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A molecular analysis of the vestigial locus in *Drosophila melanogaster*

by

Jim Williams

A THESIS

SUBMITTED TO THE FACULTY OF GRADUATE STUDIES AND

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The undersigned certify that they have read, and recommend to the Faculty of Graduate Studies and Research for acceptance, a thesis entitled A MOLECULAR ANALYSIS OF THE VESTIGIAL LOCUS IN DROSOPHILA MELANOGASTER, submitted by JAMES ARTHER WILLIAMS in partial fulfilment of the requirements for the degree of DOCTOR OF PHILOSOPHY.

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Date.....1989.....

This thesis is dedicated to my parents, for all the support and encouragement they have given me thruout my academic career.

Abstract

The vestigial locus of *Drosophila melanogaster* is involved in wing margin development. In the absence of a *vg+* gene, extensive cell death occurs in third instar imaginal discs which results in a complete loss of adult wing margin structures. Hybrid dysgenesis was used to induce a P-element *vg* allele which was used to obtain molecular clones spanning 46 kilobases (kb) of *vg* region DNA. Secondary and tertiary derivatives of this P-element allele were induced by furthur hybrid dysgenesis. The derivatives were characterized by Southern hybridization analyses and, in four cases, by DNA sequencing. The alterations found were P-element internal deletions, deletions of the insert and/or adjacent *vg* region DNA, or novel insertions of P-element sequences into existing P-element inserts. The relatively high frequency of secondary insertions into P-element sequences observed herein is unusual, since secondary insertions have seldom been recovered in other dysgenic screens. In addition, the mutant phenotypes for four of these alleles were suppressed when the alleles were stabilized in the P-cytotype. This novel suppression was observed whenever repressor-producing P-elements were present in the genome, consistent with a model in which somatic P-element repressor binding to the ends of P-element inserts can modify the effects of these inserts on target gene expression.

Deficiency analysis and molecular mapping of extant *vg* alleles defined a region of approximately 20 kb of DNA which is required for *vg*

function. A developmentally regulated 3.8 kb *vg* transcript was characterized, and shown to be spliced from exons distributed throughout this region. All the characterized classical alleles have predictable effects on this transcription unit, and the severity of this effect is directly proportional to the severity of the wing phenotype. However, an exceptional *vg* allele exists (vg^{82b27}) that produces an extreme wing phenotype, but which defines a second *vg* complementation unit. This allele is associated with a 4 kb deletion entirely within a 4.5 kb *vg* intron. Molecular and genetic evidence indicates that the vg^{83b27} mutation does not affect the 3.8 kb transcription unit, thus accounting for its ability to complement classical alleles. A second *vg* transcription unit has also been identified and mapped within the intron deletion of vg^{83b27} flies, and found to be transcribed with the opposite polarity to the 3.8 kb transcript. A model is presented, in which the *vg* gene consists of two separate transcription units, both required for wing disc development.

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List of Symbols, Nomenclature or Abbreviations

b	black, a *D. melanogaster* body colour mutant
bp	basepair
BudR	bromodeoxyuridine
Ci	Curie
cn	cinnabar, a *D. melanogaster* eye colour mutant
cpm	counts per minute
C.S.	Canton-S, a *D. melanogaster* wild type stock
Cy	Curly, a dominant *D. melanogaster* wing mutant
D. melanogaster	*Drosophila melanogaster*
D. virilis	*Drosophila virilis*
dATP	2'-deoxyadenosine 5'-triphosphate
dCTP	2'-deoxycytidine 5'-triphosphate
dTTP	2'-deoxythymidine 5'-triphosphate
di	dimorphos, a *D. melanogaster* eye mutant
DHFR	dihydro-folate-reductase
DNA	deoxyribonucleic acid
e	ebony, a *D. melanogaster* body colour mutant
EMS	ethyl methanesulfonate

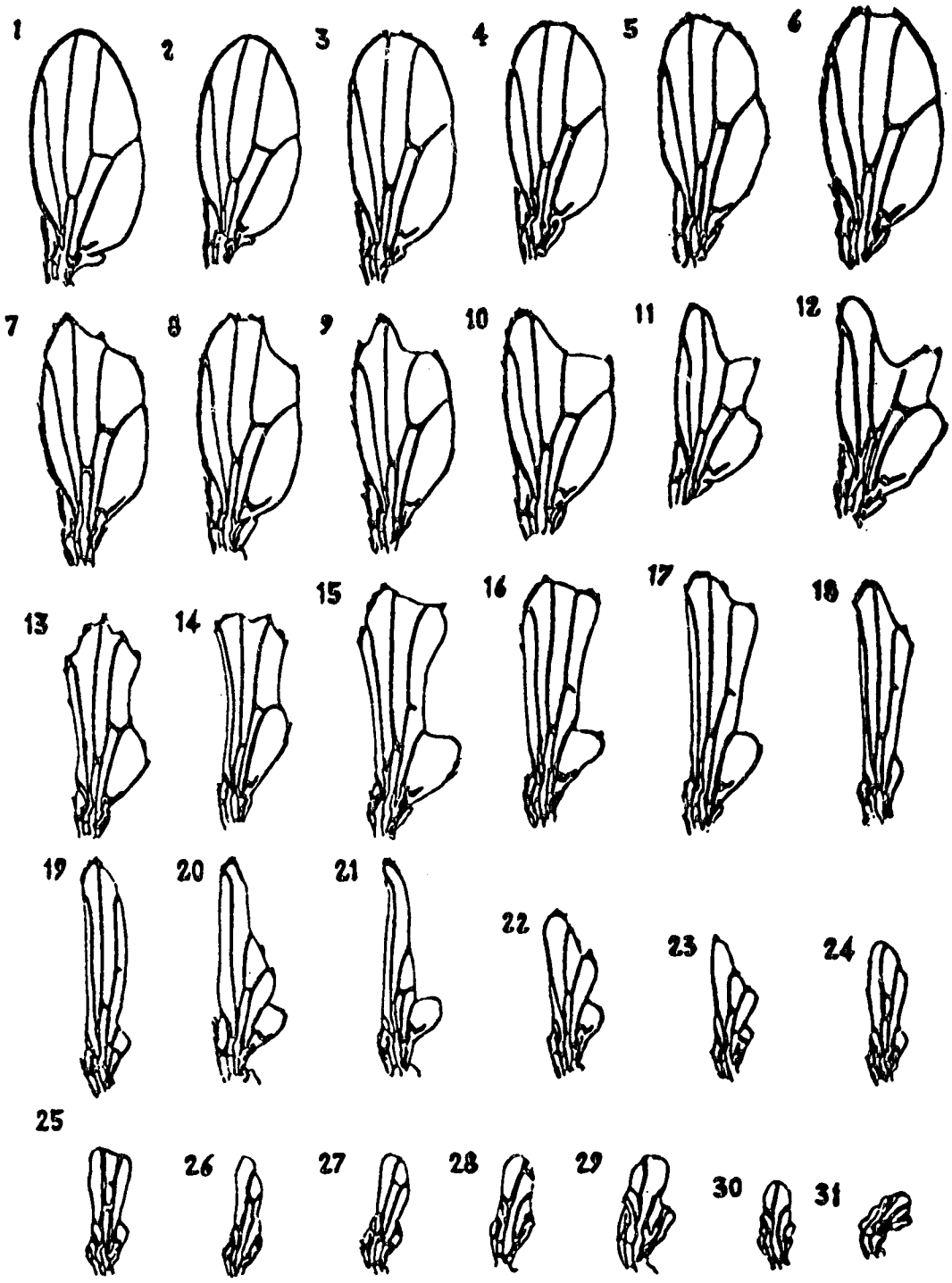
EM	electron microscope
en	engrailed, a homeotic gene of *D. melanogaster*
FudR	fluorodeoxyuridine
HGPRT	hypoxanthine-guanine-phosphoribosyl-transferase
hr	hour
kb	kilobase
mmol	millimole
mwh	multiple wing hairs, a trichome mutant of *D. melanogaster*
OR^R	Oregon-R, a wild type *D. melanogaster* lab stock
pr	purple, a eye colour mutant of *D. melanogaster*
RNA	ribonucleic acid
RP49	Ribosomal Protein 49
ss	spineless, a bristle mutant of *D. melanogaster*
ul	microliter
ug	microgram
UTP	uracil 5'-triphosphate
vg	vestigial, a wing mutant of *D. melanogaster*
vg^U	In(2R)vg^U
vg^W	In(2R)vg^W

~	approximately
λ	lambda
γ	gamma
π	*pi*

Chapter I: Introduction

Among the large number of mutations known for *Drosophila melanogaster* the vestigial (*vg*) mutant phenotype was described relatively early (Morgan, 1911; reviewed in Bridges and Morgan, 1919). The *vg* wing phenotype is well characterized, and has been used to isolate a considerable number of *vg* alleles (described in Lindsley and Grell, 1968). Strong alleles are associated with complete wing margin loss, and reduce the wings to a vestige of their normal size. However, less extreme alleles are common, each associated with a different extent of wing margin loss. This results in a series of alleles, with a phenotypic range from a cryptic effect (vg^{ni}/vg^{ni}), to a wing margin nicking or notching effect (vg^{np}/vg^{np}) to phenotypes of virtual wing elimination (vg^{nw}/vg^{nw}). We have devised a numbering system from 1-6 to categorize the wing phenotype, with 1=wild type, 2=nick wings, 3=incised wings, 4=strap wings, 5=*vg* wings, and 6=no wings (Figure I-1). Table I-1 lists all the *vg* alleles used in this study and indicates their respective wing phenotypes. Since the wing phenotypes of vg^{nw}/vg^{nw} and $vg^{nw}/Df(2R)vg^{B}$ (vg^{B} is a cytologically visible deletion of the *vg* locus) are essentially identical (6 on the phenotypic scale) vg^{nw} is considered to represent a *vg* null (amorphic) allele. Several other *vg* alleles behave similarly to vg^{nw} (Table I-1). The phenotypes of the less extreme alleles are always more extreme over a deficiency or null allele, implying that these less extreme alleles are hypomorphs (Muller, 1932). In general, heterozygotes between hypomorphic alleles show a

Figure I-1. Wing phenotypes associated with various vg alleles. The figure is reproduced from Nakashima-Tanaka (1967). The wing phenotypes associated with each number of the 1-6 phenotypic scale used in this thesis are as follows: 1=1 on the figure, 2=6 on the figure, 3=15 on the figure, 4=21 on the figure, 5=25 on the figure, and 6=31 on the figure. Of course, there are a number of phenotypic classes not indicated in our 1-6 scale. However, the crudeness of the scale allows most individual alleles to be identified by a single phenotypic number, even if some phenotypic variance exists.



Nakashima - Tanaka (1967)

Table I-1; Vestigial alleles and their phenotypic rankings

Allele genotype[a]	Homozygous[b] phenotype	Origin[c]	Source[d]
Df(2R)*vg*^{136}/SM5	L	γ	P. Lasko
Df(2R)*vg*^{56}/SM5	L	γ	P. Lasko
vg^{12}/SM5	L	γ	P. Lasko
vg^{168}/SM5	L	γ	P. Lasko
vg^{18}	5	γ	P. Lasko
tri vg^{NO2}/SM5	L	S	Bowl. Gr.
vg^{nw} *Hia*/SM5	L	S	Bowl. Gr.
vg^{nt}	1	D	Bowl. Gr.
vg (also listed as *vg*^{BG} or *vg*^{1})	5	S	Bowl. Gr.
vg^{np}	2	S	Bowl. Gr.
Df(2R)*vg*^{B}/SM5	L	S	Bowl. Gr.
In(2L)*t* In(2R)Cy/In(2R)*vg*^{U}	L	γ	Bowl. Gr.
vg^{51h25}	5	S	Amherst
In(2R)*vg*^{W}/In(2L)CyIn(2R)Cy	L	S	Umea
vg^{83b27}	5-6	γ	Alexand.
vg^{79d5}	2-3	γ and n	Alexand.
vg^{76i2}	5	γ	Alexand.
vg^{74c5}	5	γ	Alexand.
vg^{67d2}/SM5	L	γ	Alexand.
vg^{74c6}/*bPm*	L	γ	Alexand.
vg^{21}	1	hd	This study
vg$^{21\text{-}3}$	5	hd	This study
vg$^{21\text{-}4}$	4	hd	This study
vg$^{21\text{-}4Rev}$	3	hd	This study
vg$^{21\text{-}6}$	5	hd	This study
vg$^{21\text{-}7}$	4	hd	This study
vg$^{21\text{-}7Rev}$	1	hd	This study
vg$^{21\text{-}8}$	4	hd	This study
vg$^{21\text{-}9}$	L	hd	This study
vg$^{21\text{-}12}$	L	hd	This study
vg$^{83b27\text{-}R}$	6	γ	This study

[a] Allele designations are as in Lindsley and Grell (1968)
b Phenotypes are designated 1-6 as in Figure I-1, with L = lethal.
c Allele origin abbreviations are γ=gamma, S=spontaneous, n=neutrons, D= spontaneous derivative of vg, and hd=P/M hybrid dysgenesis.
d Allele source abbreviations were Bowl. Gr.= Bowling Green Stock Centre, Amherst= Amherst College, Umea=Umea Stock Centre, and Alexand.= I. Alexandrov.

wing phenotype intermediate to the respective homozygote phenotypes (Lindsley and Grell, 1968; Silber and Goux, 1978). This implies that these mutations all define one *vg* complementation unit with respect to the wing phenotype, and indicates that different threshold levels of the *vg* product are associated with discrete wing phenotypes. Since $vg^{nw}/+$ and $vg^{B}/+$ show only slight wing nicking, this implies that one vg^{+} allele is just enough to produce an essentially normal wing; that is, the locus is haplosufficient. Thus, the wing phenotypes shown in Figure I-1 would be generated by *vg* product levels from fifty percent (nicked wings) to zero percent (complete wing elimination) . This, of course, assumes that vg^{B} /+ flies in fact have fifty percent gene activity compared to vg^{+} homozygotes. An intriguing result obtained by Green (1946) implies that this may not be the case. He examined the penetrance of heterozygous $vg^{1}/+$, $vg^{1}/+/+$, and $vg^{1}/vg^{1}/+$ (vg^{1} is the original classical strong *vg* allele , assumed to be the progenitor of vg^{BG}). He found that mutant phenotypes were most common in $vg^{1}/vg^{1}/+$ and least common in $vg^{1}/+/+$. The observation that more mutants are seen in $+/vg^{1}/vg^{1}$, than $+/vg^{1}$ heterozyotes implies an antimorphic nature to vg^{1}, or that *vg* autoregulates its own expression.

Vestigial is a pleiotropic mutant, since a variety of qualitative and quantitative ancillary phenotypes are associated with *vg* alleles. These include haltere reduction, erect postscutellar bristles, female sterility, low viability, pupal lethality, reduced adult size, and developmental delay. In general, these ancillary phenotypes are associated only with

strong *vg* alleles. Thus, a notch or strap wing *vg* allele (vg^{np} or vg^{79d5}) will be wild type for these other phenes. However, these alleles heterozygous with a strong *vg* allele (vg^{nw}) do show most of these secondary phenotypes, indicating that all the *vg* phenotypes may result from a common genetic deficiency. Consistent with this conclusion is the fact that essentially all homozygous null *vg* alleles show all of the *vg* phenotypes, while essentially all homozygous weak *vg* alleles show only wing defects (see Alexandrov and Alexandrova, 1988; Lindsley and Grell, 1968). A model consistent with these observations is that the secondary phenotypes are also due to qualitative or quantitative reductions of the vg^{+} product, but have different thresholds for expression of the phenotype. In this model, the erect postscutellar and haltere reduction phenotypes would be expressed at *vg* product levels below that required to form a "4" wing. Female sterility would only be associated with "6" wing phenotypes, but reduced fertility would be seen with "4" or "5" wing phenotypes. The other secondary phenotypes have not been extensively analysed, and thus cannot be assessed. This type of model has been proposed to explain the pleiotropic effects of different apterous alleles (Stevens and Bryant, 1985).

Genetic evidence implies that *vg* is genetically complex. Two dominant *vg* alleles exist (vg^{U} and vg^{W}) which cause complete wing margin loss in the heterozygous condition (Bownes and Roberts 1981a; Ives, 1956). Both alleles are unconditionally lethal when homozygous or in trans to each other. Both are also associated with inversions which have a breakpoint in salivary chromosome interval 49D-F, the

cytological location of *vg* (Morgan et al, 1938). It is not the inversions *per se* which mediate dominance, since in the case of vg^U, a spontaneous *vg* null derivative of vg^U remains associated with the inversion (Silber and Lemeunier, 1981). The phenotypes of both vg^U and vg^W are more extreme when heterozygous with *vg* alleles, indicating the dominance is due to an antimorphic gene product rather than a neomorphic product (McGill et al, 1988). However, unlike homozygous null *vg* alleles which show poor viability and female sterility, the two dominant alleles are unconditionally lethal, either when homozygous or *in trans* to each other. The simple antimorphic model of dominance cannot account for this unconditional lethality. Finally, vg^W is also associated with dominant homeotic transformations of haltere to wing and posterior wing duplications. This is the only *vg* allele which shows homeotic effects.

A complex *vg* allele exists (vg^{83b27}) which defines a second *vg* complementation unit. Homozygous vg^{83b27} flies have extreme wing and haltere deficiencies and extreme developmental delay. However, vg^{83b27} flies are wild-type for the scutellar and female fertility phenes, even when heterozygous to a *vg* deficiency (Alexandrov and Alexandrova, 1988). Thus vg^{83b27} is an exception to the threshold model presented above, and also implies that two separate *vg* functional units exist. This is consistent with the fact that vg^{83b27} complements the wing and haltere phenotypes of a number of classical *vg* alleles. This complementation extends to essentially all recessive female fertile alleles (viable alleles) while recessive female sterile alleles (lethal

alleles) are only weakly complemented or not complemented at all (see Alexandrov and Alexandrova, 1988). This indicates that vg^{83b27} defines a second *vg* complementation group separate from the complementation group defined above. Thus, the genetics of the dominant alleles and vg^{83b27} imply that *vg* is a genetically complex locus.

A number of genetic and environmental factors have been found which modify *vg* expression. Goldschmidt (1935) reported the identification of *vg* modifier genes which he named *vg* dominigenes. These genes showed no homozygous phenotype, but enhanced the wing phenotype of *vg*/+ heterozygotes (ie. 100% of heterozygotes show scalloped wings). One major dominigene was mapped to the X chromosome and was thought to be an allele of cut (*ct*). The effect of this allele was recessive, and required the presence of two dominant autosomal genes (denoted A and B) to affect *vg* expression. The two autosomal genes alone have very little effect on *vg* expression. Goldschmidt (1937) presented more data indicating that the X-linked dominigene was indeed an allele of cut. The cut locus is characterized by recessive alleles and plays a role in wing development since classical cut alleles show incised wings. One classical cut allele, ct^6, was shown by Goldshmidt (1937) to enhance the *vg* phenotype of *vg*/+ heterozygotes. This cut allele's effects were recessive with respect to the cut phenotype, but dominant with respect to the dominigene effect. The dominigene effects are vg-specific, but not specific to one type of *vg* allele, since Goldschmidt and Honer (1937) demonstrated that other

vg alleles (vg^{ni}, vg^{no}, vg^{nw}) were also affected by the dominigenes.

Goux (1973) reported that the penetrance of different vg^{1} heterozygotes depended on which "wild type" stock the heterozygotes were derived from. This phenomenom was further characterized by Silber (1980). He reported the identification of two X-linked modifier systems, one enhancing and one diminishing the penetrance of +/*vg* heterozygotes. Flies homozygous for the enhancer system show scalloped wings in the presence of a heterozygous or homozygous wild type *vg* locus. He could not eliminate the possibility that the X-linked enhancer system was the same as the Goldschmidt dominigenes. However, crosses using a deficiency of cut led him to believe that they were not.

The identity of the A and B dominant *vg* modifiers (required in addition to the X-linked modifier) was determined indirectly by Gardner (1942). He tested whether the *vg*/+ penetrance was affected by a variety of known mutants. A large number of gene mutations were found to enhance the mutant phenotype of *vg*/+ flies. Blanc (1946) found that two of these enhancers, spineless (*ss*) and purple (*pr*), corresponded respectively to the A and B autosomal genes identified by Goldschmidt. He also characterized a black (*b*) allele as a dominant suppressor of *vg*. Blanc speculated that these autosomal dominant modifiers may be polygenic in nature and suggested that the effect on *vg* may be due to increased larval development time in enhancer mutants. This is consistent with independant observations that indicate genetic or environmental factors that prolong larval development

enhance *vg* expression. Green and Oliver (1940) showed that dominant Minute mutants which prolong larval development result in scalloping of wings in *vg*/+ heterozygotes. Overcrowding or poor nutrition both delay development, and were reported by Stanley (1935) and Child (1939) to result in more extreme *vg* phenotypes. Thus, these results are consistent with many autosomal dominant *vg* enhancers exerting their effects by delaying development. However, the strong X-linked *vg* enhancers reported by Goldschmidt (1935a) and Silber (1980) do appear to be vg-specific enhancers. As discussed above, Goldschmidt (1937) speculated that the X-chromosome dominigene was a cut allele. Notchoid alleles of the Notch locus also enhance *vg* mutant expression (L. Rabinow, pers. comm.). It would be interesting to determine what the basis for the enhancement is since Notch and cut are both neurogenic loci (Poulson, 1937; Bodmer et al, 1987).

