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A deleted *hobo* element is involved in the unstable thermosensitive vg^{al} mutation at the *vestigial* locus in *Drosophila melanogaster*

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Summary

We have described a new unstable mutant of the *vestigial* locus isolated from a natural population. From this mutant, *vestigial*^{almost} (vg^{al}), wild-type (vg^{al+}), and extreme (vg^{ext}), alleles arose spontaneously. The molecular analysis of vg^{al} shows that the mutation is due to a 1874 bp *hobo* element inserted in a *vestigial* intron. Two distinct kinds of events lead a wild-type phenotype. Three independent vg^{al+} alleles result from an excision of the *hobo* element and two other vg^{al+} alleles have further deletions of *hobo* sequence. The sequence of one of them shows a 1516 bp *hobo* insertion at the same place and in the same orientation as the 1874 bp insertion. In the vg^{ext} alleles, we found a 5′ or 3′ variably sized deletion of *vg* sequences. One of them, which has been cloned and sequenced, has a deletion finishing exactly at the left terminal repeat′ *hobo* element. The genetic implications of these different genetic structures are discussed.

1. Introduction

The *vestigial* locus of *Drosophila melanogaster* is involved in wing development. In the absence of the vg^{+} gene, extensive cell death occurs in the third-instar imaginal discs (Fristrom, 1968). The *vg* locus was cloned by Williams & Bell (1988), and a 19 kb sequence of DNA was shown to be involved in *vestigial* function. Most of the classical alleles analysed were found to be associated with deletion of *vg* sequences (vg^{nw}, vg^{56}, $su(z)2^{5}$) or insertions (vg^{np}, vg^{BG}, vg^{ni}, vg^{12}). The two dominant mutants (vg^{U} and vg^{W}) were shown to be due to inversions with one of the breakpoints located in the *vestigial* locus (Williams & Bell, 1988). A developmentally regulated 3·8 kb transcript was characterized and shown to be spliced from eight exons (Williams *et al.* 1990, 1991). The vg^{83b27} allele, induced in mutagenesis studies by Alexandrov & Alexandrova (1987), produces an extreme wing phenotype which defines a second complementation unit. This allele is associated with a 4 kb deletion entirely within *vg* intron two (Williams & Bell, 1988; Williams *et al.* 1990).

The vg^{al} allele was isolated from a natural French population. This allele is unstable; the genetic instability of the mutant is thermosensitive. At 28 °C $vg^{extreme}$ (vg^{ext}) derivatives appear and have a strong wing mutant phenotype, while at 21 °C wild-type revertants (vg^{al+}) are more common. However, the temperature effect is not absolute, as vg^{ext} could also be isolated at 25 and 21 °C, and vg^{al+} at 25 °C (Bazin *et al.* 1991). Further, whilst vg^{al} belongs to the same complementation group as the classical vg^{BG} mutant, vg^{ext} does not complement with either vg^{BG} or vg^{83b27}. Southern hybridization analyses of vg^{al}, vg^{ext}, and two independent vg^{al+} alleles, and the cloning of the vg^{al} mutation, showed that the vg^{al} mutation is due to the insertion of a deleted *hobo* element. The vg^{ext} derivative alleles appear to be caused by a deletion of *vg* sequences, since the *hobo* element is still present. Two different molecular events can lead to a wild-type revertant phenotype: either the excision of the *hobo* element as in vg^{al+1}, or a further deletion of *hobo* sequences as in vg^{al+2}.

