in several wild-type chromosomes.

Thus, *412* could conceivably be associated with every mutation that is suppressible by *su(s)* except *s*. The poor association of the *roo* element with suppressible mutations is not surprising, considering the weak effect of *su(s)* on the *roo* insertion in the mutant v^{36f}. However, because each of these genes has been assigned to an interval large enough to include other genes and because *412* and *roo* are present in relatively high copy number [30 and 100 copies, respectively, on the average (see Table 1)], it is possible that the observed association of an element with the appropriate cytological interval could be fortuitous.

Table 1. *In situ* hybridization of mutations suppressible by *su(s)*

Locus	Allele	Hybridization *in situ* 412	Hybridization *in situ* roo
s (11F1,2-12A1,2)	s^1	–	–
pr (38B4-6)	pr^1	+	–
	pr^{bw}	+	–
sp (60B13-C5)	sp^1	+	+

Larval salivary gland squashes were prepared from *s*, *pr*, pr^{bw}, and *sp* mutants. Polytene chromosomes were hybridized *in situ* with cloned 412 and roo sequences [plasmids 2042 and 2173, respectively (see ref. 36)] labeled by nick-translation with biotinylated-UTP. The 412 element hybridized to an average of 30 different genomic sites, and roo hybridized to about 100 different sites. The *s*, *pr*, and *sp* loci have been mapped to the intervals enclosed in parentheses (*s*, ref. 37; *pr*, refs. 38 and 39; *sp*, ref. 16).

DISCUSSION

Mutations that disrupt the expression of the *v* locus span a maximum distance of 2 kb of DNA. Within this region the physical map of DNA is consistent with predictions based on fine structure recombination studies of *v* (see Fig. 2). The spontaneous mutations v^1, v^2, and v^k, which have not been separated by recombination, appear to be identical insertions of the *412* element. The spontaneous v^{36f} mutation that maps to the right of v^1 (6) is an insertion of the *roo* element. The recombination distance between v^1 and v^{36f} of 0.0025 map units predicts that these two mutation sites are approximately 1 kb apart [assuming 0.01 map units = 3.8 kb of DNA (40)]. The observed distance between the sites of the v^1 and v^{36f} insertions, approximately 0.7 kb, is in good agreement with the value predicted by recombination studies. The x-ray-induced allele v^{48a} also maps to the left of v^{36f} and has not been separated from v^1 (41). We have shown that this mutation is a 200-bp deletion within the same restriction fragment that contains v^1, v^2, and v^k.

The v mRNA is transcribed from the same DNA region defined by *v* mutations. This transcript is 1.4 kb in length and thus could potentially be translated into a polypeptide of approximately 40 kDa. Although the subunit composition of *Drosophila* tryptophan oxygenase has not been determined, Baillie and Chovnick (4) have estimated that the molecular size of the enzyme is 150 kDa. Tryptophan oxygenases from *Pseudomonas acidovorans* (42) and rat liver (43) have been purified to homogeneity and are of molecular sizes 122 and 167 kDa, respectively. Both enzymes consist of four identical subunits. The length of the v transcript and the molecular size of *Drosophila* tryptophan oxygenase indicate that the active form of the *Drosophila* enzyme could also be a tetramer and that its size more closely resembles the rat liver enzyme.

The molecular analysis of *v* mutants has provided new information about the nature of suppression at the *v* locus. Earlier studies suggested that *su(s)* affects tRNA modification (44, 45) or that *su(s)* encodes a posttranslational inhibitor (46). However, our finding that the suppressible *v* alleles are insertions of the mobile element *412* illustrates that *su(s)* belongs to the class of *Drosophila* suppressors that suppress transposable element insertion mutations. Included in this group are *(su)Hw*, which suppresses gypsy insertion mutations at several different loci, *su(f)*, which suppresses a subset of the mutations suppressed by *su(Hw)*, *su(w^a)*, which suppresses a *copia* insertion mutation at the white locus, and *su(s)*, which suppresses *412* insertions at *v* and possibly at *sp* and *pr*. It is likely that suppressor of purple [*su(pr)*] also belongs to this group since the same *pr* alleles that are suppressible by *su(s)* are suppressible by *su(pr)* (16). Several of these suppressors also act as enhancers. For example, *su(f)* enhances the w^a phenotype that is suppressed by *su(w^a)* (14), and *su(s)* enhances some of the mutations that are suppressed by *su(Hw)* (16). However, none of the other *Drosophila* suppressors are known to affect the phenotypes of suppressible *v* alleles.