Genetic modifiers which suppress the *vg* mutant phenotype also exist. Silber (1980) reported the identification of an X-linked modifier system which diminishes the penetrance of +/*vg* heterozygotes. Harnley (1935) reported the identification and analysis of dimorphos (*di*), an X-linked recessive modifier of *vg*. Homozygous *di, vg* flies show partial suppression of *vg* in males (ie. strap wings) but no significant suppression in females. In the absence of *vg* alleles, dimorphos flies have no wing phenotype, but at high temperatures *di* homozygotes affect the size and surface of the eyes in both sexes (Harnley and Harnley, 1935). This implies that dimorphos affects *vg* without itself having a fundamental role in wing development. Bazin and Silber (1982) used

ethyl methanesulfonate (EMS) and bromodeoxyuridine (BudR) to isolate phenotypic revertants of *vg*^{1}. Of ten isolated revertants, two were wild type and appeared to represent true reversion events at the *vg* locus. The remainder were recessive second and third chromosome suppressors which partially suppressed the wing phenotype of homozygous *vg* flies. The suppressed flies showed strap wings, as well as increased haltere size, weight and viability. Thus, genetic factors exist which either suppress or enhance the phenotype of *vg* mutants. The analysis of these modifier genes may eventually help our understanding of the role of the vestigial gene in wing development.

Roberts (1918) reported that the size of the wings of *vg*^{1} flies increased when the flies were reared at a high temperature. This observation was extended by several investigators (Stanley 1931, 1935; Li and Tsui, 1936; McCrady and Smith, 1984) with homozygous *vg*^{1} stocks. Restrictive temperatures were established to be below 25^{o}C, while permissive temperatures were 30^{o}C and above. At these permissive temperatures, substantial increases in wing size were seen in both sexes, although wild type wings were seldom observed. As well, the haltere and scutellar bristle phenotypes were less extreme at 30^{o}C, consistent with the model that all the *vg* phenotypes are associated with the absence of a single *vg* product. The temperature effect is not specific to the *vg*^{1} stock, since similar results were obtained in such experiments on *vg*^{D} (Harnley, 1940), *vg pennant* (Harnley and Harnley, 1936) and *vg*^{no} (Akita and Nakayama,1954).

Temperature shift experiments were performed with *vg*^{1}

(Stanley, 1931, 1935; Harnley, 1936) and vg^{no} (Akita and Nakayama, 1954) to establish the *vg* temperature effective period. Both down-shift and up-shift experiments were performed. Briefly, in a down-shift experiment vials of embryos start development at restrictive temperatures, and at various developmental times, the vials are shifted to permissive temperatures. The wing lengths of the adult flies were determined, to establish how long development has to proceed at restrictive temperatures before the restrictive phenotype is established. The up-shift experiments are simply the reciprocal experiment. These studies established that the time of action of *vg* with respect to the wing phenotype is approximately the second to third larval instar molt. Interestingly, Stanley (1935) determined that the postscutellar bristle phenotype has a later temperature effective period (during the pupal stage). If *vg* has one product, this result implies that this product is essential at different times and locations in the developing fly.

Since *vg* is required for wing development and this requirement is during the second and third larval periods, investigators began examining the wing discs in third instar larvae to determine if any abnormalities exist in vg discs. Goldschmidt (1935) and Waddington (1940) both observed that *vg* wing discs were smaller than their wild type derivatives. Goldschmidt proposed that the reduction of the adult wing was a consequence of degradation of the affected wing structures during development. Waddington concluded that the missing wing regions failed to develop at all. An electron microscope (EM) study by Fristrom (1968) indicated that abnormally high levels of cell death

occur in wing discs of *vg* larvae in the presumptive wing blade region of the disc (the region which is absent in adult wings). This argues that Goldschmidt's theory of degeneration of wing structures is correct. Several subsequent studies utilizing EM or histochemical staining to detect cell death have corroborated and extended Fristrom's initial observation (Fristrom, 1969; James and Bryant, 1981; Bownes and Roberts, 1981a,b; O'Brochta and Bryant, 1983). These studies all determined that the regions of cell death in the *vg* wing disc correspond exactly with the presumptive structures deleted in the adult wing. As well, this cell death occurs throughout the third instar period, corresponding to the cold sensitive period determined by temperature shift experiments. Thus, cellular degeneration within *vg* wing discs can account for the wing phenotype of *vg*. O'Brochta and Bryant (1983) demonstrated that cell death in vg^1 and vg^U follow different processes. However, this may be due to different genetic backgrounds in the two strains. Interestingly, James and Bryant (1981) observed a small amount of scattered cell death in early third instar wild-type discs. They proposed that *vg* affects wing shape by exaggerating the extent of normal cell death.

Although not experimentally proven, it is generally assumed that the *vg* haltere phenotype is analogous to the wing phenotype, and is thus due to extensive cell death in the haltere disc. The female sterility and postscutellar phenotypes have not been extensively analysed, so the basis of these traits is unknown. However, it is likely that these phenotypes are due to other defects than cell death in the wing and

haltere discs. The observation that the postscutellar phenotype has a later temperature sensitive period (Stanley, 1935) is consistent with this theory. One of the quantitative phenotypes of *vg* is developmental delay. Borot and Goux (1981) found that the increased developmental time of vg^1 is due mainly to increased duration of the third larval instar. This may represent a link between the cell death (wing) phenotype and developmental delay phenotypes.

It would be of interest to determine the basis of *vg* cell death, since little is understood about programmed cell death in general. Two major theories exist to account for *vg* cell death.The first is based on the observation of Silber (1980) that *vg* mutants (vg^1, vg^{np}, vg^{51h25}) are resistant to inhibitors of purine nucleotide metabolism (ie. aminopterin and fluorodeoxyuridine-FudR) and have an altered purine nucleotide pool. Silber and Becker (1981) demonstrated that extracts from adult vg^1, vg^{np}, vg^U and *vg*/+ flies show hypoxanthine-guanine-phosphoribosyl-transferase (HGPRT) activity, while wild type flies don't. This enzyme is present in wild type flies, but is only detected after dialysis or treatment with activated charcoal (Becker, 1978). When *vg* and wild type extracts are treated with activated charcoal, similar HGPRT activities are observed. This implies that *vg* mutants lack an inhibitor of HGPRT and don't affect the actual enzyme levels. This is corroborated by the author's observation that HGPRT levels in *vg* flies are decreased at 30°C, the temperature at which the wing phenotype becomes less severe. The enzyme dihydro-folate-reductase (DHFR) also has enhanced levels in *vg* stocks (J. Silber, pers. comm.). Thus, *vg*

mutants appear to lack inhibitors of enzymes involved in purine nucleotide biosynthesis. The aminopterin and FudR resistance of *vg* stocks are consistent with the enzyme studies, since these compounds are inhibitors of purine nucleotide biosynthesis. Silber and Becker (1981) speculate that this altered metabolism may be the cause of *vg* wing disc cell death. Examples of mutants which decrease pyrimidine nucleotide biosynthesis exist, and these may show strong wing phenotypes (Norby, 1970). However, of the known purine biosynthesis mutants, very few have wing phenotypes, and those show only weak effects. As well, purine biosynthesis mutants often have eye color phenotypes, which are never seen in *vg* alleles (for a review, see Nash and Henderson, 1982). Most of the purine and pyrimidine pathway mutants reduce enzyme activity and therefore biosynthesis. Vestigial stocks have increased levels of at least two purine biosynthetic enzymes (HGPRT and DHFR), implying that *vg* mutants lack a complex inhibitor of purine metabolism, and don't affect structural genes responsible for these enzymes. It is difficult to predict the phenotype of a mutant which enhances purine nucleotide biosynthesis enzymes. Thus, it is uncertain if the biochemical defects associated with *vg* do, in fact, account for the cell death seen in *vg* wing discs.

The second theory to account for *vg* cell death is based on experiments which examined the regulative properties of *vg* wing discs. Bownes and Roberts (1981b) performed a series of experiments examining the ability of *vg* wing discs to regenerate wing margin structures deleted by cell death. They found that *vg* wing discs cannot

regenerate margin structures under a variety of experimental conditions. Metamorphosed young *vg* wing discs (before cell death occurs) produced no wing margin structures, indicating that *vg* discs cannot produce wing margin structures even before cell death occurs. Vestigial wing discs were transferred to adult abdomens to permit extra growth, and then metamorphosed in larval hosts. No margin structures were formed, indicating that, even with extra growth, *vg* discs cannot form wing margin. The dead cells in *vg* discs do not physically prevent regeneration since physical removal of these cells and culturing the discs in adults and metamorphosing as above does not result in margin structure regeneration. Finally, experiments mixing fragments of *vg* wing discs with ebony multiple wing hairs (*e mwh*) wing disc fragments intercalated wing structures of both genotypes. However, structures affected in *vg* wings (wing margin, etc.) were always made from *e mwh* cells and not *vg* cells. Thus, *vg* wing discs are unable to differentiate margin cells under a variety of experimental conditions. The authors speculated that *vg* discs have abnormal positional information and that the cell death observed is a consequence of this position-specific defect in cell determination. Thus, experimental evidence exists which supports at least two models accounting for the cell death observed in *vg* discs.

From the great amount of experimental data published on *vg* since 1918 and discussed above, several general findings can be stated. Vestigial mutants are pleiotropic, affecting wing, haltere and postscutellar bristle development, as well as causing quantitative defects

such as female sterility, reduced viability, reduced adult weight and extended first and third larval instar developmental times. The wing phenotype is the most distinctive and well characterized phenotype. This phenotype is temperature sensitive in that most alleles are less extreme at 30°C than at 18°C or 25°C. Using temperature sensitivity as an assay, the time of action of the *vg* gene on the wing phenotype was established to be at the second to third larval instar molt. This period corresponds to the time when extensive cell death begins within the wing imaginal disc. This cell death extends through the third instar, and accounts for the adult wing margin structures which are deleted. Two theories to explain this cell death have been proposed. The absence in *vg* mutants of an inhibitor of certain purine metabolism enzymes has been demonstrated, and proposed to be the cause of the cell death. Other data support models in which the cell death is due to aberrant wing disc positional information. As well, *vg* displays genetic complexity. Although most *vg* alleles are recessive and define one complementation unit, two dominant *vg* alleles exist, as well as a recessive mutant which defines a second vg complementation unit.

To analyse this genetic complexity as well as facilitate future investigations into the basis of *vg* cell death, it was decided to molecularly clone the *vg* locus. Although no molecular data existed for *vg*, some analyses provided clues about the physical complexity of the locus. Carlson et al (1980) established the pseudoallelic nature of *vg* by generating an intragenic recombination map between the vg^{1}, vg^{nw} and vg^{np} alleles. The recombination frequencies between these alleles

indicated that *vg* is a large gene; approximately 20 kilobases (kb) using the kb/genetic map unit ratios defined for previously cloned *Drosophila* genes (Bender et al., 1983; Cote' et al., 1986). This is consistent with the analysis of Schuchman et al (1980), which showed that *vg* is hypermutable by EMS. Interestingly, more mild alleles than extreme alleles are generated utilizing EMS as a mutagen.

The following four chapters describe the molecular cloning and analysis of the vestigial locus. Chapter II describes the molecular cloning of *vg* and the mapping of the lesions associated with several extant alleles. Chapters III and IV describe the generation and analysis of a variety of secondary and tertiary P-element alleles. Chapter V describes transcriptional analysis of *vg*, and the isolation and characterization of two *vg* transcription units.

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Chapter II: Molecular organization of the vestigial region in *Drosophila melanogaster*[1]

Introduction

Among the large number of mutations known for *Drosophila melanogaster* the vestigial (*vg*) mutant phenotype was described relatively early (Bridges and Morgan, 1919), and a considerable number of alleles have been isolated (described in Lindsley and Grell, 1968). Some *vg* mutants reduce the wings to a vestige of their normal size, but considerable phenotypic variation is exhibited by the extant alleles of this locus. The phenotypes range from a cryptic effect (e.g., vg^{ni}/vg^{ni}), to an effect of slight nicking of the wing margins (e.g., vg^{np}/vg^{np}), to phenotypes of virtual wing elimination (e.g., vg^{nw}/vg^{nw}) and homozygous lethality (e.g., vg^{U}/vg^{U}). Classical *vg* alleles may also show a variety of other phenotypic effects including haltere reduction, developmental delay, low viability, female sterility, high penetrance when homozygous, or even homeosis (described in Lindsley and Grell, 1968; or Bownes and Roberts, 1981a). Most of the *vg* alleles are recessive under standard laboratory conditions although many act as weak dominants under specific conditions (ie. low temperature or in a Minute background). In general, any factor (environmental or genetic)

1. A version of this chapter has been published. Williams J., and J. B. Bell. 1988. EMBO J. 7: 1355-1363.

that increases the length of the developmental cycle will modulate *vg* expression towards a more severe phenotype (reviewed in Green, 1946). Alleles also exist which always behave as strong dominants, as well as alleles which show a complex complementation pattern with other *vg* alleles.

Several studies have implicated excessive cell death in regions of the imaginal wing disc as the physiological cause of the mutant phenotype (Fristrom, 1969; James and Bryant, 1981; Bownes and Roberts, 1981a,b; O'Brochta and Bryant, 1983), but the reason for this cell death remains uncertain. Bownes and Roberts (1981b) have shown that *vg* wing discs are unable to regenerate margin structures under a variety of experimental conditions, and they postulated that *vg* wing discs have an altered positional information system. Thus, the cell death could be a consequence of an earlier position-specific defect in cell determination. An alternative hypothesis was advanced by Silber and Becker (1981) who speculated that aberrant purine metabolism is the cause of the *vg* mutant phenotype. They showed that *vg* mutants have much higher HGPRTase and 5' nucleotidase activities than do wild type flies.

The molecular cloning of the vg^+ gene as well as various mutant alleles was made feasible by the development of P-element tagging, which has been used successfully in cloning many other *Drosophila* structural genes (e.g. Bingham et al., 1981; Searles et al., 1982). The cloning of the vg^+ gene by this means is reported here. The cloning of the locus serves as an entry point into the molecular characterization of

the wild type versus the mutant alleles of *vg*. Data on the partial characterization (at the DNA level) of several extant *vg* alleles of spontaneous or induced origin are presented. These results are consistent with the pattern emerging that many of the spontaneously derived mutant alleles of *Drosophila* genes are due to gross sequence changes resulting from insertions or deletions of up to several kilobases of DNA (Zachar and Bingham, 1982; Bender et al., 1983a; Cote et al., 1986). Data are also presented that orient the molecular map of the *vg* locus with respect to the classical genetic fine structure map (Carlson et al., 1980). The characterization of the lesions associated with two dominant *vg* alleles and one complex *vg* allele implies interesting regulatory mechanisms for this locus. The new data accumulated in this study should eventually lead to an understanding of the wild type function of the locus and hopefully will help to resolve various hypotheses explaining the functional deficiency of *vg* mutants.

Materials and Methods

Stocks and dysgenic screens: *Drosophila melanogaster* cultures were grown at 24°C and maintained on a synthetic medium (Nash and Bell, 1968). The genotypes, origin, and sources of all strains used in this study are presented in Table II-1.

Materials: Restriction enzymes and other DNA modifying enzymes were obtained from BRL or Pharmacia and used according to the manufacturer's instructions. All radioisotopically labeled compounds were purchased from New England Nuclear. Oligolabeled probes were made with [^{32}P]dCTP (3000 Ci/mmol), RNA probes were labeled with [^{32}P]UTP (3000 Ci/mmol), while nick translations for *in situ* hybridizations utilized [^{3}H]dTTP (50-80 Ci/mmol). The *Drosophila* embryonic cDNA clone banks were gifts from T. Kornberg.

DNA Manipulations: Culturing and storage of bacterial or lambda phage, preparation of DNA, restriction analyses, and plasmid subcloning were performed according to standard methodology (Maniatis et al., 1982). Genomic *Drosophila* DNA for Southern hybridizations and genomic libraries was prepared according to Ish-Horowitz et al., (1979), and repurified by spermine precipitation (Hoopes and McClure, 1981).

Table II-I: *Drosophila melanogaster* Strains

Genotype[a]	Origin	Source
Df (2R) vg^{136}/SM5	γ	P. Lasko
Df (2R) vg^{56}/SM5	γ	P. Lasko
vg^{12}/SM5	γ	P. Lasko
vg^{168}/SM5	γ	P. Lasko
vg^{18}	γ	P. Lasko
vg^{21}	hybrid dysgenesis	This study
tri vg^{NO2}/SM5	spontaneous	Bowling Green Stock Centre
vg^{nw} Hia/SM5	spontaneous	"
vg^{ni}	spontaneous derivative of vg	"
vg (vg^{BG})	spontaneous	"
In(2L)t In(2R) Cy/In(2R) vg^{U}	γ	"
Df(2R)vg^{B}/SM5	spontaneous	"
vg^{np}	spontaneous	"
vg^{51h25}	spontaneous	Amherst College
In(2R) vg^{W}/In(2L)Cy In(2R)Cy	spontaneous	Umea Stock Centre
vg^{83b27}	γ	I. Alexandrov
vg^{79d5}	γ and neutrons	"
vg^{76i2}	γ	"
vg^{74c5}	γ	"
vg^{67d2}/SM5	γ	"
vg^{74c6}/bPm	γ	"
sc z w^{15};Su(z)(2)^{5}/CyO	induced?	Chao-ting Wu
π2 (P-cytotype)	---	W. Engels
Oregon-R (M-cytotype)	---	T. Wright
Canton-S (M-cytotype)	---	R. Hodgetts
In(2L)Cy In(2R)Cy *al dp b px cn vg c a px bw mp sp*		J. Kennison

[a] Allele designations are as in Lindsley and Grell (1968)

Genomic Libraries: All genomic libraries were constructed in EMBL-3 according to Frischauf et al., (1983) and Maniatis et al., (1982). Conditions for partial digestion of genomic DNA with Sau3a were established, optimally cut *Drosophila* DNA was purified in 0.4% agarose gels by electrophoresis onto dialysis membranes and 15-20 kb fragments were collected on dialysis membranes inserted in the gel, ethanol precipitated and resuspended in 5 ul TE buffer. Four ul of vector DNA (2ug) was added, the mixture ligated, and packaged. Typical results yielded 6 x 10^5 pfu/reaction. Genomic libraries and the cDNA libraries were transferred to biodyne membranes (Pall) and prepared for hybridization by standard methodology. The vg^{nw} and vg^{56} libraries were also constructed as above except that genomic DNA was digested with Sal1, rather than Sau3a, prior to ligation into appropriately prepared EMBL3 arms.

Southern and Northern Hybridizations: All gels for Southern or Northern hybridization analyses were blotted onto GenescreenPlus membranes using the capillary blot protocol recommended by the manufacturer (DuPont). RNA was extracted, purified by oligo-dT chromatography, and run on 1.5% formaldehyde agarose gels as in Gietz and Hodgetts (1985). For Northern gels 4 ug RNA/lane was used, while for Southern gels 5ug DNA/lane was used. Size markers for Southern gels were BamHI digested EMBL3, and size markers obtained from mixing the digestion products of Pπ25.1 treated with a variety of enzymes and calibrated by comparison to the known sequence (O'Hare

and Rubin, 1983). Hybridization conditions for all plaque lifts, Southerns, and DNA-probed Northerns were as in Klessig and Berry (1983). Southerns and DNA-probed Northerns were washed according to Genescreen Plus specifications. DNA probes were made from restriction fragments resolved on low melt agarose gels and oligolabeled by the method of Feinberg and Vogelstein (1983). RNA probes for Northerns were prepared from restriction fragments cloned into Bluescribe (Vector Cloning Systems) using the transcription protocol of Melton et al., (1984), and their methods for hybridization conditions and washes of RNA-probed Northerns were also used. The prehybridization and hybridization temperature was 65°C.