Hobo elements participate in a third hybrid dysgenesis system (the others being I-R and P-M), which have some similarities with P element (Blackman *et al.* 1987; Yannopoulos *et al.* 1987; Louis & Yannopoulos, 1988; Blackman & Gelbart, 1989; Calvi *et al.* 1991). A complete and functional *hobo* element is 3 kb long, possesses two terminal inverted repeats of 12 bp and generates an 8 bp duplication at its insertion site

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(Streck *et al.* 1986; Calvi *et al.* 1991). Blackman *et al.* (1989) have shown that the *hobo* HFL1 element is able to mediate germline transformation and is an autonomous and fully functional element. Mobilization of *hobo* occurs not only in dysgenic crosses, but also in intrastrain crosses (Blackman *et al.* 1987; Yannopoulos *et al.* 1987; Lim 1988), producing molecular rearrangements such as inversions, deletions or new *hobo* insertions, close to the resident element. Such rearrangements could be a consequence of recombination between two neighbouring *hobo* elements. In this study we show that the vg^{al} mutation is due to a 1874 bp *hobo* insertion in the third *vestigial* intron. The derivative vg^{extl} allele is due to a deletion of 2·4 kb of DNA, and other vg^{ext} alleles have smaller deletions originating from the same position. The vg^{al} mutation can also revert to wild type. Two different vg^{al+} alleles are characterized; one is dominant when heterozygous with a deletion of the *vg* locus, while the other is only partially dominant. We also observed two different molecular events which can produce wild-type reversions. They are either an excision of the *hobo* element or a partial deletion of the *hobo* sequences. For example, the vg^{al+2} wild-type revertant is due to a deletion of 358 bp located in the central part of the 1874 bp *hobo* element. Herein we discuss the observations that some of the vg^{al+} revertants are due to a further deletion of *hobo* sequences, whereas various deletions of the adjoining *vg* sequences lead to a vg^{ext} phenotype (no wing at all and female sterility).

2. Material and methods

(i) D. melanogaster *stocks and culturing*

D. melanogaster cultures were grown at 25 or 21 °C and maintained on standard corn, yeast and sugar medium. The wild-type strain used was OregonR and the *vestigial* mutant strains were vg^{B}: *Df(2R)49D3–4; 50A2–3/CySM5* (Bowling Green *Drosophila* Center) and vg^{al} isolated in a natural population, from France (Bazin *et al.* 1991). The revertant wild-type strains (vg^{al+}) were isolated independently from vg^{al} cultured at 21 °C: vg^{al+1} and vg^{al+6} or at 25 °C: vg^{al+2}, vg^{al+3}, vg^{al+4}. The derivative $vg^{extreme}$ strains were isolated from vg^{al} cultured at 25 °C. The $vg^{extreme}$ (vg^{ext1}, vg^{ext3}, vg^{ext5}, vg^{ext7}, $vg^{extV-75}$ and $vg^{extVII-24}$) homozygotes display a very pronounced mutant phenotype: no wing, no haltere and the females are sterile. The vg^{ext6} allele is a recessive lethal mutation. Therefore, the vg^{ext} stocks are maintained as heterozygotes with a balancer chromosome.

(ii) *DNA manipulation*

The culturing and storage of bacteria or lambda phage, preparation of DNA, and plasmid subcloning were performed by standard methods (Maniatis *et al.* 1982). Genomic *D. melanogaster* DNA for Southern hybridizations and genomic libraries was prepared by the method of Ish-Horowicz *et al.* (1979) and repurified by spermine precipitation (Hoopes & McClure, 1981). All gels for Southern hybridization analyses were blotted on to Genescreen Plus membranes using the capillary blot protocol recommended by the manufacturer (Dupont). Four Southern gels, 5 μg of DNA/lane were used. After hybridization the filters were washed according to Genescreen Plus specifications. DNA probes were made from restriction fragments resolved on low-melting agarose gels. For the vg^{al}, vg^{al+2} and vg^{extl} libraries, genomic DNA was digested entirely with *Eco*R I and fragments between 2 and 4 kb, purified within 0·5% agarose gels and electroelution on to dialysis membranes, were cloned in λGT10 and subcloned in bluescribe (Williams & Bell, 1988). All DNA sequencing was performed by double-stranded DNA sequencing of inserts cloned into Bluescribe (Chen & Seeburg, 1985).