Our *in situ* hybridization studies indicate that *412* may be associated with the suppressible *sp* and *pr* alleles but not the suppressible *s* mutation. It is possible that at the *s* locus only a small portion of the *412* element is present, and the homology is undetectable under our hybridization conditions. Alternatively, the *s* mutation could have a completely different origin from the other suppressible alleles. The *s* mutation is analogous to rudimentary alleles suppressible by *su(Hw)*. Unlike the other mutations suppressible by *su(Hw)*, the *gypsy* element is not apparently associated with suppressible rudimentary alleles (15).

Tryptophan oxygenase activity of v^{36f} is slightly elevated in the presence of *su(s)*, although this amount of activity is not adequate to suppress the mutant phenotype (3, 10). The fact that v^{36f} is an insertion of a different *copia*-like element raises the possibility that *su(s)* is capable of suppressing both *412* and *roo* insertions, although to different degrees. Our *in situ* hybridization studies indicate that only the suppressible *sp* allele could possibly be a *roo* insertion, and the association of *roo* with the chromosome interval containing the *sp* mutation could be fortuitous. Minor effects, such as the observed interaction between *su(s)* and v^{36f}, may commonly occur with other suppressors but are not detectable, because they do not affect phenotype. On the other hand, it is possible that *su(s)* does suppress *roo* insertions but the position of the v^{36f} insertion within the *v* gene results in a less significant effect.

Suppressible transposable element insertion mutations have been found in organisms other than *Drosophila*. *Ty* element insertions at the *his4* locus of yeast (47) and an endogenous retrovirus insertion at the dilute locus of the mouse (48) are suppressible. In all cases, the transposable elements involved in suppression have structures similar to mammalian retroviruses. Like the other *Drosophila* mobile elements involved in suppression, *412* is a member of the *copia*-like family of elements (31). This 7.5-kb element consists of a core sequence bounded by a 480-bp direct repeat (long-terminal repeat) at each end. There are approximately 30–40 copies of *412* distributed throughout the genome, the sequences being closely conserved at each site. Polyadenylylated RNAs measuring 7.5, 2.2, and 1.6 kb in length are transcribed from *412* (49). The largest RNA is a full-length transcript that probably is initiated in one long-terminal repeat and is terminated in the other (50). All *copia*-like elements have similar structural features, although there is no sequence homology between the various elements.

The understanding of how these suppressors revert mutant phenotypes would be facilitated by understanding how transposable element insertions interfere with gene expression to cause mutant phenotypes. The manner by which disruption of gene expression occurs is likely to depend on the position of the insertion within a gene. To date only a few suppressible mutations have been analyzed in detail at the molecular level. Suppressible transposable element insertions appear to be restricted to noncoding gene regions. For example, the suppressible w^a mutation is a copia insertion within an intron (51, 52), and the suppressible *Ty* element insertion in the yeast *his4-917* mutant is in the 5′ noncoding region (47). The suppressible *v* alleles have not yet been localized precisely with respect to the v transcript; however, the alleles v^1, v^2, and v^k are leaky (53), which is suggestive of mutations in noncoding regions. Transcription of the mutant w^a apparently differs from transcription of *v 412* insertion mutants. Transcription of white in the mutant w^a apparently terminates within the *copia* element causing the production of aberrant transcripts (51, 52), and *su(w^a)* partially relieves transcription termination within the element (52). In the case of *v* mutants with *412* insertions, detectable v transcripts do not accumulate. It is possible that the v transcript is not synthesized at a detectable level in these mutants, or the transcripts synthesized by mutant genes are unstable. Alternatively the *412* insertion could be located in an intron downstream of a small 5′ exon, and the aberrant RNA could be small or have little homology to the probe.

Based on measurements of tryptophan oxygenase activity, we anticipate that *su(s)* only partially relieves the effects of the *v* mutations since the activities in suppressed v^1 and v^k are 10–20% of wild type (3). Apparently, this amount of activity is sufficient to produce a normal phenotype. On the other hand, tryptophan oxygenase activity in *su(s)* v^{36f} mutants, only 4% of the wild-type level (3), is insufficient to correct the eye color defect.

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