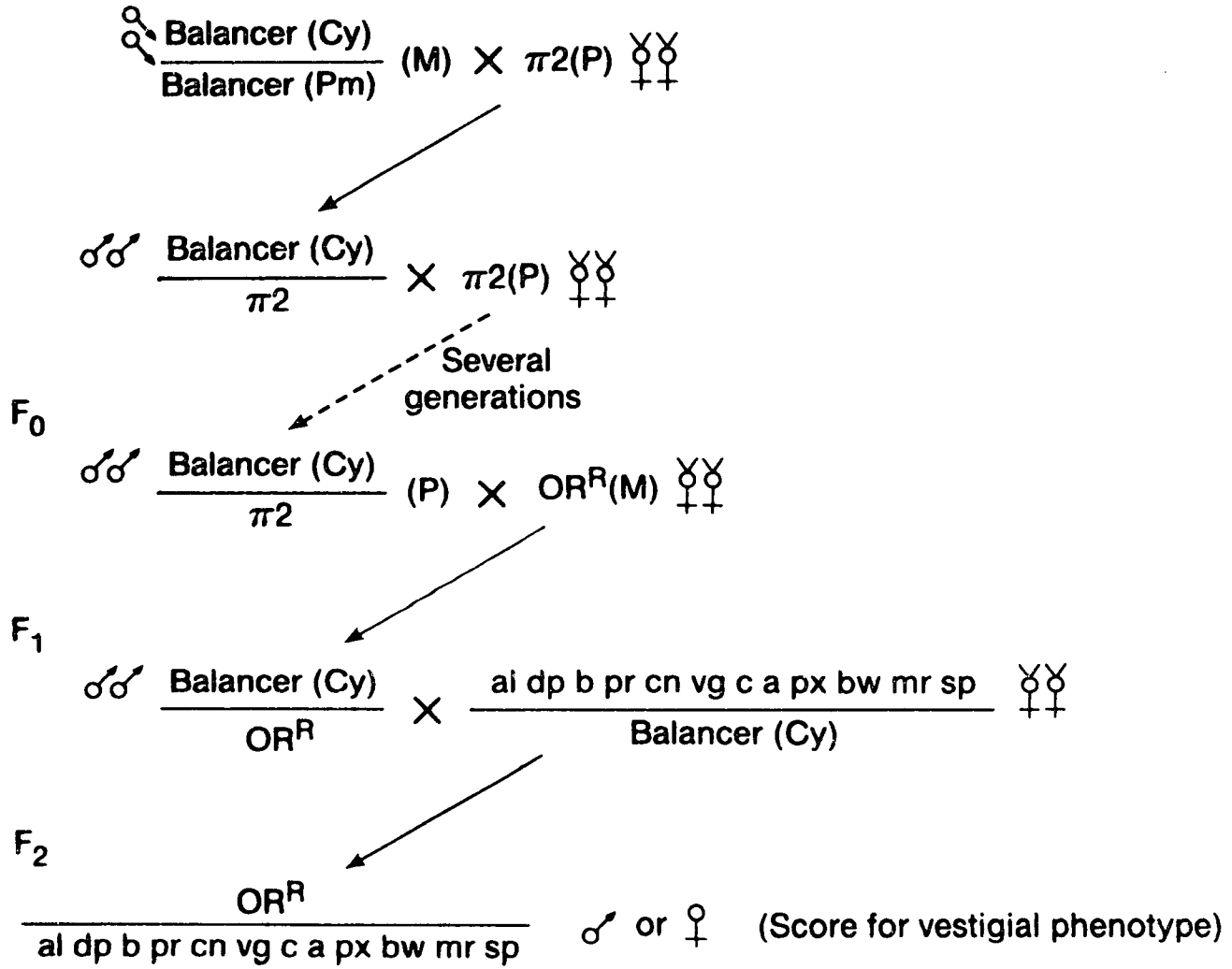
In Situ Hybridizations: The protocol of Pardue and Gall (1975) as modified by Glew et al., (1986) was used for *in situ* hybridizations. Labeled probes were nick translated as in Maniatis et al., (1982). At least 5×10^5 cpm of [^{3}H]dTTP labeled probe (5×10^6 - 10^7 cpm/ug DNA) were used for each slide subjected to *in situ* hybridization.

Results

Cloning of the vg^+ gene:

The *vg* gene has been cytologically mapped to salivary chromosome interval 49D-F by deficiency analysis (Morgan et al., 1938). Due to the relative paucity of rearrangement breakpoints in this region and the lack of any known gene product for the vg^+ locus, transposon tagging appeared to be the method of choice to clone the gene. A P/M dysgenic cross was performed between P-cytotype balancer stock (π2/SM5,Cy) males and Oregon-R (M cytotype) females (Figure II-1). The F_1 OR^R/SM5,Cy dysgenic progeny were mated to *vg* flies (M cytotype) and wing-defective F_2 progeny were analyzed. The use of the π2/SM5,Cy balancer stock allows the selection of transposon insertions onto the OR^R second chromosome by selecting only the Cy^+ F_2 progeny. A total of 100 independent wing-defective flies were isolated from a total of approximately two million Cy^+ flies analyzed. These mutants were tested for complementation of the vg^{BG} allele and one was found to be allelic. The majority were X-linked dominant and recessive wing defectives and were not analyzed further. The *vg* mutant (henceforth called vg^{21}) is a cryptic allele in that it is homozygous wild type, but it exhibits a weak *vg* phenotype (the heterozygote has a '3' wing phenotype; see Figure I-1) when heterozygous with vg^{BG} (*BG* denotes Bowling Green, the source of this *vg* stock). *In situ* hybridizations of radiolabeled P-element sequences to salivary gland

Figure II-1: Flow chart for the isolation of P/M hybrid dysgenesis induced *vg* alleles. The use of the π2 /SM5, Cy balancer stock allows the selection of transposon insertions onto the OR^R second chromosome by selecting only the Cy^+ F2 flies. Both dysgenic male and female flies were used in the F1 generation. Allele designations are as in Lindsley and Grell (1968).


Balancer (Cy)
Balancer (Pm)
(M) × π2(P)
Balancer (Cy)
π2
× π2(P)
Several
generations
F0
Balancer (Cy)
π2
(P) × OR^R(M)
F1
Balancer (Cy)
OR^R
×
al dp b pr cn vg c a px bw mr sp
Balancer (Cy)
F2
OR^R
al dp b pr cn vg c a px bw mr sp
or
(Score for vestigial phenotype)

chromosomes of vg^{21} larvae revealed several regions that hybridized to P-elements, including 49D-F (Figure II-2). The vg^{21} strain was backcrossed to vg^{BG} (M cytotype) for several generations to eliminate most of the extraneous P-elements and a genomic library was constructed (Methods) and screened with a P-element probe. *In situ* analysis showed that 8 of 150 positive clones hybridized to the *vg* region (Figure II-3). Restriction endonuclease analysis of four of the vg region λ clones yielded a map comprising 34kb of DNA from the region of the P-element insert. Unique DNA, flanking the P-element sequences, was identified and used to clone vg^{+} sequences from an OR^{R} genomic DNA library. These sequences, in turn, were subjected to restriction endonuclease analysis and a final map covering about 46kb of DNA from the *vg* region was constructed (Figure II-4). A comparison of the OR^{R} restriction map to that of vg^{21} revealed that the only difference was an approximately 650 bp insert in vg^{21}, indicated in Figure II-4. This insert hybridizes only to P-element sequences from the ends of the pπ25.1 element (O'Hare and Rubin, 1983), so it is an internally-deleted P-element. The insert position is designated as co-ordinate O on the restriction map presented. *In situ* hybridization analysis and reverse Southern analysis detected no other repetitive element within the 46 kb DNA interval (results not shown).

Localization of *vg* gene in cloned region, i) by deficiency analysis:

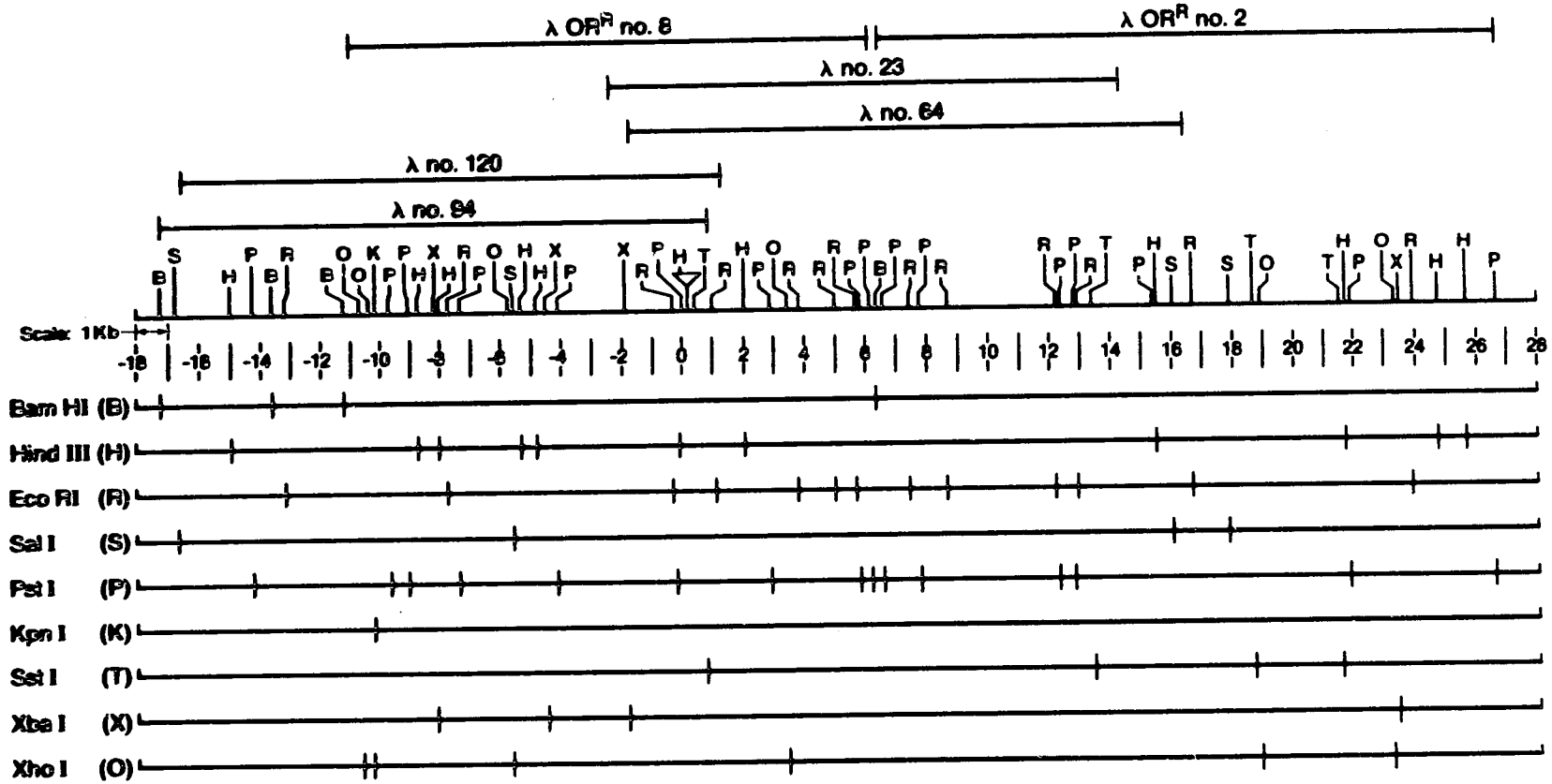
A deficiency map of the *vg* region is available (Lasko and Pardue,

Figure II-2: *In situ* hybridization of a P-element probe to vg^{21} salivary gland chromosomes. P-element sequences in vg^{21} were localized cytologically by hybridizing [^{3}H] labeled pπ25.1 (O'Hare and Rubin, 1983) to vg^{21} salivary chromosomes. The cytological location of *vg* (49D-F) is indicated with an arrow. The other prominently labeled region is another site of P-element integration unrelated to vg. Exposure time was one week.

Figure II-3: *In situ* hybridization of a *vg* region DNA clone to salivary gland polytene chromosomes. Lambda 120 (From Figure II-4) was localized cytologically by hybridizing [^{3}H] labeled λ 120 to OR^{R} polytene chromosomes. Some cytological landmarks are identified by number. Exposure time was one week.

48A

Figure II-4: Restriction map of the vestigial region. A restriction map of 46 kb of the *vg* region is shown. The extents of four recombinant vg^{21} lambda clones (lambda's 120, 94, 64 and 23) and two recombinant OR^R clones (OR^R#2 and OR^R#8) are indicated above the map. The location of the vg^{21} insert is designated as coordinate 0, with the P-element shown as a triangle above the map at this point. Centromere proximal is left, centromere distal is right.


λ OR^R no. 8
λ OR^R no. 2
λ no. 23
λ no. 64
λ no. 120
λ no. 94
Scale: 1Kb
-18 -16 -14 -12 -10 -8 -6 -4 -2 0 2 4 6 8 10 12 14 16 18 20 22 24 26 28
Bam HI (B)
Hind III (H)
Eco RI (R)
Sal I (S)
Pst I (P)
Kpn I (K)
Sst I (T)
Xba I (X)
Xho I (O)

1988) which contains four deletions useful for physically locating the *vg* gene within the cloned region. Df(2R)vg^B deletes the *vg* locus, two centromere-proximal lethal complementation groups, and several polytene chromosome bands distal to *vg*. *In situ* hybridization analyses show that none of the recombinant *vg* region clones hybridized to the deficiency-bearing chromosome of Df(2R)vg^B/vg^+ heterozygotes (Figure II-5), indicating that the entire cloned region is within the limits of this deficiency. Df(2R)vg^{136} deletes *vg* and several distal loci, but it complements the two proximal complementation groups that are missing in Df(2R)vg^B. Genomic Southern analyses of Df(2R)vg^{136} DNA (data not shown) indicate that the centromere proximal endpoint of the deficiency is at +17 on the restriction map (Figure II-1) but in addition there is a small inversion extending from +5 to +17. A genomic library of Df(2R)vg^{136} was constructed and a recombinant clone which spanned the breakpoint was obtained. This clone was restriction mapped and Southern blots of various restriction digests were probed with *vg* region sequences. The restriction map and the vg region homology were consistent with the above interpretation of genomic Southern data that a 12 kb inversion is situated next to the proximal deletion breakpoint in Df(2R)vg^{136} flies (data not shown). Df(2R)vg^{56} is a small deletion which removes several lethal complementation groups distal to *vg* but which almost completely complements *vg*. Since this deficiency gives only a very weak *vg* phenotype (i.e., only slight nicking when heterozygous with vg^{BG}), it likely does not delete DNA essential to *vg* function. Genomic Southern analyses indicate that the

Figure II-5: *In situ* localization of a *vg* region clone within a *vg* region deficiency. Tritium labelled λ 120 (From Figure II-4) was hybridized to Df(2R)vg^B/SM5, Cy polytene chromosomes. Some cytological landmarks (including Df(2R)vg^B) are identified by number. Exposure time was one week. Hybridization to only the balancer chromosome of vg^B/SM5, Cy was also seen utilizing λ 64 and λ OR^R #2 (Figure II-4) as the labelled probe (data not shown).

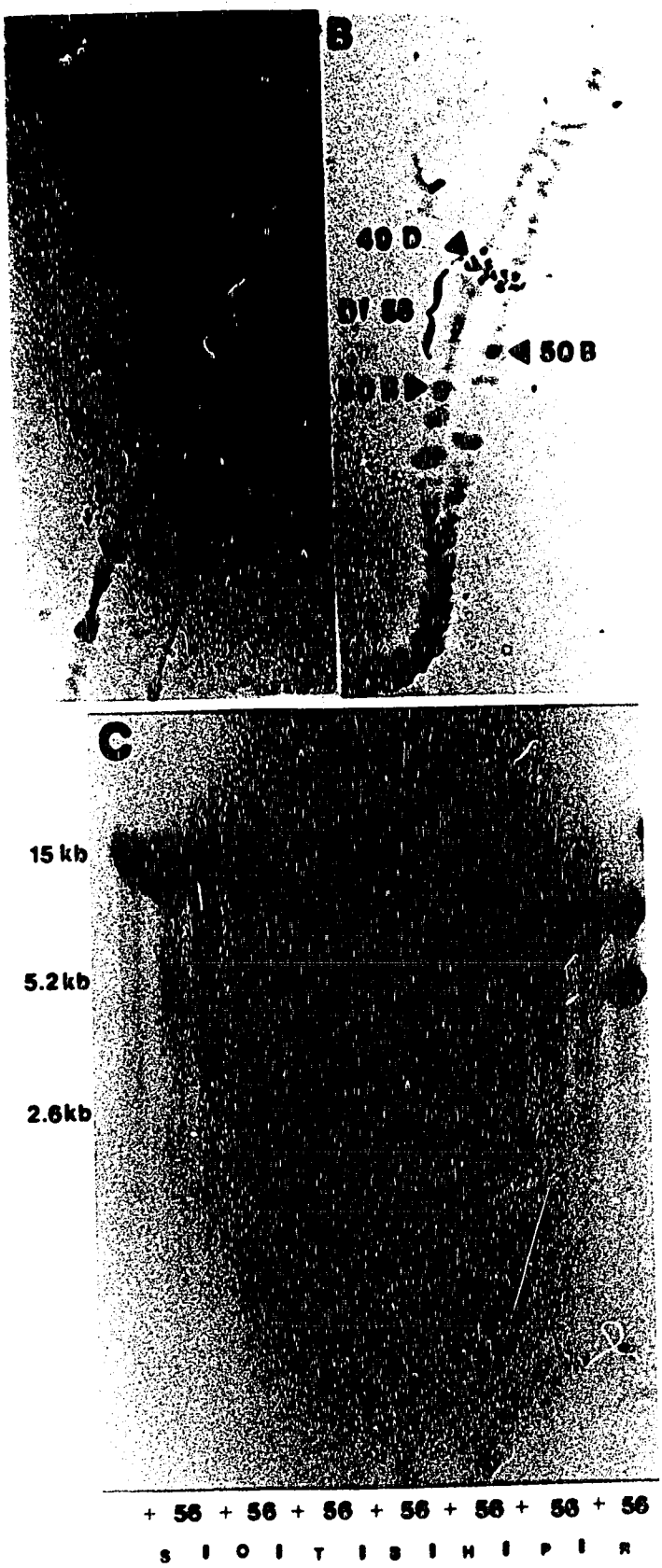
Df

deficiency breakpoint is at +19 on the restriction map (Figure II-4). The aberrant Sal1 fragment detected (Figure II-6C) was cloned and restriction mapped, and gels containing digested DNA were blotted and probed with *vg* region sequences. The restriction map was consistent with the genomic Southern predictions, in that the Southern analyses of the cloned breakpoint showed *vg* region DNA up to +19 linked to normally noncontiguous distal DNA (data not shown). When this breakpoint clone was radiolabeled and hybridized to salivary gland chromosomes from Df(2R)vg^{56} flies, the results show, as expected, that the breakpoint clone also contains DNA from the distal end of the deficiency (Figures 2-6A and 2-6B). This analysis is important since it places a centromere distal limit on the *vg* locus (at about +19), while the Df(2R)vg^{136} analysis shows that sequences essential to *vg* function are encoded within the 14 kb of DNA proximal to the Df(2R)vg^{56} breakpoint. *Su(z)2*^{5} is a small deficiency deleting polytene bands distal to *vg* and gives an intermediate *vg* phenotype (Ting Wu, pers. comm.; and this study). This indicates that while it has a greater effect on *vg* function than Df(2R)vg^{56}, it does not act as a null allele and thus retains some *vg* function. Southern analysis of this allele shows that it breaks at approximately +16 (data not shown). This is consistent with the Df(2R)vg^{56} results which indicate that the centromere distal end of the *vg* gene is proximal to +19 on the restriction map (Figure II-4).

ii) by mutant analysis:

An intragenic recombination map between 3 *vg* alleles (vg^{BG},

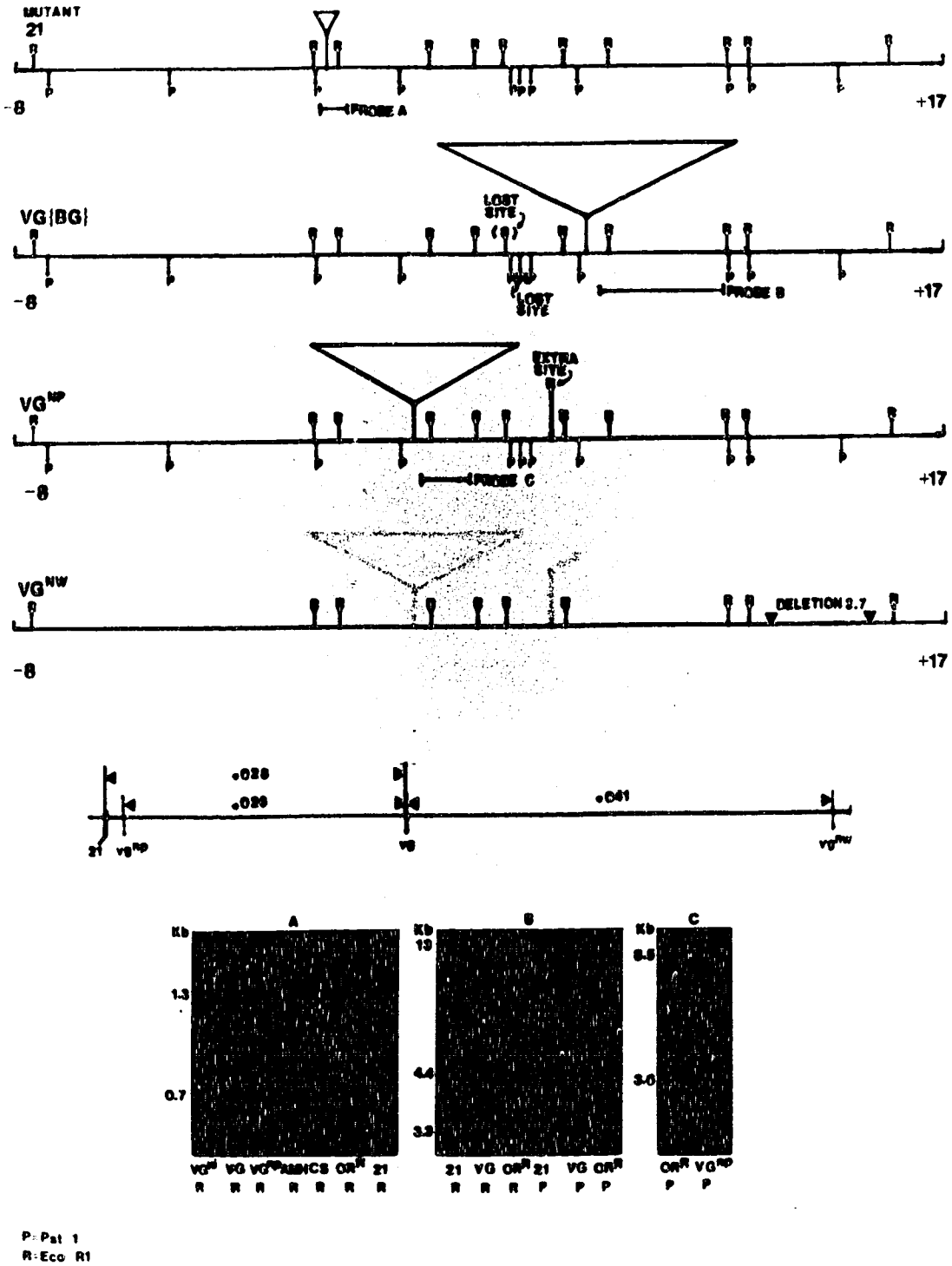
Figure II-6: Demonstration of the position of the Df(2R)vg^{56} proximal breakpoint. Panels A and B demonstrate *in situ* hybridizations to Df(2R) vg^{56}/SM5,Cy polytene chromosomes; by a recombinant clone spanning the Df (2R) vg^{56} proximal breakpoint in Panel A and by λ OR^{R}-2 (Figure II-4) in Panel B. The weak hybridization to the *vg* region in panel A is expected since the breakpoint clone is not entirely composed of non-vg region DNA (11 kb/12 kb). Exposure time was three days. Panel C portrays a genomic Southern hybridization to restriction digests of Df(2R)vg^{56} DNA (56) or the Canton-S parental strain (+). Restriction enzyme designations are as in Figure II-4. The probe used is a Sal1/Xho1 DNA fragment from +18 to +19 (Figure II-4). Subsequently, the blotted filter was stripped and reprobed with a Sst1/Xho1 DNA fragment from +21 to +23 (Figure II-4) and only the DNA bands from the balancer chromosome hybridized (results not shown). The arrows indicate the novel restriction fragments created by the deficiency.