3. Results

The vg^{al} mutation results from an internally deleted *hobo* element inserted into the 1·4 kb *Eco*R I fragment of the *vestigial* locus (Fig. 1) (Bazin *et al.* 1991). DNA sequencing of this fragment showed that the insertion is located in the third *vg* intron, 462 bp 5′ to the beginning of the 4th exon. The insertion also generated an 8 bp TACTACAT duplication (Fig. 2). A large number of base changes were found in the *vg* sequences compared to a wild-type allele (Fig. 2). These are probably due to the fact that vg^{al} was isolated from a natural population, and that most intronic sequences are not functionally conserved. The data show that the vg^{al} mutation is an insertion of an internally deleted *hobo* element. The only difference detected between the sequence of *hobovg*^{al} (*hvg*^{al}) and the published sequence of a functional complete *hobo* element called HFL1 (Calvi *et al.* 1991) is an internal deletion (1086 bp) between positions 995 and 2082, with a '**G**' inserted at the deletion junction.

(i) *Molecular analyses of independent* vg^{al+} *revertant strains*

Molecular analysis of five independent vg^{al+} revertant strains was undertaken by comparing them to vg^{al} and vg^{+} (Or^{R}) strains, utilizing probes covering the whole *vestigial* locus. In all cases the results indicate that there is a single alteration in the relevant vg^{+} 1·4 kb *Eco*R I fragment. In vg^{al} this fragment is 3·4 kb long, due to the *hobo* insertion. The vg^{al+1}, vg^{al+3} and vg^{al+6} strains show the same pattern of hybridization as vg^{+} when the 6·5 kb probe is used (see Fig. 1) indicating an excision of the *hobo* element. However, the relevant *Eco*R I fragment in vg^{al+2} and vg^{al+4} is now 3·1 kb (Fig. 3), indicating a partial deletion only of DNA. To localize this deletion, the 3·1 kb vg^{al+2} *Eco*R I fragment was cloned in bluescribe (pvg^{al+2}). A restriction map

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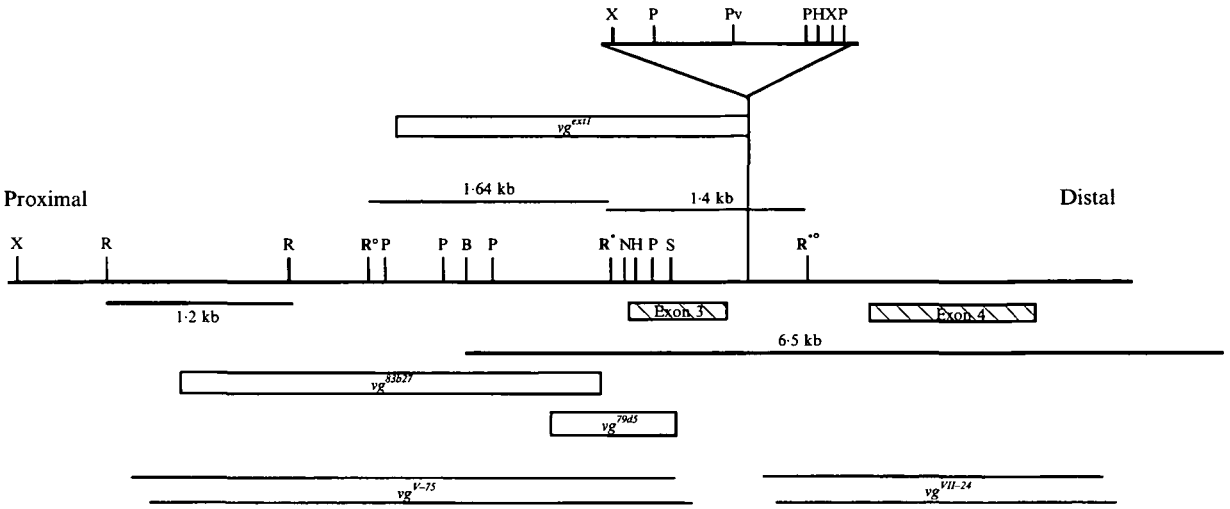