B
40 D
50 B
C
15 kb
5.2 kb
2.6 kb
+ 56 + 56 + 56 + 56 + 56 + 56 + 56
S I O I T I B I H I P I R

vg^{np} and vg^{nw}) has been constructed (Carlson et al. 1980), and is shown in Figure II-7. We obtained genetic recombinants between vg^{21} and vg^{BG} to place vg^{21} on the genetic fine structure map as well. The vg^{21} allele maps centromere-proximal to vg^{BG}, very close to vg^{np} (Figure II-7). Since the number of recombinants recovered between the alleles (Carlson et al, 1980; this study) was low, the genetic distances are only approximate. However, proximal-distal orientation of the alleles is accurate. The molecular mapping of the four *vg* alleles revealed the nature of their respective lesions within the cloned region. The vg^{21} alteration as described above is a small insertion of about 650 bp of P-element sequences into the locus. The vg^{BG} and vg^{np} alleles were characterized by genomic Southern analyses for detection of any alterations. Two restriction site polymorphisms were detected, but since these are also seen in a revertant of vg^{BG} (ie., vg^{nt}) they are unlikely to be the lesions responsible for vg^{BG}. An 8 kb insertion which mapped to +8 on the restriction map was observed in all isolates of vg^{BG} (Figure II-7). The genetic recombinants between vg^{21} and vg^{BG} have been shown to have a molecular cross over between the respective inserts. The recombinants recovered were vg^{+} and lacked both inserts (data not shown). Since this insert was altered in a revertant of vg^{BG} (see below), it seems likely that this 8 kb insertion is the causative lesion for vg^{BG}. The vg^{np} allele was found to have an insert of 5 kb located at +3 on the restriction map (Figure II-7). The only other alteration detected is a restriction site polymorphism which has also been noted with other *vg* alleles, so it appears likely that the insertion

Figure II-7: Concordance of some *vg* alleles from the molecular map with the genetic fine structure map. The molecular lesions associated with each allele, and their relative positions, are indicated in the respective lines for each allele. Southern analyses demonstrating three of these lesions are shown in the panels at the bottom. The probes used for the Southerns are EcoRI fragments indicated as A, B, and C on the respective molecular maps above. The arrows indicate novel fragments generated by the respective *vg* alleles. The abbreviations for the various restriction enzymes and alleles are the same as in Figure II-8. A genetic map showing recombination distances between the alleles is also shown (Carlson, et al., 1980; and this study). The genetic ordering of these alleles and the recombination distances between them is in accordance with molecular locations of the respective lesions.

SOUTHERN ANALYSIS OF VG(BG) AND VG-NIPPED



P: Pst 1
R: Eco R1

is the vg^{np} lesion. Southern analyses of DNA from vg^{nw} flies indicate that a deletion of about 3 kb is present. Subsequent cloning and restriction analyses localized it between +14 and +17 on the restriction map (Figure II-7). This was the only detectable genomic change in vg^{nw} flies within the cloned region. This evidence, plus similar deletions in several other lethal *vg* alleles (results below), indicate that the 3 kb deletion is likely the mutant lesion for vg^{nw}.

As is demonstrated in Figure II-7, the positioning of the above *vg* alleles on the molecular map of the cloned region is consistent with both genetic data (recombination distances and proximal/distal orientation) and the deficiency analyses, which define the region essential for vg^{+} gene function as the same region where the above mutants are distributed. This provides strong evidence that the molecular lesions that are mapped are the lesions responsible for the respective mutant effects. The distances between the alleles in kb DNA/recombination unit are similar to those estimated previously for other cloned *Drosophila* genes (Bender et al., 1983b; Cote et al., 1986). In the present study, both deficiency and mutant recombination analyses served to orient the cloned region (i.e., proximal-distal) and to define a region of about 19 kb where *vg* mutants map. The centromere proximal limits of the locus have not yet been rigorously determined, although the identification of *vg* nonspecific transcripts (see below) provide strong circumstantial evidence for placing this limit just proximal to coordinate 0.

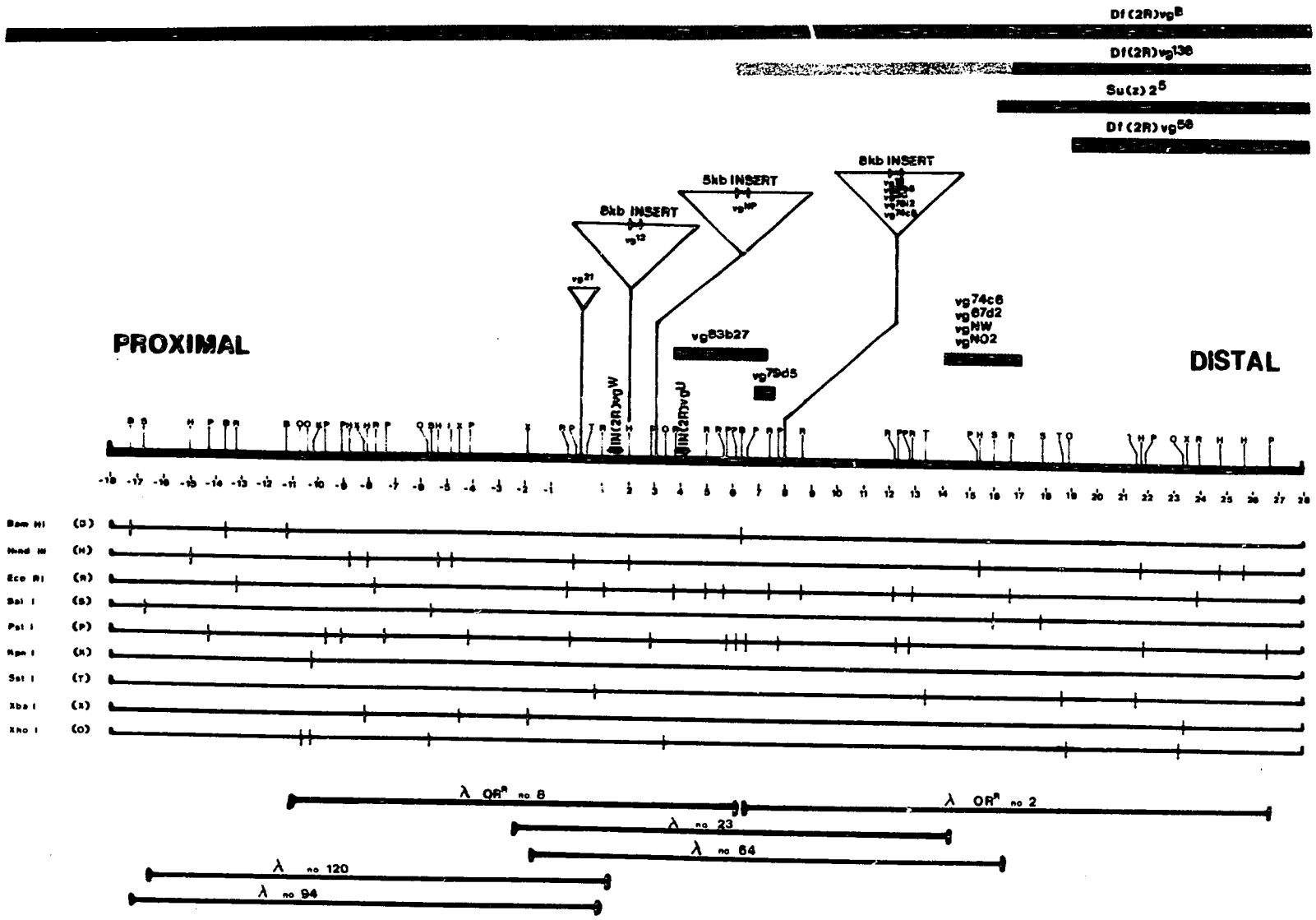
After the initial analyses above, which defined the approximate

limits within which *vg* mutants map, several other recessive viable and recessive lethal alleles were positioned on the map by genomic Southern analyses to detect the molecular lesions associated with each mutant. The lesions found are summarized in Figure II-8. The alterations indicated are the only lesions seen in the entire 46 kb cloned region. The results show that all recessive and lethal *vg* alleles (other than large deficiencies extending into the region) show lesions localized between coordinates 0 and +17. In all cases the alterations detected were insertions or deletions of DNA, except vg^{168} for which no alteration was detected. Small deletions or insertions (<200 bp) would not have been detected in these analyses, so it remains possible that smaller alterations may be associated with vg^{168} or any of the other *vg* alleles analyzed.

Analysis of dominant and complex *vg* mutants:

Two *vg* alleles behave as dominants (i.e., the entire wing margin is gone when one dose of the mutant allele is present) under all known growth regimens and both of these mutants are associated with cytologically detectable inversions. In both cases one end of the inversion is close to the cytological location of *vg*, so it is likely the inversions disrupt normal function and cause the dominant *vg* phenotype. Figure II-9A and II-9B show *in situ* hybridizations of two different *vg* region EMBL3 λ clones to the salivary gland chromosomes of flies heterozygous for the In(2R)vg^{U} inversion (henceforth designated

Figure II-8: Molecular map of the *vg* region derived from OR^R with the physical locations of analyzed *vg* alleles added. The EMBL3 λ clones derived from the *vg* region are shown at the bottom with restriction enzyme maps above. Restriction fragments < 200 bp are not shown. All of the restriction maps, except Kpn1 and Xba1, were confirmed by genomic Southern hybridizations to OR^R and Canton S DNA and showed no restriction polymorphisms between the two strains. The positions of various *vg* alleles are shown above the composite restriction map, while the positions of the chromosome deficiencies analyzed are shown at the top as black bars. The lightly shaded portion of Df(2R)vg^{136} designates inverted DNA. The 0 coordinate is arbitrarily assigned to the insert position of vg^{21}, which was used to clone the region. Negative values proceed in the centromere proximal direction while positive values proceed distally.


Df(2R)vg^B
Df(2R)vg^136
Su(z)2^5
Df(2R)vg^58
8kb INSERT
5kb INSERT
8kb INSERT
vg^12
vg^MP
vg^21
vg74c6
vg67d2
vgNW
vgNO2
vg83b27
vg79d5
PROXIMAL
DISTAL
Bam HI (D)
Hind III (H)
Eco RI (R)
Sal I (S)
Pst I (P)
Kpn I (K)
Sst I (T)
Xba I (X)
Xho I (O)
λ OR^R no 8
λ OR^R no 2
λ no 23
λ no 64
λ no 120
λ no 94

as vg^U). Since λ120 hybridizes only to the proximal end of the inversion, while λ64 hybridizes to both ends, the proximal end of the inversion must be within the λ64 clone. Southern analysis of DNA from this region localized the breakpoint to +4 on the restriction map (data not shown). Figure II-9C shows *in situ* hybridization of lambda 64 to both ends of the In(2R)vg^W inversion (henceforth designated as vg^W). *In situ* hybridization of a more distal clone (OR^R2) showed hybridization only to the distal end of the inversion (data not shown) localizing the breakpoint to within λ64. Genomic Southern analysis located the breakpoint at about +2 on the restriction map, and the proximal breakpoint was cloned. Figure II-9D shows *in situ* hybridization of this breakpoint clone to wild type salivary bands 48A and 49D providing confirmation that the breakpoint was cloned. Genomic Southern analyses of DNA from flies heterozygous for vg^U and vg^W detected no other alterations within the *vg* region, although as mentioned previously alterations of <200 bp likely would not have been detected. The map positions determined for the vg^U and vg^W breakpoints within the *vg* region are recorded on Figure II-8.

At least one mutant of *vg* is known which displays a complex complementation pattern (Alexandrov and Alexandrova, 1988; our unpublished observations). This mutant (vg^{83b27}) is homozygous viable, but with an extreme *vg* phenotype. However, when heterozygous with several *vg* alleles, the phenotype is wild type. Thus, vg^{83b27}

Figure II-9: *In situ* localization of vg^U and vg^W breakpoints in the *vg* region. Salivary gland chromosomes from vg^U/Balancer (Table II-1) larvae were probed with [^{3}H] labeled λ120 (A) or λOR^R#2 (B) (see Figure II-4 for origin of two probes used). Panel (C) depicts a λ64 probe hybridized to vg^W/Balancer (Table II-1) salivary gland chromosomes. Panel (D) shows hybridization of a [^{3}H] labeled λ clone (which spans the vg^W proximal breakpoint) to OR^R. Certain landmark polytene chromosome bands are indicated on the figures.


A
40 D
In
50Cl
B
R

defines a second *vg* complementation unit. Southern hybridization analysis (Figure II-10) of DNA from the *vg* region of vg^{83b27} flies detected only a 4 kb deletion extending from +4 to +8 on the restriction map (Figure II-8).

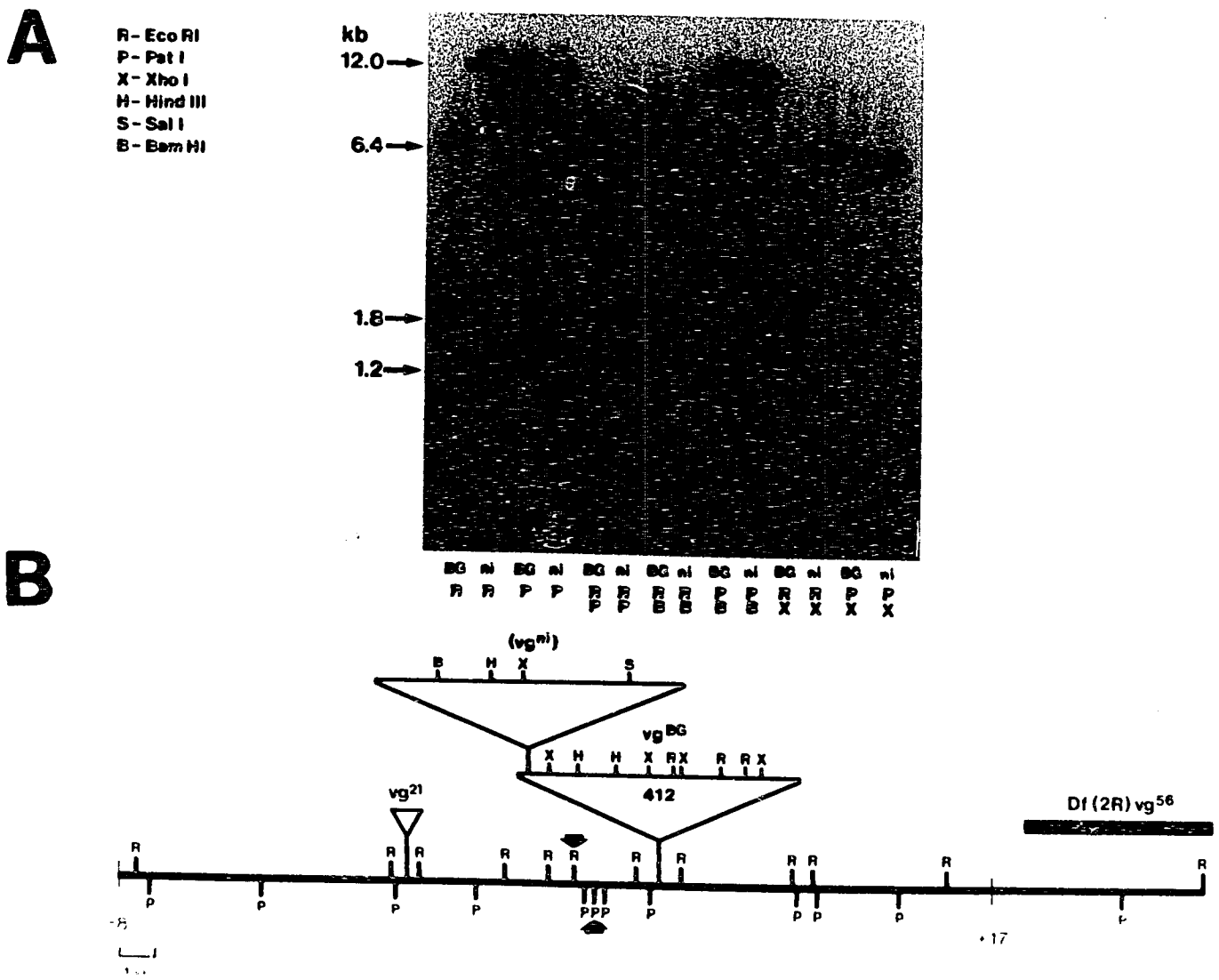
Analysis of vg^{ni}:

The vg^{ni} mutation is a nearly complete phenotypic revertant of vg^{BG} (Lindsley and Grell, 1968). Genomic Southern analyses (Figure II-11) indicate that this revertant has a further insertion into the proximal end of the vg^{BG} insert. A genomic recombinant DNA library was made from the vg^{ni} strain, and 3 overlapping clones were isolated which spanned the *vg* region from coordinates 0 to +15 (Figure II-8). This includes the vg^{ni} alteration. Restriction mapping suggested that vg^{ni} is an 8 kb insert into the vg^{BG} insert. The restriction map of the vg^{BG} insert matches that of the 412 mobile element (Shephard and Finnegan, 1984). To confirm this, Southern blots of restriction digests of the vg^{ni} λ clones were probed with a radiolabeled 412 element. This probe hybridized strongly to the vg^{BG} insert which in DNA from vg^{ni} flies was separated into two segments; a strong distal signal and a weak signal proximal to the actual vg^{ni} insert. This confirms that the vg^{BG} insert is indeed a 412 element, and indicates that the vg^{ni} insert is within the 412 element (Figure II-11). A restriction fragment entirely within the vg^{ni} element was hybridized to OR^R salivary gland chromosomes. The results indicated that the vg^{ni} insert is moderately repetitive (data not shown) with greater than 50 sites hybridizing to the

Figure II-10: Definition of the limits of the *vg*^{83b27} deletion. The position of the deletion is indicated above the restriction map and the five probes used to map the deletion are also shown. Southern blots of EcoR1 and Pst1 digested DNA from Canton-S wild type (CS) or *vg*^{83b27} flies hybridized with each of the probes is shown below. Aberrant bands are recognized with probes 1 and 5, which define the limits of the deletion. Probes 2, 3, and 4 recognize no DNA in the *vg*^{83b27} lane, indicating that this DNA is absent in the mutant.

PROBE
vg21
vgBG
83b27
Df(2R)vg56
R
P
1
2
3
4
5
83b27
CS
Kb
3.0
2.4
2.1
1.2
0.74
0.6
0.64

Figure II-11: Molecular analysis of the vg^{ni} revertant of vg^{BG}. A. Southern blots of restriction digests of DNA from vg^{BG}(BG) and vg^{ni}(ni) were probed with a 1.4 kb EcoR1 fragment from +8 (Figure II-4). Arrows indicate aberrant restriction fragments seen in the revertant, showing further insertion into this region. B. Composite restriction map (omitting Pst1 sites) from three overlapping λ clones derived from vg^{ni}. The inserts are oriented in the manner indicated from the analysis described in the text. The arrows on the restriction map identify polymorphisms common to vg^{BG} and vg^{ni}.

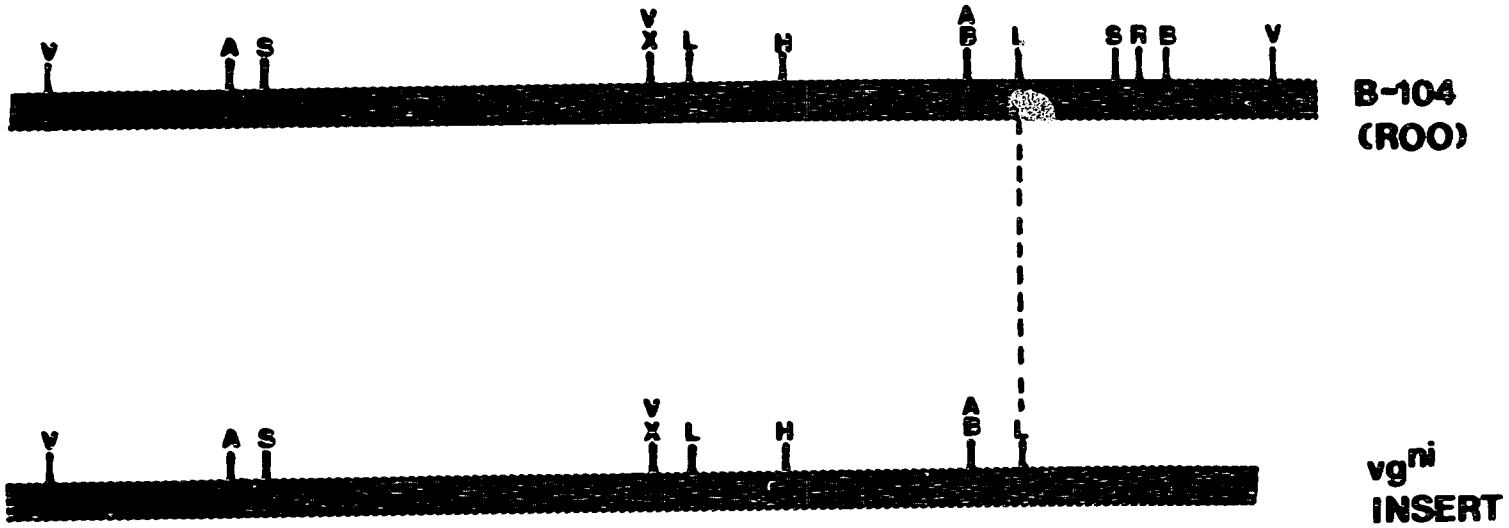

A
R - Eco RI
P - Pst I
X - Xho I
H - Hind III
S - Sal I
B - Bam HI
kb
12.0
6.4
1.8
1.2
BG ni BG ni BG ni BG ni BG ni BG ni BG ni
R R P P R P R B P B R X P X
B
(vg^ni)
B H X S
vg^BG
X H H X RX R R X
412
vg^21
Df (2R) vg^56
R P
17

probe. A more complete restriction map (Figure II-12) of the vg^{ni} insert revealed an almost perfect match with the roo (Scherer et al., 1982) element. The only difference is a small deletion of roo sequences at the proximal end of the vg^{ni} element. Thus, it appears that vg^{ni} is an insertion of 8 kb of a roo element into the proximal end of the 412 element insert of vg^{BG}.

Centromere proximal limits by identifying non-*vg* transcripts:

Transcription analysis of the cloned region identified several relatively abundant transcripts, centromere proximal to coordinate 0 (Figure II-8), which are present throughout ontogeny (Figure II-13B). However, none were identified in the expected region from 0 to +19 which our previous mutant analyses identified as essential to vg^{+} function. Utilizing embryonic cDNA clone banks (Poole et al, 1985), cDNAs were subsequently identified and mapped in this region (Figure II-13 A, C). Three groups of cDNA's were isolated; of which one was probe A (see Figure 13A) specific (cDNA1), one was probe B specific (cDNA2) and the third hybridized to probes B and D (cDNA3). Hybridization of cDNA1 and cDNA2 to first instar larval RNA is shown in Figure II-13C. This demonstrates that the cDNA's do not crosshybridize and each recognizes a specific transcript from the region. The smaller transcript (1.2 kb) recognized by probe B (see Figure II-13B is specifically detected by cDNA3 (data not shown). Thus, three separate transcription units are present in the -1 to -11 interval. The cDNA1 and cDNA2 (most *vg*-proximal) transcription units were analyzed to see if

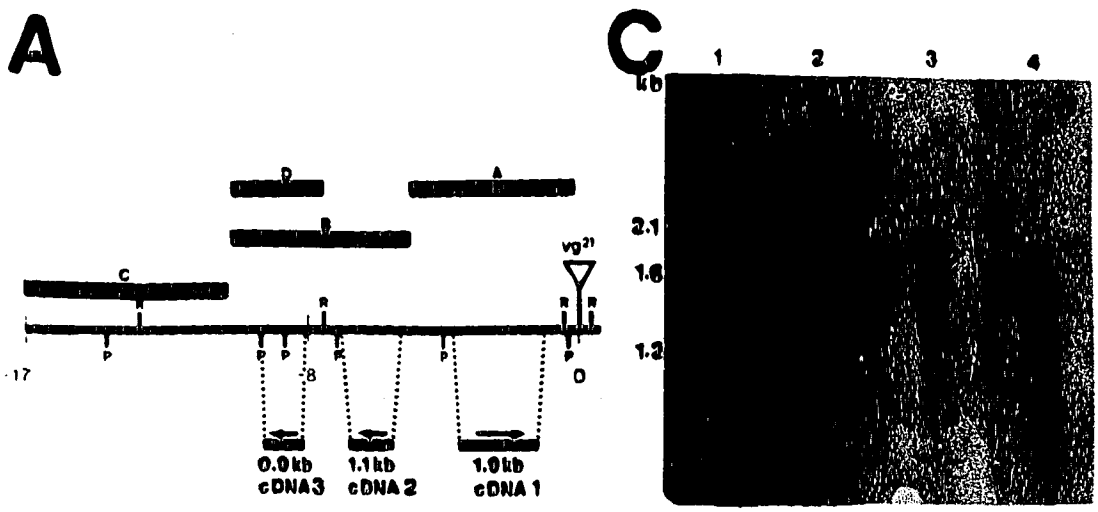
Figure II-12: Restriction map comparisons of the roo (B104) transposon and the vg^{ni} insert. The roo map is drawn from Scherer et al (1982). The vg^{ni} map matches that of roo except for the 3' end which may have undergone a deletion in the vg^{ni} insert.

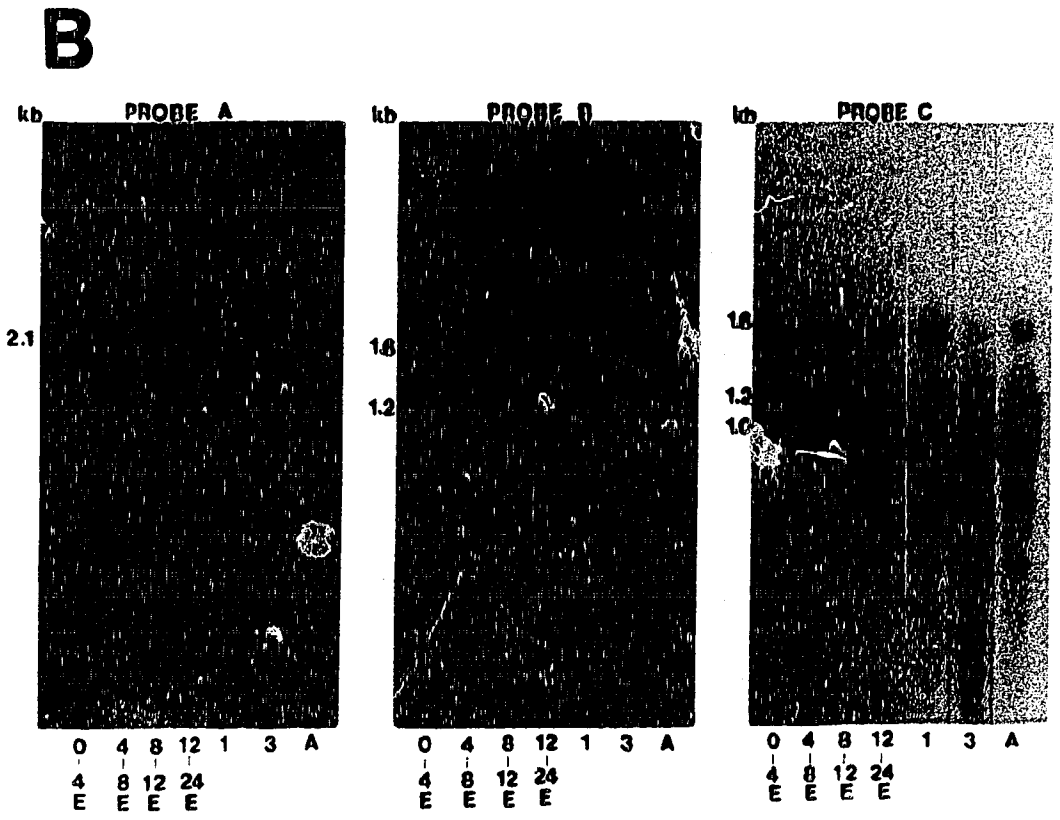


1Kb

V=Ava I
A=Xba 1
S=Sal 1
X=Xho 1
L=Bgl II
H=Hind III
B=Bam HI

Figure II-13: Analysis of transcription units proximal to *vg*. A. The proximal *vg* region map is shown with probes A-D indicated above. The locations of the three cDNA classes are given below. cDNA1 is probe A specific, cDNA2 is probe B specific and cDNA3 is probe B and D specific. cDNA localization was established by hybridizing the longest cDNA inserts of each cDNA class to Southerns of various restriction digests of EMBL3 clones from the *vg* region. The direction of transcription was determined by probing Northerns with strand-specific RNA probes. B. Developmental Northerns were probed with the indicated oligolabeled probes. The RNA size markers were Drosophila rRNA and mouse B-globin mRNA. The multiple transcripts recognized by probe C have not been analyzed further. Stages of development used were 0-4 hr embryonic (0-4E), 4-8 hr embryonic (4-8E), 8-12 hr embryonic (8-12E), 12-24 hr embryonic (12-24E), first instar larvae (1L), third instar larvae (3L) and adult (A) C. Hybridization of labeled cDNAs to Northern blots of first instar mRNA. The oligolabeled probes are cDNA1 (lane 1), cDNA2 (lane 2), probe A (lane 3) and probe B (lane 4).


A
D
A
B
C
vg²¹
R
P
-17
-8
0
0.0kb
cDNA3
1.1kb
cDNA 2
1.0kb
cDNA 1
C
kb
1
2
3
4
2.1
1.6
1.2


B
kb
PROBE A
2.1
0
4
8
12
1
3
A
4E
8E
12E
24E
kb
PROBE D
1.6
1.2
kb
PROBE C
1.6
1.2
1.0

their expression is altered in *vg* mutants (results not shown). No alteration of expression, in levels or size of transcripts, was seen with any of the *vg* mutants examined in first instar, third instar, or adult stages. Thus, we conclude that these are not likely to be *vg* transcripts. However, it is possible that the transcripts are spatially altered in *vg* mutants, similar to that seen with engrailed breakpoint mutations (Drees et al., 1987). If these transcription units are, indeed, unrelated to *vg* function they provide strong circumstantial evidence for the centromere-proximal limits of vg^+ function close to coordinate 0 within the cloned region. If this is the case, functional *vg* transcripts within the 0 to +19 interval (Figure II-8) must be expressed at very low levels and/or in a small number of cells. Since examples of genes within genes and overlapping transcription units have been documented (Henikoff et al., 1986: Spencer et al., 1986), we can not yet exclude the possibility that sequences important to *vg* function are proximal to one or more of these adjacent transcription units. A detailed analysis of *vg*-specific transcription is the subject of Chapter V.

Discussion

Using P-element transposon tagging, the vestigial (*vg*) region of the *Drosophila melanogaster* genome was isolated. Molecular analyses of *vg* deficiencies, together with the molecular mapping of several *vg* mutations and cDNA analyses, established that the cloned 46 kb interval contains the vg^+ gene and defined a smaller region, of approximately 19 kb, required for vg^+ function.

Vestigial mutants cause wing margin loss and haltere reduction due to cell death in the respective imaginal discs. Previous genetic analyses indicate that four general classes of *vg* mutations exist: recessive viable alleles, recessive lethal alleles, completely dominant alleles, and complex alleles. Recessive viable *vg* alleles show variable elimination of wing margin structures when homozygous. Weak recessive viable alleles (e.g., vg^{np}) may exhibit only slight nicking of the wing margins, while extreme recessive viable alleles (e.g., vg^{BG}) have the entire wing margin eliminated. Combinations of strong and weak alleles yield intermediate wing phenotypes, indicating that the mutants in this class are probably hypomorphic mutants. Several of these recessive viable *vg* alleles were characterized and located on the molecular map of the *vg* locus in this study. These include: vg^{21}, vg^{np}, vg^{79d5}, vg^{18}, vg^{51h25}, vg^{BG}, vg^{76i2}, and vg^{74c5}. Of these, the latter five appear phenotypically and molecularly indistinguishable. Since these five mutants all show the same two restriction site

polymorphisms and all have an 8 kb DNA insert at +8 on the restriction map, it is likely that they are merely different isolates of only one independent event. There seems to be no correlation between the severity of the *vg* mutant phenotype and whether the lesion is due to an insertion or a deletion.

Molecular analysis of vg^{ni} indicates that this revertant results from a roo insertion into the 412 insert of vg^{BG}. Presumably the roo element disrupts some function of the vg^{BG} 412 insert which is causing the vg phenotype. It is unknown what the disrupted function is, but likely possibilities are transcription, polyadenylation or splicing problems generated by the 412 inserts. Sequence analyses to determine the exact site of the roo element insertion into the 412 element may indicate which 412 function is disrupted in the vg^{ni} revertant. This type of revertant has been reported (Campunzano et al., 1986; Collins and Rubin, 1982; Levis et al., 1982; Mizrokhi et al., 1985; Mount et al, 1988), but in the previous examples the revertants were either small inserts or caused only slight changes in phenotype. The vg^{ni} revertant demonstrates that parts of the *vg* region are relatively insensitive to insertions *per se*, since the vg^{ni} revertant of vg^{BG} is nearly wild type for *vg* function yet now has 16 kb of DNA inserted into the locus. An attractive hypothesis is that most recessive viable *vg* alleles are intronic mutations which cause a mutant phenotype by reducing *vg* expression but which do not alter the gene product itself. This would account for the hypomorphic nature of recessive mutants and is consistent with the vg^{ni} analysis.

Several recessive lethal *vg* alleles, that are not associated with cytologically visible deficiencies, were examined in this study. These were: vg^{12}, vg^{168}, vg^{nw}, vg^{no2}, vg^{67d2}, and vg^{74c6}. The latter four have molecularly indistinguishable deletion lesions within the *vg* region. It is difficult to know if they represent only one independent event or if they exemplify a hot spot for deletions. The three distinct kinds of molecular lesions (i.e. in vg^{nw}, vg^{168}, and vg^{12}) are respectively a deletion, a possible point mutant, and an insertion. Comparison of these lesions to the types of alterations identified among the recessive viable mutations shows no inherent bias towards the type of mutant (i.e., insertion or deletion) or position of the mutant in the locus for producing a lethal or viable allele. It will be of interest to see if future functional analyses (ie. cDNA mapping) indicate that the lesions associated with the recessive lethal alleles affect exons, while the recessive viable alleles are associated with lesions which either affect introns, or only have minor affects on exons.

Almost all spontaneous or induced *vg* alleles fall into the recessive viable or recessive lethal classes described above. However, a few behave as completely dominant or complex mutants. The dominant *vg* alleles, vg^{U} and vg^{W} are recessive lethals, but also cause a strong *vg* phenotype when heterozygous with vg^{+} alleles. The *vg* phenotype of the dominant alleles is stronger when heterozygous with other *vg* alleles, indicating an antimorphic nature to the dominant alleles, at least with respect to the wing phenotype (see Chapter I). Thus, since vg^{+} is essentially haplo-sufficient, (i.e. heterozygous deficiencies of the region

give only slight nicking of the wings) dominant *vg* alleles may be envisioned as somehow blocking the expression of a wild type allele in heterozygous flies. When these mutants were analyzed molecularly, both were found to have an inversion breakpoint splitting the locus in two. Since these are the only mutants analyzed which split the locus, it is likely that this disruption generates an aberrant *vg* product which mediates the dominance. This could be by producing an aberrant 5' gene product or an aberrant 3' gene product or both. Alternatively, it is possible that the inversions themselves cause the dominant phenotype, by disrupting normal chromosome synapsis in a transvection-like process (Lewis, 1954). The generation and analysis of phenotypic revertants of the dominant alleles could help resolve this question. Another intriguing question, if transvection can be discounted, is how the aberrant product mediates a dominant phenotype. It may be, perhaps, by a negative complementation-like process or by an unusual type of gene fusion. Identification and analysis of *vg* RNA transcripts of the gene and cDNA analyses should produce some understanding of this process. Another complication of the dominant alleles is found in vg^W. This allele, in addition to being a *vg* dominant, is also a homeotic dominant causing transformation of dorsal metathorax to mesothorax and posterior wing duplication (Bownes and Roberts, 1981a). This homeotic effect could be a *vg* specific effect, or could be due to a breakpoint effect caused by the other end of this particular inversion. Since the proximal end of the vg^W inversion is cytologically inseparable from engrailed (Bownes and Roberts, 1981a, and Figure II-9), this is a

tantalizing possibility. The cloning of the vg^{W} breakpoints has allowed the isolation of DNA from the engrailed region which is disrupted by this inversion and should facilitate the utilization of P-element mediated transformation to attempt to define DNA fragments which give the dominant homeotic effect.

One extant complex *vg* allele, which does not fit any of the three previous classifications, was also analyzed. This mutant, vg^{83b27}, is homozygous viable with an extreme *vg* phenotype. However, in trans with several other *vg*-containing chromosomes, complementation is observed (Alexandrov and Alexandrova, 1988; and our unpublished observations). Thus, vg^{83b27} defines a second *vg* complementation unit. All of the recessive viable *vg* alleles analyzed in this study are completely or almost completely complemented by vg^{83b27}, while the chromosome deletion alleles (i.e., Df(2R)vg^{B} and Df(2R)vg^{136}), the recessive lethal alleles, and the dominant alleles are not complemented. The molecular analysis of vg^{83b27} revealed a 4 kb deletion of DNA from the central region of the locus. Thus, the vg^{83b27} mutant complements with *vg* alleles that map both proximal and distal to the vg^{83b27} lesion. It is unusual for a deletion mutant to complement other alleles, especially since in this case the homozygous deletion is an extreme allele. However, the observation that recessive viable alleles are complemented while recessive lethals are not, supports the notion that the viables are hypomorphic mutants and the lethals are structurally aberrant mutants of the locus. It is possible that the deletion removes one or more exons from the gene and produces a product which in

some way enhances the expression of the allele on the homologue. In this way, hypomorphic alleles would be complemented, while structurally aberrant alleles (i.e., lethals) would not. This may be envisioned as a reverse process to that seen in the case of the dominant alleles. Clearly, further analysis of these two interesting classes of *vg* alleles will yield valuable insight into how the vg^+ gene is normally regulated.

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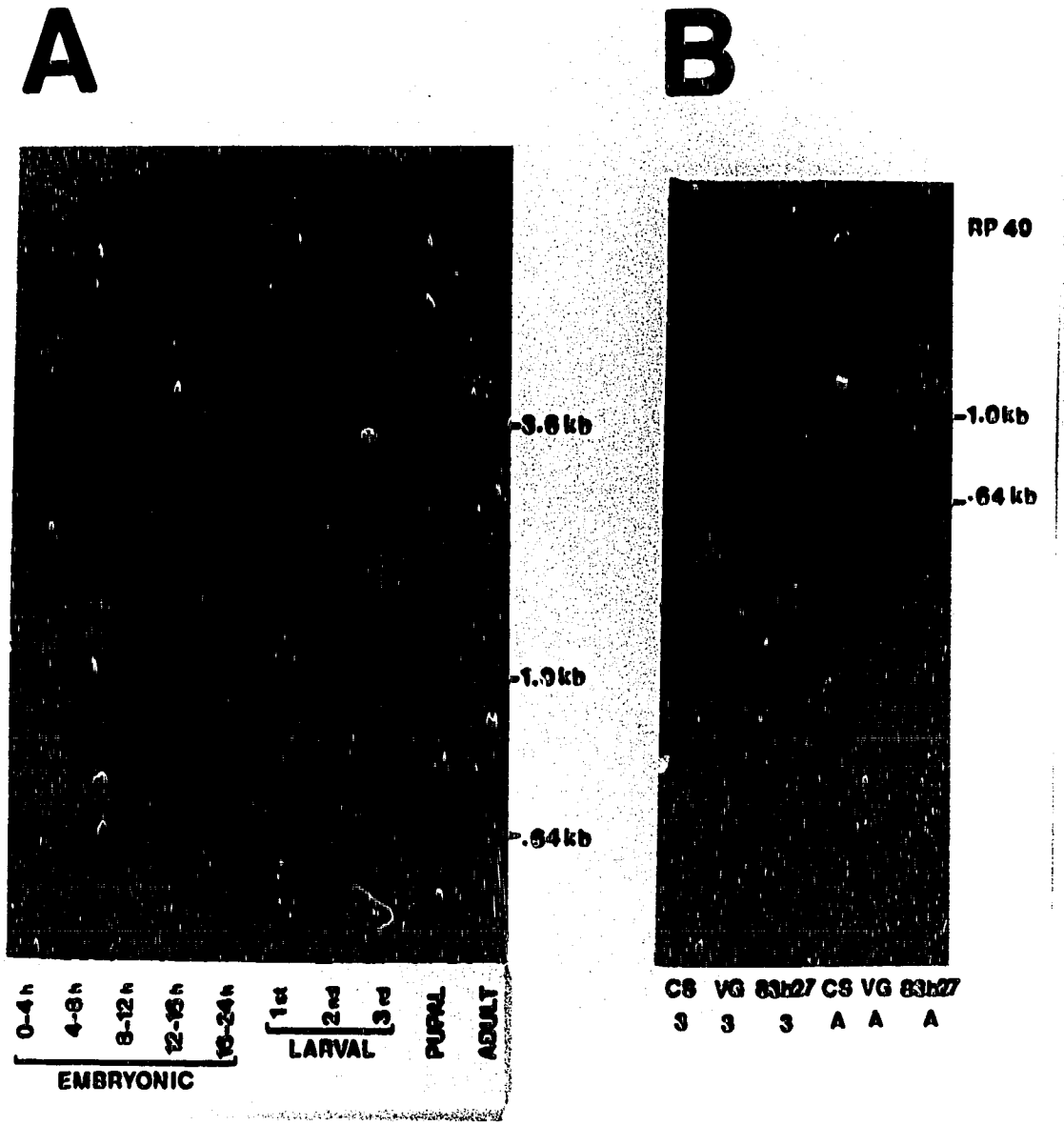
Addendum

Although the preceding paper is similar to the final version of Chapter II (as published, Williams and Bell, 1988) previous unrevised versions contained one important addition. This included identification of adult transcripts, which are *vg* specific and altered in a *vg* allele. The tentative conclusion was that these transcripts may represent a functional *vg* transcript, but that further analysis would be needed to confirm this. However, the reviewers felt the results were too preliminary to publish in EMBO, and required that this section be deleted from the manuscript. Chapters III and IV, which included analysis of these transcripts, had already been accepted. At the time, I was working on isolating cDNA's representing these transcripts, and felt that subsequent publications would include these analyses, which would clarify the analyses presented in Chapters III and IV (following). Unfortunately, experimental results cannot always be predicted, and the functional analysis did not verify this assumption. Thus, for clarity, the deleted adult transcript characterization is included at this point, so that the analysis presented in Chapters III and IV may be fairly evaluated.