Fig. 1. Partial physical map of the *vestigial* locus. The labelled open boxes designate the known extent of various *vg* deletions. The hatched boxes below the restriction map denote the exons 3 and 4. The open boxes designate the vg^{V-75} and vg^{VII-24} deletions (which endpoint is not defined). The triangle designates the *hobo* insertion involved in the vg^{al} mutation. The vg^{al} 3·4 kb and vg^{al+2} 3·1 kb *Eco*R I fragments were cloned between the designated sites: **R***. The relevant cloned vg^{ext1} 2·5 kb *Eco*R I fragment lies between the *Eco*R I sites designated: R°. The probes used for Southern analyses were the 1·2 kb *Eco*R I fragment and the 6·5 kb *Bam*H I–*Sst* I fragment. The restriction sites on the map are abbreviated as follows: R, *Eco*R I; P, *Pst* I; X, *Xho* I; B, *Bam*H I; Pv, *Pvu* II; H, *Hinc* II; S, *Sma* I and N, *Bgl* II.

CTGCAGCTAATAACACTGCAACAGATACGGGATACAAGTACACC
GG(G)AAAATGATACGTTGCTCAGATAG(G)TTAAATTAATTAAT
GGTCAGGGT*.....*(not sequenced)*.....
GTGAGCAAGGATCACTTGGGTACATCCCTAATGATGGCGATCTA
GATCCCAAAAGGAAACTTTCAAATAGTCATTGTTTGAAATTATC
TGAATTGCAAGTTGTTGTTTAGTTTTAGCTTTACTATAACTAAA
AACACGACTGTCATTAATTAGTTACTGAGTAAAGAGAACAATCA
TTTTAAAATAGATATGATGATTTGTTTAACTTTAGAGATCGTTT
CCATTTAGCCCTTCCACTAATTAATACATTAGTGTCTCAAT***TAC***
TACAT * CAGAGAACTGCA ...(*hobovg^{al}*)
....TGCAGTTCTCTG***TA(G)TACAT***TATAAAGTTCAG(C)TCCAAAG
GTGAACATA(G)TAGCAAAAGTATTGCT(A)CCAAAAT(T)AAAGT
ATAGTCGCTATAAATGTAATCAATAATTCATCAGCTAAACACTT
TGTTTACACGCGTTC(G)TTC(G)AAACGCTTTAAA(G)CAATGAAT
TT(T)ATTAGTTTTCATGTGCGTGTTCATTGATATTGTCAATGTCA
ATGTTTGCATAACATTTATTTTTTGGCAGCACACGGAAAATTCA
TGCAAGTGAAAAAGCCCATAGTGGGGAAGAGCGCGATAGTCAT
CGCACACTCGTAGCTAATTAATTTGAAAATTCTTGAAATTTCTG
ACGAAGCACTCGCATTCCAAACCAGTTAGCATTCAATAAATTAT
ATCATATTTTCCCGTTGGCGAATTCGCCATTACTTAGCGATTATT
TAATAGTTTTTCCGCTTGCCTTTTCTCTCGCCCTGTCTGATTTCC
CAGCACGCCTGGTGGG.

Fig. 2. The limits of the vg^{ext1} deletion are indicated by asterisks. The deletion extends from 88 bp after the first underlined *Pst* I site of the vg^{+} 1·64 *Eco*R I fragment (see Fig. 1) to the *hobo* insertion site. The localizaton of the hvg^{al} insertion is also shown: the **GTG** and **CAG** indicate the limits of the third *vg* intron where the hvg^{al} insertion takes place, generating an 8 bp duplication which is underlined, *TACTACAT*. CAGAGAACTGCA...(in open face lettering) are the *hobo* terminal repeats, the $hobovg^{al}$ sequence is not shown. The polymorphic bases compared to vg^{+} Or sequence, in the neighbouring *vg* sequence are noted in parentheses (.).

was made and compared with that of the 3·4 kb *Eco*R I fragment of vg^{al}. These fragments differ only in the size of the central *Xho* I fragment, which is 1·5 kb in pvg^{al} and 1·2 kb in pvg^{al+2}. The sequence of the $hobovg^{al+2}$ element (hvg^{al+2}) shows that it is almost identical to, and in the same orientation as, the hvg^{al} sequence. The only difference is a further internal deletion of 358 bp, so that the total internal deletion now extends from positions 938 to 2380.