These data should be read from the end of the results section in Chapter II. They immediately follow the data which demonstrate that several transcription units are present in the 0 to -17 interval, but these transcripts are not affected by *vg* mutants. No transcripts were

identified in the 0 to +20 interval using oligo-labelled DNA probes generated from subclones from this interval. The 0 to+15 region was then re-examined using RNA probes in a further attempt to identify *vg* transcripts (all methods are as in Chapter II). RNA probes give high signal/noise ratios and may detect transcripts not seen with the less sensitive oligolabeled DNA probes (Melton et al, 1984). A 1.0 kb RNA probe produced from a clone spanning the +6 to +7 area of the restriction map (Figure II-4) hybridized to *in vivo* produced transcripts to indicate the expression profile seen in Figure II-14A. Since RNA probes are highly sensitive, it was possible that the hybridizing transcripts were not from the *vg* region. RNA was extracted from first instar, third instar and adult stages of vg^{BG} and vg^{83b27}. Since vg^{83b27} is a homozygous deletion including the entire region of the 1.0 kb RNA probe, it should serve as a negative control for *vg* region specificity. Figure II-14B shows the results of Northern analyses of these mutants compared to a vg^{+} control. The first instar (data not shown) and third instar transcripts are recognized in the deletion mutant and their size and levels are not altered compared to vg^{BG} and vg^{+}. Thus, these transcripts are probably not *vg* specific and may be analogous to the nonspecific transcripts detected by RNA probes of plasmid sequences (Cavener et al, 1986). However, the adult transcripts are *vg* specific, and do show alterations in vg^{BG}, so they are likely candidates for vg^{+} transcripts.

Figure II-14: Transcripts from the *vg* region. A. A Northern blot of RNA from the indicated developmental stages was probed with an RNA probe produced by an EcoR1/BamH1 fragment from +6 to +7 on the physical map (Figure II-4). Size markers (not shown) were *Drosophila* rRNA and mouse globin mRNA. B. Northern blot of Canton S (CS), vg^{BG} (VG), and vg^{83b27} (83b27) at two developmental stages (third instar, 3; and adult, A) probed with the same probe as in panel A. This Northern was rehybridized with a ribosomal protein probe - RP-49 (O'Connell and Rosbach, 1984) to roughly quantify the amount of mRNA/lane. This serves as an internal control and shows that the differences in *vg* transcription are real rather than merely fluctuations in RNA loaded/lane. The orientation of the RNA probe indicates that *vg* transcription is from centromere distal to centromere proximal (see Figure II-4).


A
B
3.6kb
1.9kb
.64kb
0-4h
4-8h
8-12h
12-16h
16-24h
1st
2nd
3rd
LARVAL
PUPAL
ADULT
EMBRYONIC
RP 49
1.0kb
.64kb
CS VG 83h27 CS VG 83h27
3 3 3 A A A

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Chapter III: Molecular analysis of hybrid dysgenesis induced derivatives of a P-element allele at the *vg* locus[1]

Introduction

Hybrid dysgenesis mediated by P-elements is a powerful tool for studying gene expression in *Drosophila*. It is induced in the progeny of crosses between P-cytotype males and M-cytotype females, but not in the reciprocal cross. P-strains contain multiple copies of chromosomal P-elements while M-strains lack functional P-elements (Rubin et al, 1982). Dysgenic crosses cause mutations due to P-element insertions into genes or imprecise excisions from genes with pre-existing P-element sequences. The functional P-element is a 2.9 kb transposon which encodes a transposase required for transposition (Laski et al, 1986; Rio et al, 1986). Most P-elements in a P-strain are smaller than the 2.9 kb element and are derived from this element by internal deletion (O'Hare and Rubin, 1983). P-elements have 31 bp terminal repeats on each flank which are required for transposition (Karess and Rubin, 1984) and 8 bp of chromosomal DNA is duplicated at the insertion site (O'Hare and Rubin, 1983). The internally deleted P-elements are unable to produce their own transposase, but they are mobilized when supplied with transposase produced by complete

1. A version of this Chapter has been published. Williams, J., S. S. Pappu, and J. B. Bell. 1988. Mol. Cell. Biol. 8: 1489-1497.

P-elements (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Transpositional activity occurs only under dysgenic conditions since P-strains also encode a repressor which prevents transposition in P-cytotype flies. It is unknown what the structure of the repressor is or exactly how it works, but it appears to be encoded by P-element sequences (reviewed in Engels, 1983).

P-element mutagenesis can be used to produce primary or secondary mutants at a locus. Primary mutants are usually insertions of P-element sequences into a gene, and are particularly useful in facilitating the initial cloning of genes. A variety of loci have been cloned in this manner (Bingham et al, 1981; Maine et al, 1985; Searles et al, 1982; Chapter II). Secondary mutants arise when pre-existing P-element alleles are induced to undergo further dysgenic activity (at rates as high as 10^{-2} to 10^{-3}; O'Hare and Rubin, 1983; Tsubota and Schedl, 1986). The secondary mutants are often revertants, but may also be more extreme derivatives of the original allele. They have been extensively studied at the rudimentary (Tsubota and Schedl, 1986), RPII215 (Searles et al, 1986; Voelker et al, 1984), and yellow loci (Chia et al, 1986). Secondary mutants have been shown to be due to either precise or imprecise P-element excisions (Chia et al, 1986; O'Hare and Rubin, 1983; Searles et al, 1986; Tsubota and Schedl, 1986; Voelker et al, 1984), internal deletions within the resident P-element (Daniels et al, 1985; Searles et al, 1986; Tsubota et al, 1985), deletion of DNA adjacent to the resident P-element (Daniels et al, 1985; Tsubota and Schedl, 1986), or inversions with a breakpoint within the P-element

(Engels and Preston, 1981; Tsubota and Schedl, 1986). Thus, P-elements within a locus can be potent mutators. The examples described above all involve P-elements inserted into the promotor region of the respective genes, which appears to be a preferential target among recovered P-element insertions (reviewed in Kelley et al, 1987).

In this study, an analysis of secondary and tertiary P-element induced mutations at the vestigial (*vg*) locus is reported. The *vg* gene is required for normal wing formation. Strong *vg* alleles cause extensive cell death in the third instar wing imaginal discs resulting in complete loss of adult wing margin (Fristrom, 1969). However, weak *vg* alleles cause less severe wing loss and various phenotypic gradations exist between strong and weak *vg* alleles. Thus, secondary *vg* alleles can be selected which are only subtly different from the parent allele. Chapter II described the cloning of the *vg* locus using P-element tagging. The region was partially characterized at the DNA level, and an adult *vg* specific transcript was identified (Chapter II, Addendum). The primary P-element induced mutation was shown to be due to P-element sequences inserted into the 3' region of the gene with respect to this transcript. Here the generation of a series of secondary and tertiary derivatives of this *vg* allele that were induced by further hybrid dysgenesis is reported. These alleles were examined by DNA cloning and DNA sequence analysis. The results provide additional corroborating examples of previously described types of secondary P-element induced mutations and in addition identify an unusual form of secondary event. The results also provide evidence for models of

transcriptional interference as a mechanism by which P-element insertions disrupt target gene expression.

Materials and Methods

Drosophila stocks and culturing: *Drosophila melanogaster* cultures were grown at 24°C, and maintained on a synthetic medium (Nash and Bell, 1968). Allele designations are as in Lindsley and Grell (1968).

Materials: Restriction enzymes and other DNA modifying enzymes were obtained from BRL or Pharmacia and used according to the manufacturer's instructions. All radioisotopes were purchased from New England Nuclear. Oligolabeled probes were labeled with [^{32}P] dCTP (3000 Ci/mmol), RNA probes were labeled with [^{32}P]UTP (3000 Ci/mmol), while DNA sequencing utilized [^{32}P]dATP (800 Ci/mmol). The RP49 clone was a gift from M. Rosbach.

DNA manipulations: Culturing and storage of bacteria or lambda phage, preparation of DNA and plasmid subcloning were performed according to standard methodology (Maniatis et al, 1982). Genomic *Drosophila* DNA for Southern hybridizations and genomic libraries was prepared according to Ish-Horowicz et al. (1979), and repurified by spermine precipitation (Hoopes and McClure, 1981).

Genomic libraries: The *vg$^{21\text{-}3}$* and *vg$^{21\text{-}4\text{-}Rev}$* libraries were constructed in EMBL-3 according to Frischauf et al. (1983). Bam H1 restricted *Drosophila* DNA was electrophoresed in 0.4% agarose gels

and 18-20 kb fragments were retained by electroelution onto dialysis membranes, ethanol precipitated, and resuspended in 5 ul TE buffer. Four ul of vector DNA (1 ug/2 ul) was added, ligated, and packaged as described (Maniatis et al, 1982). The *vg*$^{21-4}$, *vg*$^{21-7}$ and *vg*$^{21-7-Rev}$ libraries were constructed in λGT10. The genomic DNA was digested with EcoR1 and size selected as above on 1% agarose gels. Purification, ligation, and packaging were as indicated above. EMBL-3 libraries were plated on NM-539 and λGT10 libraries were plated on C600 Hfl. Genomic libraries were transfered to biodyne membranes (Pall) and prepared for hybridization by standard methodology.

Southern and Northern hybridizations: All gels for Southern or Northern hybridization analyses were blotted onto Genescreen Plus membranes (Du Pont) using the capillary blot protocol recommended by the manufacturer. RNA was extracted, purified by oligo-dT chromatography, and electrophoresed on 1.5% formaldahyde gels as in Gietz and Hodgetts (1985). For Northern gels, 4 ug poly A$^+$ RNA/lane was used, while for Southern gels 5 ug DNA/lane was used. Size markers for Southern gels were obtained from mixing the digestion products of pπ25.1 treated with a variety of enzymes and calibrated by comparison to the known sequence (O'Hare and Rubin, 1983). Hybridization conditions for all plaque lifts, Southerns, and DNA-probed Northerns were as in Klessig and Berry (1983). Southerns and DNA-probed Northerns were washed according to Genescreen Plus

specifications. DNA probes were made from restriction fragments resolved on low melt agarose gels and oligolabeled by the method of Feinberg and Vogelstein (1983). RNA probes for Northerns were prepared from restriction fragments cloned into Bluescribe (Vector Cloning Systems) using the transcription protocol of Melton et al. (1984), and their methods for hybridization and washing of RNA-probed Northerns were also used. The prehybridization and hybridization temperature was 65°C.

DNA sequencing: All sequencing was performed by the dideoxy method (Sanger et al, 1977) from inserts in M13mp18 and M13mp19. Since all of the P-element mutants analyzed herein had alterations within a 200 bp genomic Sst1/Pst1 fragment (Figure III-6), the sequencing was limited to this region. The Oregon-R sequence was determined by sequencing both strands of the 200 bp Sst1/Pst1 fragment. The *vg*^{21} insert was characterized by sequencing both strands of the Pst1/Hind III, Hind III/Sca1, and Sca1/Sst1 fragments, and single strands of the Pst1/Sst1 fragment to establish overlap at the Hind III and Sca1 sites (See Figure III-6). Both strands of the following subclones (see Figure III-6) were sequenced to map the lesions in the *vg*^{21} derivatives: *vg*$^{21\text{-}4}$, Hind III/Sca1, Sca1/Sst1, Pst1/Hind III; *vg*$^{21\text{-}7}$, Pst1/Hind III, (one strand),Hind III/Sst1; *vg*$^{21\text{-}4\text{-}Rev}$, Sst1/Sca1, Sca1/Pst1; *vg*$^{21\text{-}7\text{-}Rev}$, Pst1/Hind III, (one strand), Hind III/Sal1, Sal1/Sca1; Sca1/Sst1.

Results

Induction of *vg*^{21} derivatives by hybrid dysgenesis:

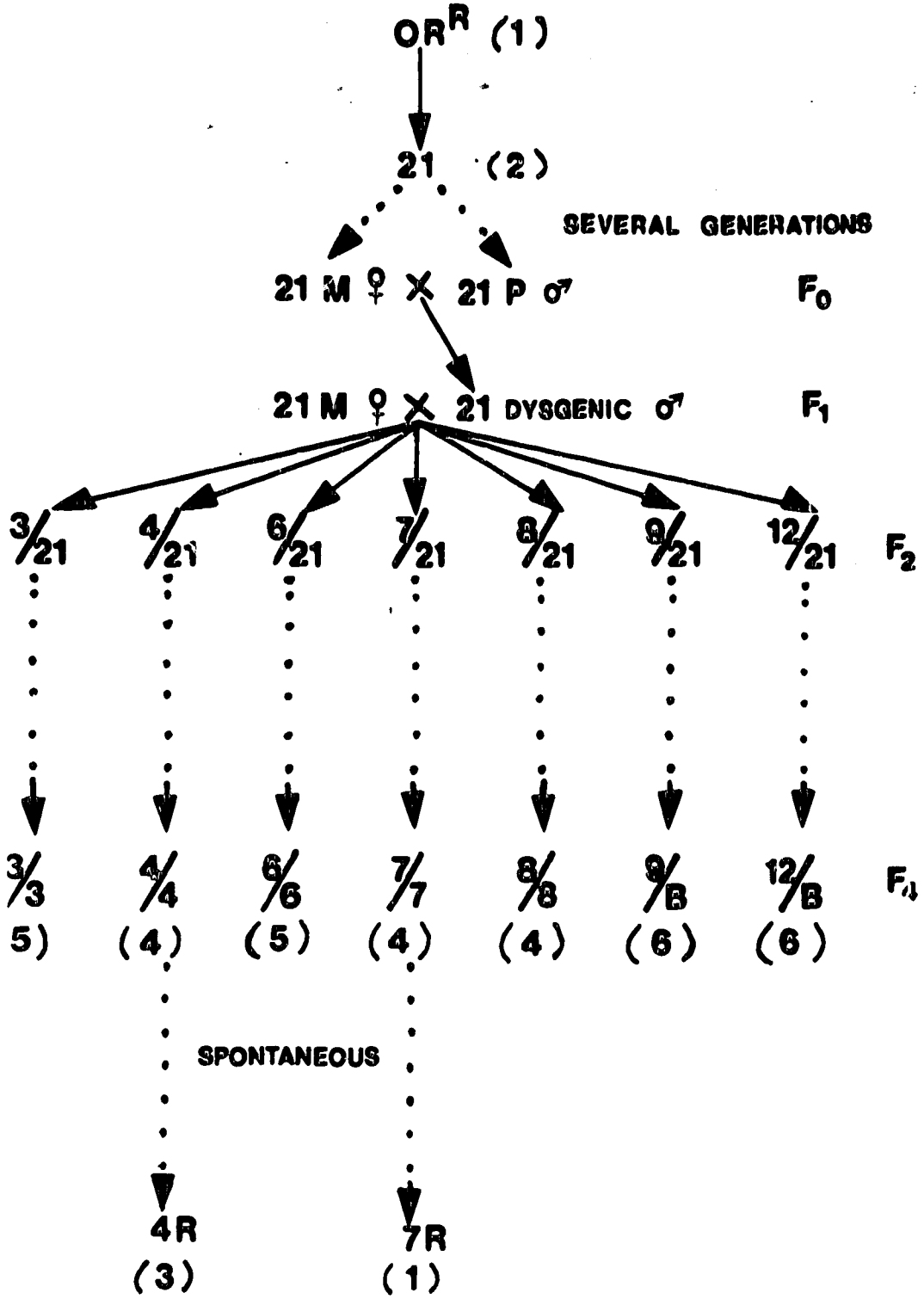
The molecular cloning of the *vg* locus has facilitated a detailed analysis of the DNA lesions associated with several mutants and the identification of an adult *vg* specific transcript (Chapter II and Chapter II Addendum). A map of the *vg* region is shown in Figure III-1 and the alterations associated with three *vg* alleles are indicated. A region of 19 kb (coordinates 0 to +19) has been defined which is required for normal *vg* function. The *vg* locus was cloned using a hybrid dysgenesis induced P-element allele. This *vg* allele (henceforth called *vg*^{21}) was caused by the insertion of an internally deleted P-element into a Oregon-R (OR^{R}) wild type chromosome. The phenotype of *vg*^{21} is cryptic (i.e., homozygous wild-type), but it shows a weak *vg* phenotype when heterozygous with a strong *vg* allele (i.e., *vg*^{BG}).

The cryptic phenotype of *vg*^{21} lends itself to the selection of more extreme hybrid dysgenesis induced derivatives. When initially isolated, the *vg*^{21} stock was backcrossed to an M-cytotype strain (*vg*^{BG}) for several generations to stabilize the allele in an M-cytotype. For the present study, a *vg*^{21} P-cytotype strain was re-established by multiple passages of the *vg*^{21} chromosome through a P-cytotype balancer strain derived from the π2 stock (a strong P-cytotype stock from W. Engels). A dysgenic cross was then performed as illustrated in Figure III-2.

Figure III-1: The *vg* region of *Drosophila melanogaster*. The alterations associated with the three *vg* alleles cited in this study are indicated. The vg^{21} allele has a 650 bp insertion of an internally deleted P-element, while vg^{BG} is an 8 kb insertion of a 412 element. The vg^{83b27} allele is associated with a deletion whose limits are shown by the black bar. The open bar denotes the region defined as essential for *vg* function. The arrow indicates the direction of *vg* adult transcription. The data are from Chapter II and Chapter II Addendum. The solid bar labelled "a" represents the fragment which was used as a probe for the Northern hybridization presented later.

PROXIMAL
-17
vg21
0
vg83b27
a
+6
vgBG
412
+19
DISTAL
+28

Figure III-2: Flow chart of the isolation of dysgenic derivatives of vg^{21}. The vg^{21} allele was induced by hybrid dysgenesis in Oregon-R flies as described previously (Chapter II). The F_2 wing defective mutants ($vg^{21\text{-}m}/vg^{21}$) were made homozygous by backcrossing single flies to vg^{21} (M-cytotype) and self-crossing the $vg^{21\text{-}m}/vg^{21}$ offspring to isolate $vg^{21\text{-}m}/vg^{21\text{-}m}$ progeny. In the case of $vg^{21\text{-}9}$ and $vg^{21\text{-}12}$ the homozygotes were lethal, so stocks of these were established and maintained utilizing a balancer chromosome. Stocks of $vg^{21\text{-}4Rev}$ and $vg^{21\text{-}7Rev}$ were established as described in text. The vg^{21} derivative alleles are designated above as 3 = $vg^{21\text{-}3}$ et cetera; while OR^R = Oregon-R, M = M cytotype, P = P cytotype, and B = Balancer Chromosome. The wing phenotype of each allele is indicated by a number in brackets below or beside the genotypic abbreviations. The numbering system proceeds from weak to strong alleles where 1 is wild type and 6 is a null allele (see Chapter I).


OR^R (1)
21 (2)
SEVERAL GENERATIONS
21 M ♀ × 21 P ♂
F0
21 M ♀ × 21 DYSGENIC ♂
F1
3/21
4/21
6/21
7/21
8/21
9/21
12/21
F2
3/3
4/4
6/6
7/7
8/8
9/B
12/B
F4
5)
(4)
(5)
(4)
(4)
(6)
(6)
SPONTANEOUS
4R
(3)
7R
(1)

Thirty-two *vg* phenotype flies were isolated from 1.5 x 10^4 F_2 flies that were screened. A control cross of the same strains using non-dysgenic conditions yielded no *vg* flies among 1 x 10^4 screened, demonstrating that the 32 *vg* mutants above were induced by hybrid dysgenesis. Very high sterility was observed among these F_2 *vg* mutants as only 12 of them were fertile. Of these, seven that were known to be of independent origin were chosen for further study and stocks of these were established. Preliminary genetic analysis indicated that all seven derivatives of vg^{21} are, indeed, *vg* alleles.