At the genetic level, we have shown that two types of vg^{al+} alleles exist. The vg^{al+1}, vg^{al+4} and vg^{al+6} alleles display a wild-type phenotype when crossed with vg^{B} (vg^{B} is a complete deletion of the *vg* locus), whereas the vg^{al+2} and vg^{al+3} alleles showed a 'notched' phenotype (results not shown). These results do not correlate simply with the molecular alteration observed, since we found a 3·1 kb *Eco*R I fragment in both vg^{al+2} and vg^{al+4} (Fig. 3), and yet these alleles were different at the phenotypic level when crossed with vg^{B}. Moreover, vg^{al+2} and vg^{al+3} gave the same notched phenotype in the heterozygotes with vg^{B}, but differ at the molecular level.

(ii) vg^{ext} *analyses*

A previous analysis of the vg^{ext1} mutation by Southern hybridization identified that there is a deletion of *vestigial* sequences within two neighbouring *Eco*R I fragments (1·4 and 1·64 kb) (Bazin *et al.* 1991). The relevant *Eco*R I from vg^{ext1} fragment (i.e. missing these deleted sequences) was cloned in bluescribe (pvg^{ext1}) and a restriction map was prepared. Several genomic *vg* restriction sites are missing: *Pst* I, *Hinc* II, *Bgl* II, *Sma* I and *Eco*R I from the 1·4 kb *Eco*R I fragment and two *Pst* I sites and *Bam*H I from the 1·64 kb *Eco*R I fragment (see Fig. 1). The *hobo* element is still present and had the same characteristics as hvg^{al}. The DNA sequence of the vg^{ext1} proximal region (Fig. 2) shows that the deletion extends from 88 bp

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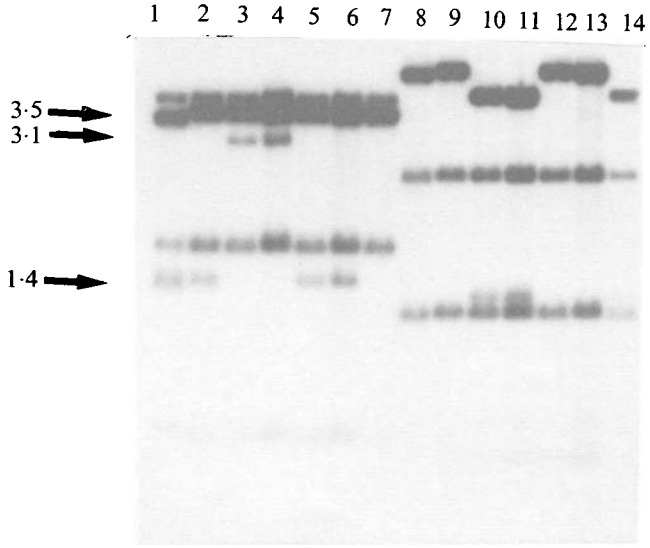

Fig. 3. Southern blot analysis of Or^R, vg^{al+3}, vg^{al+4}, vg^{al+2}, vg^{al+6}, vg^{al+1} and vg^{al} strains. The DNA, digested with *Eco*R I (lanes 1–7) or *Pst* I (lanes 8–14), was hybridized with the 6·5 kb probe (Fig. 1): Or^R lanes 1 and 8, vg^{al+3} lanes 2 and 9, vg^{al+4} lanes 3 and 10, vg^{al+2} lanes 4 and 11, vg^{al+6} lanes 5 and 12, vg^{al+1} lanes 6 and 13 and vg^{al} lanes 7 and 14.

after the *Pst* I site to exactly the *hobo* insertion site. The 8 bp duplication is missing, but there is no alteration in the left terminal *hobo* sequence.

We analysed five independent vg^{ext} strains to see if they all resulted from *vestigial* deletions. The DNA was digested with *Xho* I and hybridized with the 1·2 kb *Eco*R I *vestigial* fragment to determine the size of the *Xho* I fragment in the area with the *hobo* insertion (Fig. 1). This fragment 17 kb long in vg^+ was as against 4·7 kb in vg^{al}, because the *hobo* insertion contains a *Xho* I site. In vg^{ext3} and vg^{ext6}, the 4·7 kb fragment typical of vg^{al} was observed, showing that there is no detectable change in the vg^{ext3} and vg^{ext6} mutations in this region, apart from the *hobo* insertion (data not shown). Paradoxically, vg^{ext3} is female sterile and the wing phenotype is dramatically reduced, whilst vg^{ext6} is a recessive lethal. This result may be explained by a small inversion or deletion in the *vestigial* sequences, which was not detected in our analyses, or by a point mutation in the exonic sequences. In the vg^{ext1} mutation we found a 2·5 kb fragment approximately as expected. The equivalent fragment is 3·5 kb in vg^{ext5} and vg^{ext7} (data not shown), indicating smaller deletions than in vg^{ext1}. We also analysed two additional vg^{ext} alleles: $vg^{extV-75}$ and $vg^{extVII-24}$. These mutations result from the loss of *vg* sequences located 5′ to the *hobo* insertion in the case of $vg^{extV-75}$ and 3′ to the *hobo* insertion in the case of $vg^{extVII-24}$. The exact end points of the deletions were not located, and we do not know if there are any changes to the 5′ or 3′ *hobo* terminal inverted repeats (Fig. 1).

All of these results show that the size of the deletion of genomic *vg* sequences varies in the vg^{ext} mutations,

Table 1 *Thoracic phenotypic analyses of* vg^{83b27}, vg^{al}, $vg^{extV-75}$ *and* $vg^{extVII-24}$ *homozygous flies*

Strain	Wild-type thorax	Thorax abnormalities		
		Dorsal (%)	Legs (%)	Total
vg^{83b27}	277	4 (1)	0	281
vg^{al}	93	0	0	93
vg^{ext7}	49	18 (20)	24 (26)	91
$vg^{extV-75}$	185	14 (6)	26 (12)	225
$vg^{vgVII-24}$	399	55 (11)	31 (6)	485

The number of flies in each category was scored. The parentheses are percentages that the respective group makes up of the total flies scored with that genotype.

and that they can be located 5′ or 3′ to the *hobo* insertion. At the phenotypic level, all the extreme mutations express a very atrophied wing and the females are sterile. The ovaries are partially developed but no eggs are laid. In addition, some asymmetric thoracic abnormalities are observed. These abnormalities may alter either the scutellum or the thoracic ventral face. In the latter case the legs are modified; in extreme cases there are only five legs. In order to test if these abnormalities are in any way correlated with the extreme wing phenotype, we analysed the thoracic region of several homozygous strains: vg^{83b27} as a control, vg^{al}, vg^{ext7}, $vg^{extV-75}$ and $vg^{extVII-24}$ (Table 1). The results show that the thoracic abnormalities are not correlated with wing size per se, since vg^{83b27} has no wing (Alexandrov & Alexandrova, 1987; Williams & Bell, 1988) and no significant thoracic abnormality. In the $vg^{extV-75}$ and $vg^{extVII-24}$ strains we observed opposing thoracic phenotypes ($\chi^2 = 9$; 2 ddl, $P < 0{\cdot}05$). The $vg^{extV-75}$ phenotype mainly affects the legs, whilst $vg^{extVII-24}$ affects the dorsal part of the thorax. Since these two strains differ only by the *vg* sequence deletions, it would be interesting to test whether these results are correlated. Since the extreme alleles studied herein are derived from the vg^{al} allele, which is caused by an insertion into intron 3, it appears that the wing phenotype and female sterility are correlated with the loss of exon 3 (vg^{ext1} and $vg^{extV-75}$) or exon 4 ($vg^{extVII-24}$). This is similar to the situation in the vg^{nw} phenotype, which is female sterile and results from a deletion of downstream exons (Lindsley & Zimm, 1992; Williams & Bell, 1988).