Analysis of two lethal vg^{21} derivatives:

The only two vg^{21} derivatives from the present screen that are homozygous lethal are $vg^{21\text{-}9}$ and $vg^{21\text{-}12}$. Both of these alleles behave genetically as *vg* nulls. Since these alleles are unconditional lethals, while null *vg* alleles show escaper flies we feel this lethality is not *vg* specific. Southern hybridization analyses of Sal1 restricted DNA from flies heterozygous for the respective lethal alleles and each of two different *vg* alleles are shown in Figure III-3A. The results indicate that the $vg^{21\text{-}12}$ derivative has a deletion encompassing the region defined by the two probes used, while the $vg^{21\text{-}9}$ derivative does not have this region deleted. The rationale for this conclusion is based on the observation that the vg^{21} chromosome yields a 23 kb Sal1 DNA fragment which is split into two fragments in $vg^{21\text{-}3}$ (described below). Since only the vg^{21} specific band is seen in the $vg^{21\text{-}12}/vg^{21}$ lane, and

Figure III-3: Molecular analysis of lethals vg^{21-9} and vg^{21-12}. Allele designations are as in Figure III-2. The probes used for the Southern blots in panels A and B are indicated in panel C. A. Southern blots of Sal1 restricted DNA from flies of the indicated genotypes were made; blot 1 is hybridized with probe A while blot 2 is hybridized with probe B. The Sal1 band in the 12/21 lane is larger than expected, probably due to excess DNA loaded in that lane. B. Southern blots with DNA from flies of the indicated genotypes were hybridized with either probe C (blot 1) or probe D (blots 2 and 3). Arrows within the photographs indicate novel fragments seen in the vg^{21-9} lanes. C. The restriction map of the region with the utilized probes indicated. The vg^{21} insert and the vg^{21-3} derivative insert are also shown, with the extra restriction sites in the vg^{21-3} insert indicated. Restriction enzyme designations are: Sal1 = S, Pst1 = P, EcoR1 = R, Xho1 = X.

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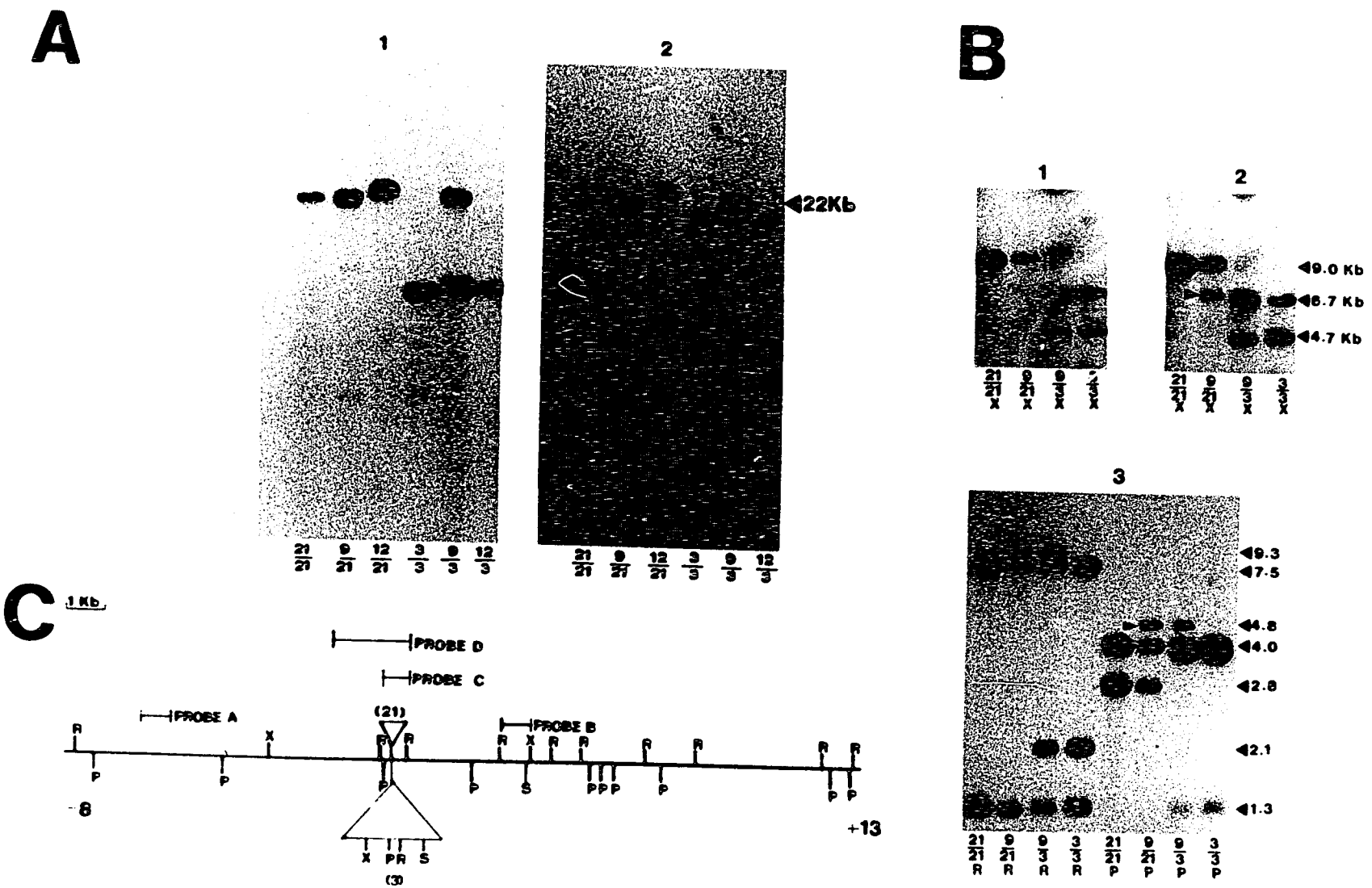

A
1
2
22Kb
21/21
9/21
12/21
3/3
9/3
12/3
B
1
2
9.0 Kb
6.7 Kb
4.7 Kb
3
9.3
7.5
4.8
4.0
2.8
2.1
1.3
C
1 Kb
PROBE D
PROBE C
PROBE A
PROBE B
(21)
-8
+13
X PR S
(3')

only the vg^{21-3} specific bands are seen in the vg^{21-12}/vg^{21-3} lane (using either probe), then the vg^{21-12} chromosome must have a deletion including at least the +6 to -7 region. Probe C of Figure III-3 also does not recognize vg^{21-12} specific restriction fragments (results not shown). Of course, this analysis does not preclude the possibility that vg^{21-12} is associated with other more complex rearrangements. However, the demonstrated deletion of *vg* region DNA is sufficient to be responsible for the *vg* null phenotype observed, so we examined this allele no further.

Similar logic is used to interpret the results presented in Figure III-3B. In this case, the vg^{21-9} allele is heterozygous with either vg^{21} or vg^{21-3}, and DNA from these flies was restricted with either Xho1, Pst1, or EcoR1, and analyzed by Southern hybridization. Since vg^{21} and vg^{21-3} exhibit different sized restriction fragments for each of the three digests (see below), the presence of vg^{21-9} specific restriction fragments is easily detected. Panels 1 and 2 demonstrate that vg^{21-9} has a deletion which removes the probe C region but not the probe D region. Panel 3 shows further characterization of this deletion using EcoR1 and Pst1 Southern blots. The results are consistent with vg^{21-9} having a deletion of 2 kb which removes the vg^{21} insert and *vg* region DNA both proximal and distal to it, including the entire .7 kb EcoR1 fragment within which the P-element is inserted.

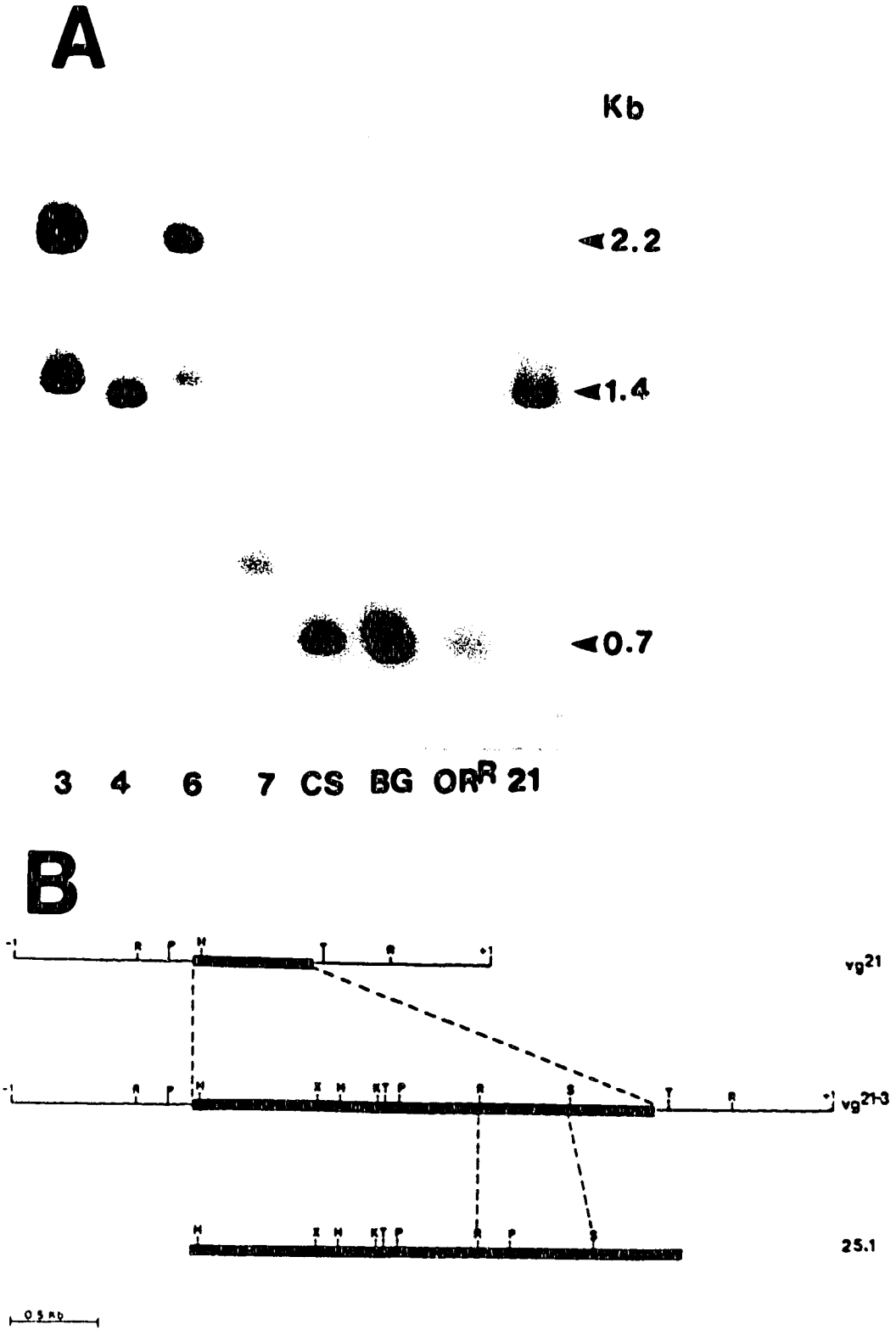
Analysis of viable vg^{21} derivatives:

Five of the vg^{21} derivatives (vg^{21-3}, vg^{21-4}, vg^{21-6}, vg^{21-7}, and

vg^{21-8}) are homozygous viable. Genomic Southern analysis of Pst1 or EcoR1 restricted genomic DNA from these mutant strains was performed using probes from throughout the cloned region (-18 to +28). The only alterations found were in the same genomic EcoR1 and Pst1 fragments within which the vg^{21} insert is located. The vg^{21-7} and vg^{21-8} derivatives have deletions within the fragments while vg^{21-3} and vg^{21-6} are complex, with additional DNA inserted into the fragments (Figure III-4A). The vg^{21-4} derivative contains a small deletion (< 50 bp) within the same region as above.

In order to determine genetically whether the physical alterations associated with the vg^{21} derivatives are associated with the more extreme *vg* phenotypes that were selected, intragenic recombination analyses were done. The vg^{21} allele has already been genetically mapped in relation to vg^{BG} (Chapter II), and is positioned approximately .028 map units centromere proximal to vg^{BG}. The relevant vg^{21} derivative flies were crossed to a multiply marked second chromosome stock (*al dp b pr cn vg c a px bw mr sp*/In(2L)Cy In(2R)Cy) obtained from J. Kennison, and F_1 mutant *vg*/marker chromosome females were backcrossed to vg^{BG} males. Southern hybridization analyses were used to confirm that the *vg* allele on the marker chromosome is the vg^{BG} allele (a strong *vg* allele obtained from Bowling Green Stock Center) physically characterized in Chapter II. One vg^{+} recombinant was isolated for each of vg^{21-3}, vg^{21-4}, vg^{21-6}, and vg^{21-7} out of 7730, 2660, 9508, and 8898 flies scored respectively

Figure III-4: Genomic Southern analysis of several viable *vg21* derivatives and restriction map analysis of vg^{21} and $vg^{21\text{-}3}$. A. The genomic southern blot of EcoR1 restricted genomic DNA of the indicated genotypes was probed with the .7 kb EcoR1 fragment at coordinate 0 of the restriction map (Figure III-3). Arrows denote aberrant size fragments seen in the vg^{21} derivatives. Strain designations are as in Figure III-2. B. Restriction map analysis of vg^{21} and $vg^{21\text{-}3}$. The P-element insertions of the respective alleles are indicated by a bar, and flanking DNAs are thin lines. The restriction map of pπ25.1, a complete P-element (O'Hare and Rubin, 1983) is shown at the bottom. Restriction enzymes are abbreviated as in Figure III-3, except that HindIII is designated as H, Sst1 as T, and Kpn1 as K.


A
Kb
2.2
1.4
0.7
3 4 6 7 CS BG ORR 21
B
vg21
vg21-3
25.1
0.5 Kb

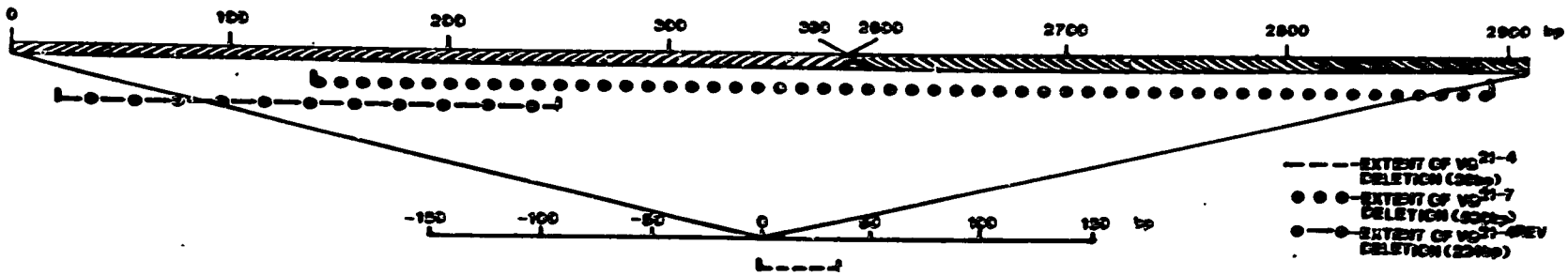
from each cross. The vg^+ progeny, upon testing, were all found to have only the proximal flanking markers which indicates that all of the derived *vg* alleles mapped closely proximal to the *vg* allele of the marker chromosome. These results indicate that the physical lesions observed with the above four vg^{21} derivatives are probably the causative lesions of the more extreme *vg* phenotypes, since they genetically map to the same general location where the lesions are observed.

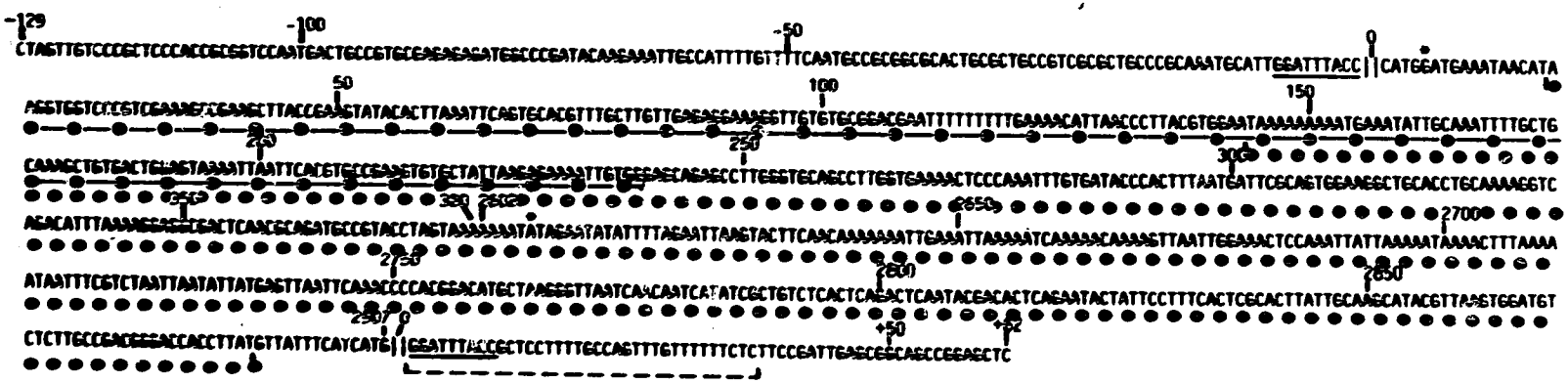
Further physical analyses helped to define more precisely the alterations associated with these vg^{21} derivatives. Since DNA from the $vg^{21\text{-}3}$ allele releases two EcoR1 fragments (Figure III-4A), the Bam H1 DNA fragment from -11 to +6 was cloned for this derivative to ensure that the entire insert was obtained (see Materials and Methods). The cloned fragment was restriction mapped and gels with various restriction digests were Southern blotted and probed with pπ25.1 (O'Hare and Rubin, 1983) to detect P-element sequences (data not shown). The results, depicted diagramatically in Figure III-4B, show that the original small vg^{21} insert now is a 2.6 kb insert at the same site. All the restriction fragments within the insert hybridize to P-element sequences, and the restriction map matches perfectly with that of a complete P-element (O'Hare and Rubin, 1983) except for a small internal deletion near the 3' end. Thus, $vg^{21\text{-}3}$ seems to be a novel type of conversion-like event of the small vg^{21} insert into a much larger insert through an as yet unknown process. The nature of $vg^{21\text{-}6}$ has not yet been rigorously determined, but it appears to be similar in

type to vg^{21-3}. Since these alleles are of independent origin, this indicates that the secondary insertion of DNA in vg^{21-3} is not an isolated event.

The affected EcoR1 fragments of vg^{21-4} and vg^{21-7} were cloned (See Materials and Methods) and mapped by further restriction endonuclease analyses. The vg^{21-7} derivative was shown to have deleted most of the DNA between the HindIII and Sst1 sites of vg^{21}, while a small (about 40 bp) deletion between the Hind III and Sst1 sites was detected in the vg^{21-4} derivative (data not shown). The exact nature of the molecular lesions in vg^{21-4} and vg^{21-7} was determined by DNA sequencing of vg^{21} and these two derivatives (see Materials and Methods). The results (Figure III-5) show that the vg^{21} mutation is due to a 687 base pair insertion of an internally deleted P-element into the 200 bp OR^R Pst1/Sst1 restriction fragment. A single base insertion in the 5' terminal repeat is present in the vg^{21} insert compared to the published P-element sequence (O'Hare and Rubin, 1983). Since, to the best of our knowledge, no other examples of base changes in the terminal repeats have been characterized, it is difficult to predict the effects on transposition. The sequence of the Pst1/Sst1 OR^R fragment indicates that vg^{21} is a clean insert which has a novel 9 bp duplication of OR^R DNA flanking the insert (Figure III-5). It is possible that the extra base in the terminal repeat may affect transposition causing a 9 bp duplication. Conversely, there may have been an 8 bp duplication followed by insertion of one nucleotide at the junction. The sequencing of the two vg^{21} derivatives shows that vg^{21-7} has deleted 529 bp of

Figure III-5: DNA sequence analysis of vg^{21} and three derivatives. In the top half is a schematic diagram of the vg^{21} insert with the extent of the DNA deletions in three vg^{21} derivatives as indicated: - - - - - - , ($vg^{21\text{-}4}$), ... , ($vg^{21\text{-}7}$), and - -- -- -- -- --, ($vg^{21\text{-}4Rev}$). The sequence of the vg^{21} insert, numbered according to O'Hare and Rubin (1983), with the indicated flanking *Drosophila* genomic DNA from the *vg* region is shown in the bottom half. The three vg^{21} derivatives, with the relevant sequences deleted are indicated as above. The $vg^{21\text{-}4Rev}$ derivative also contains the $vg^{21\text{-}4}$ deletion, which is expected since $vg^{21\text{-}4Rev}$ was derived from $vg^{21\text{-}4}$. The nine base pairs duplicated at the insertion site are underlined. Asterisks denote extra bases in the P-element inserts which differ from the published sequence (O'Hare and Rubin, 1983). The $vg^{21\text{-}4Rev}$ derivative has three bases (GAT, not shown) inserted at the internal deletion end point, while $vg^{21\text{-}7}$ has two bases inserted at the breakpoint (GG, not shown). This type of alteration has been observed before (O'Hare and Rubin, 1983; Searles et al, 1986). The genomic Pst1 site is at -139 (not shown), while the genomic Sst1 site is at +62.