4. Discussion

The unstable vg^{al} mutation is due to the insertion of a deleted *hobo* element into the third intron of the *vestigial* gene. The $hobovg^{al}$ element has a 1086 bp internal deletion (from bp 996 to bp 2081) as compared to the complete *hobo*HFL1 (Calvi *et al.* 1991). We observed one additional base in the $hobovg^{al}$ sequence, namely a guanidine (G) at the position 996 break

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point. We did not observe any homology with the 8 bp consensus sequences described by Streck *et al.* (1986). The vg^{al} derivatives at the site produce different phenotypes according to the length and structure of the *hobo* element involved. For example, a wing mutant phenotype is associated with vg^{al}, which has a 1874 bp insertion, whereas vg^{al+2} is wild type and has a 1516 bp insertion at the same site. The molecular difference between these two alleles is 358 bp deletion in the centre of the *hobo* element. The differences leading to the two phenotypes could be due to either *hobo* or *vg* transcription, which results in a differing length or quantity of *vg* mRNA.

Several independent vg^{ext} mutations arose spontaneously in the vg^{al} stock and were analysed. The vg^{ext1} mutation was cloned and was shown to have a 2·5 kb deletion of *vg* sequences extending 5′ from the *hobo* insertion. This deletion ends precisely at the site of the *hobo* insertion and excises the 8 bp duplication from the mutant, leaving the *hobo* element intact. The deletion completely removes exon 3 of the *vg* gene. It partly overlaps with the vg^{83b27} deletion, and completely overlaps with the vg^{79a5} deletion (Fig. 1). The vg^{ext} mutations display a strong mutant wing phenotype and female sterility in the homozygous state. This sterility is not observed in the vg^{83b27} and vg^{79a5} mutants. On the basis of our data we suggest that vg^{ext1} sterility is associated with the alteration of exon 3 of the *vg* gene. Moreover, vg^{ext1} does not complement either vg^{83b27} or vg^{BG}. This lack of complementation with vg^{83b27} is consistent with the loss of intron 2 sequences in vg^{ext1}. The vg^{83b27} allele has a lesion entirely within intron 2 and is the only *vg* allele known to complement any of the others. The deletion of exon 3 sequences in vg^{ext1} is sufficient to explain its inability to complement vg^{BG}.

Two independent molecular events can lead to a wild-type revertant. In the vg^{al+1}, vg^{al+3} and vg^{al+6} revertants, our results suggest a complete excision of the *hobo* element, whilst in other revertants (like vg^{al+2}), there is a change in the structure of the $hobovg^{al}$ element, such as a partial deletion. In the latter case, we cannot exclude the possibility that there was first an excision of the *hobo* element, and then the insertion of a new deleted *hobo* element in the same place. However, the hvg^{al} and the hvg^{al+2} elements are in the same orientation, tending to argue against an excision and a new insertion. This in turn implies that a partially deleted element is capable of being further deleted, and that there is a part of the *hobo* element which could be particularly sensitive to deletion, as both vg^{al+2} and vg^{al+4} seem similar at the molecular level (Fig. 3).

It has been shown that rearing temperature is an important factor in hybrid dysgenesis systems (P-M and I-R). however, nothing is known regarding the effect of temperature on the occurrence of specific molecular events. We have already shown that breeding temperature can enhance the probability of phenotypic wild-type revertants (20 °C) or extreme derivatives (28 °C) (Bazin *et al.* 1991). The vg^{al} mutant seems to be a particularly interesting model for the study of the molecular effects of environmental factors such as temperature on *hobo* transposition. It also provides a way of generating different *vg* alleles for the study of *vg* function and of the second complementation group defined by vg^{83b27}.

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