0
100
200
300
390
2800
2700
2800
2900 bp
-150
-100
-50
0
50
100
150 bp



DNA (to within 14 bp of the 3' end of the vg^{21} P-element insert), while $vg^{21\text{-}4}$ has deleted 36 bp of *vg* locus DNA, immediately adjacent to but entirely outside the vg^{21} insert. Both derivatives retain the extra base in the 5' terminal repeat. The 9 bp duplication is retained in $vg^{21\text{-}7}$ but in $vg^{21\text{-}4}$ the 3' portion is, of course, deleted.

Analysis of $vg^{21\text{-}4Rev}$:

A spontaneous partial revertant ($vg^{21\text{-}4Rev}$) was isolated from the $vg^{21\text{-}4}$ stock (Figure III-2) which exhibits strap-like wings in homozygotes instead of the more extreme vg wing phenotype of $vg^{21\text{-}4}$. Figure III-6A shows a Southern blot hybridization of Pst1 restricted DNA from flies homozygous for $vg^{21\text{-}4}$, $vg21\text{-}4Rev$, and the original $vg^{21\text{-}4}$ stock (which contains a mixture of $vg^{21\text{-}4}$ and $vg^{21\text{-}4Rev}$ flies). The pure $vg^{21\text{-}4}$ and $vg^{21\text{-}4Rev}$ stocks were selected from the original $vg^{21\text{-}4}$ stock by either repeated selection for *vg* extreme or *vg* weak phenotypes, or selection of weak and extreme *vg* phenotypes from flies heterozygous over various other *vg* alleles (labeled 1 and 2, respectively in Figure III-6A). The results show that the mixed stock contains two aberrant Pst1 bands; one the same size as the $vg^{21\text{-}4}$ band seen previously (Figure III-3) and one which is 200 bp smaller. DNA from both homozygous $vg^{21\text{-}4Rev}$ stocks is associated with this smaller band. The -11 to +6 Bam H1 fragment, which contains the affected region, was used to clone the $vg^{21\text{-}4Rev}$ allele (see Materials and Methods). Restriction digests of the insert showed that the only alteration in the region was the 200 bp deletion described above. A schematic

representation of the generation of this derivative allele, starting from vg^{21}, is shown in Figure III-6A. Since the revertant is allelic to *vg* and associated with a DNA alteration in the same region as all of the previous derivative alleles characterized above, we feel that this alteration is the lesion responsible for the revertant phenotype. The insert region of $vg^{21\text{-}4Rev}$ was sequenced to precisely localize the deletion endpoints and these results are shown in Figure III-5.

Analysis of $vg^{21\text{-}7Rev}$:

A spontaneous phenotypic revertant ($vg^{21\text{-}7Rev}$) was isolated from the $vg^{21\text{-}7}$ stock (Figure III-2) which almost completely complements vg^{BG}. Southern hybridization analysis of Pst1 digested DNA from $vg^{21\text{-}7Rev}$ is shown in Figure III-6B and indicates that this derivative allele, surprisingly, is associated with a further insertion into the $vg^{21\text{-}7}$ insert, and is not the expected deletion of $vg^{21\text{-}7}$ P-element sequences. Further Southern blot analyses indicated that this alteration was within the same EcoR1 fragment that contained the other vg^{21} derivatives discussed above (data not shown). The altered EcoR1 fragment was cloned and restriction mapped (Materials and Methods). Figure III-6B summarizes these results and shows a schematic representation of the generation of this allele from the vg^{21} allele. The $vg^{21\text{-}7Rev}$ insert was sequenced and the comparison of this sequence with the parent insert is given in Figure III-7. The $vg^{21\text{-}7Rev}$ allele results from a novel insertion of a 1.1 kb P-element into the $vg^{21\text{-}7}$ insert. The data are most consistent with the $vg^{21\text{-}7Rev}$ derivative

Figure III-6: Southern hybridization analyses and interpretative flow diagrams of $vg^{21\text{-}4Rev}$ and $vg^{21\text{-}7Rev}$. Panels A and B show Southern blots of Pst1 restricted DNA from flies of the indicated genotypes hybridized with probe C from Figure III-3. Strain designations are as in Figure III-2, and BG is vg^{BG}. The 1 and 2 designations indicate independent lines of $vg^{21\text{-}4}$ and $vg^{21\text{-}4Rev}$ established by two different selection methods (see text). At the bottom of each panel are flow diagrams indicating the physical alterations that have taken place, proceeding from vg^{21} through to either $vg^{21\text{-}4Rev}$ or $vg^{21\text{-}7Rev}$. The cross-hatched bars denote P-element sequences. Restriction enzyme designations and restriction maps are as in Figure III-4. The alterations shown for $vg^{21\text{-}4Rev}$ and $vg^{21\text{-}7Rev}$ were derived from DNA restriction analyses and DNA sequencing of clones of these alleles.

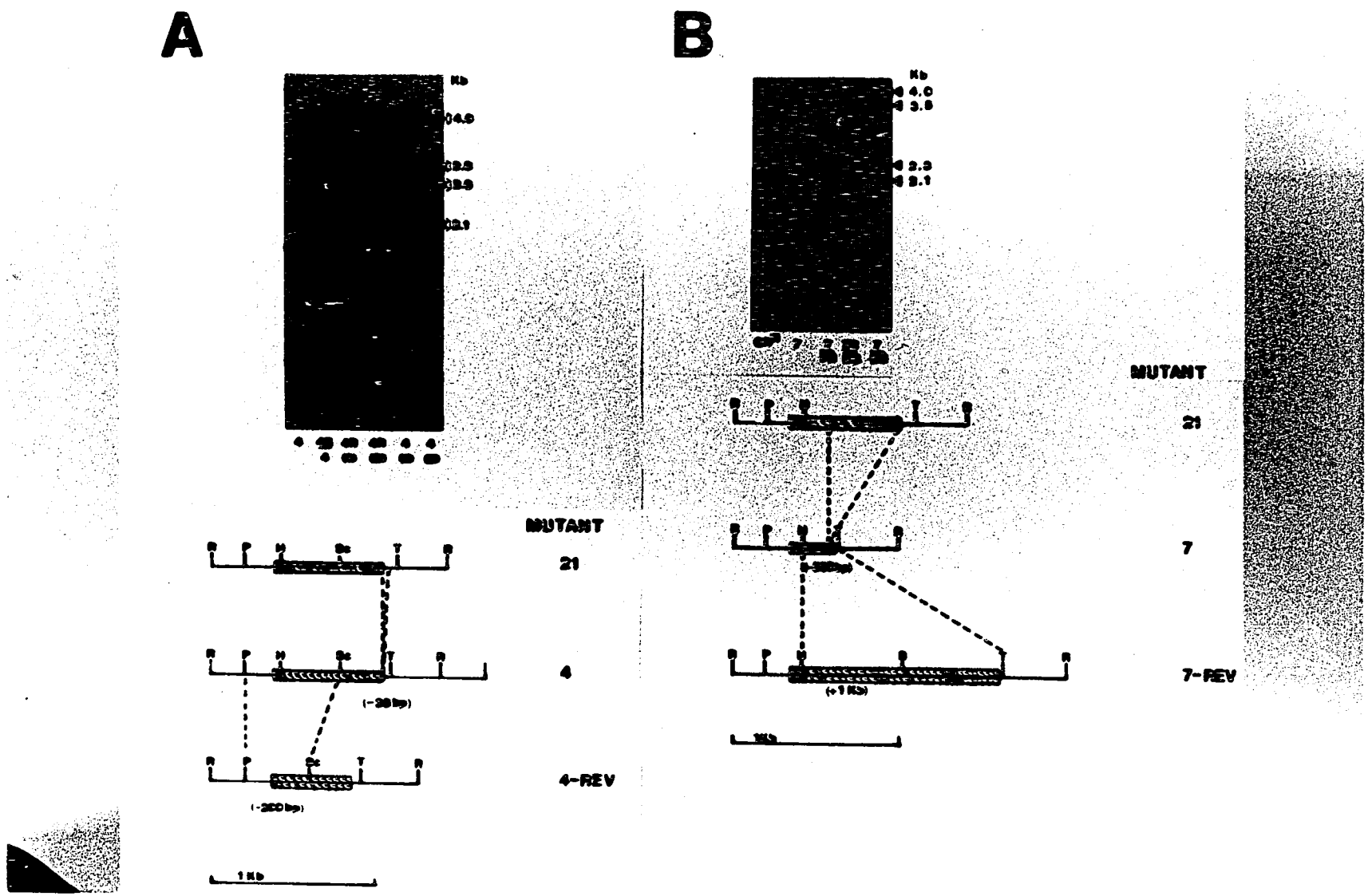
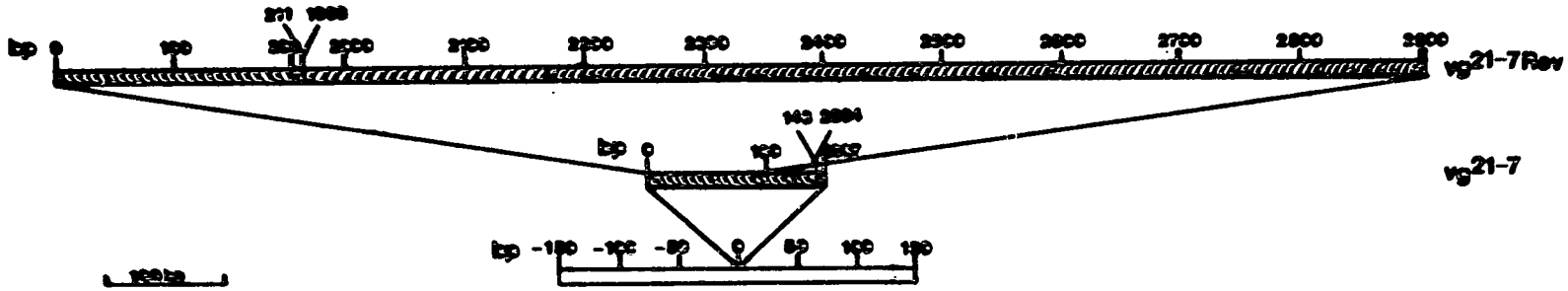
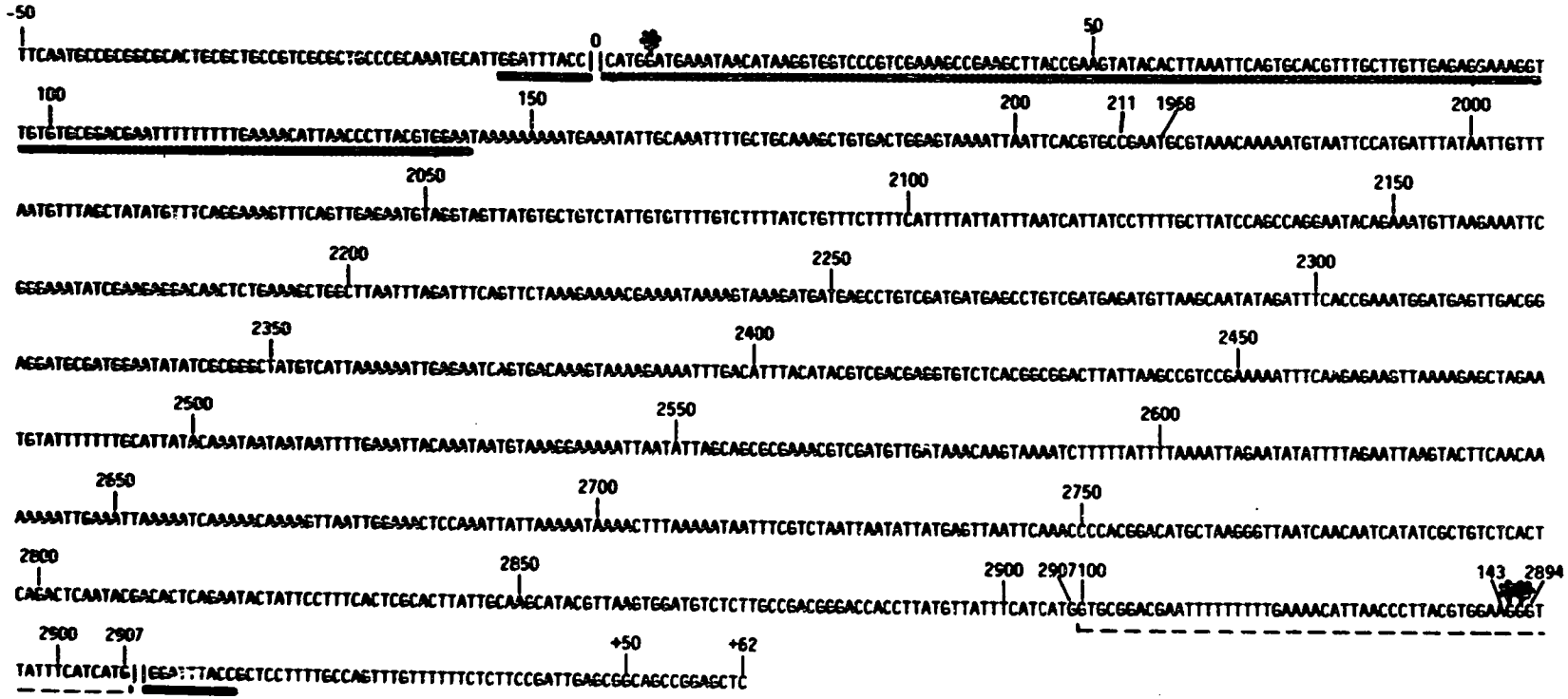

A
B
MUTANT
21
4
4-REV
7
7-REV
1 Kb

Figure III-7: DNA sequence analysis of $vg^{21\text{-}7Rev}$. At the top is a schematic representation, derived from sequencing, of the event that produced $vg^{21\text{-}7Rev}$. The DNA sequence of $vg^{21\text{-}7Rev}$ is indicated at the bottom. P-element sequences of $vg^{21\text{-}7}$ origin are indicated by a dashed line, while a hatched line denotes P-element DNA derived from either $vg^{21\text{-}7}$ or $vg^{21\text{-}7Rev}$. The 9 bp duplication of flanking *vg* region DNA is indicated with a solid bar, while an asterisk indicates extra bases seen in both $vg^{21\text{-}7}$ and $vg^{21\text{-}7Rev}$ differing from the published sequence (O'Hare and Rubin, 1983), which was used for the numbering here. The 3 base pairs at the junction of the 211-1968 internal deletion are unlabelled since they correspond to both the 212-214 and 1965-1967 nucleotides in the published sequence.


bp
vg21-7Rev
vg21-7
bp
bp


-50
0
50
100
150
200
211
1958
2000
2050
2100
2150
2200
2250
2300
2350
2400
2450
2500
2550
2600
2650
2700
2750
2800
2850
2900
2907
100
143
2894
2900
2907
+50
+62

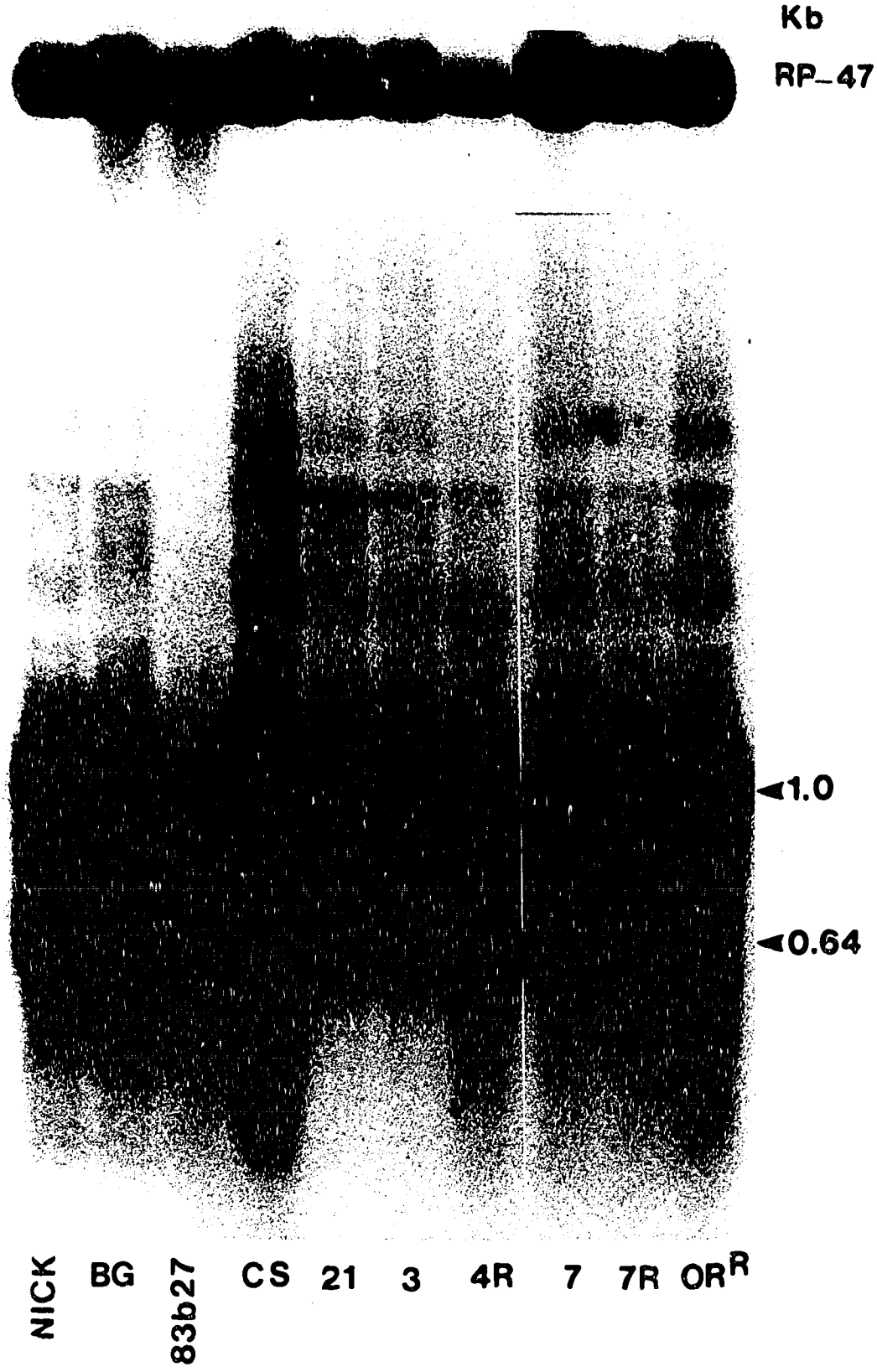
resulting from a P-element integration into vg^{21-7} at the 3' end (at base 100) and a homologous recombination with the vg^{21-7} insert at the 5' end. For this reason, the exact integration site at the 5' end is not shown in Figure III-7; the P-element sequences of either possible origin (vg^{21-7} or $vg^{21-7Rev}$) are indicated. No base changes are seen in the portion of vg^{21-7} which is retained in $vg^{21-7Rev}$ (Figure III-7). The 9 bp duplication and the extra base in the 5' terminal repeat are also retained in $vg^{21-7Rev}$.

vg transcript analysis:

Adult *vg* specific transcripts have been identified (Chapter II, Addendum). In order to examine how the series of P-element inserts affect adult *vg* expression, adult poly A^+ RNA was prepared from vg^{BG}, vg^{83b27}, OR^R, and the homozygous viable P-element induced alleles reported here, and examined by Northern blot analysis (Figure III-8) using the *vg*-specific probe identified in Figure III-1. No transcripts are detected in the vg^{83b27} lane, which demonstrates that the transcripts are *vg* specific since vg^{83b27} is a homozygous deletion of the probe region (see Figure III-1). The blot was subsequently rehybridized with a RP49 probe (O'Connell and Rosbach, 1984) to control for differences in the amount of RNA/lane. The results indicate that the mutants affect the level of adult *vg* transcripts without altering the size.

The *vg* transcript levels in the vg^{21-3} lane do not appear reduced relative to vg^{21}. This is unexpected, since vg^{21-3} is a more severe *vg* allele than vg^{21}. However, since we have not sequenced vg^{21-3}, it is

Figure III-8: Effects of P-element inserts on *vg* expression. A Northern blot of adult poly A^+ RNA from flies of the indicated genotypes was hybridized with a *vg* antisense RNA probe made from the restriction fragment indicated in Figure III-1. Size markers (not shown) were *Drosophila* rRNA and mouse B-globin mRNA. The blot was stripped and reprobed with an oligolabeled ribosomal protein gene probe (RP49) to allow rough quantification of the amount of RNA loaded/lane (O'Connell and Rosbach, 1984). The results of this second hybridization are shown at the top of the figure. The mutant alleles are labelled as in Figure III-2, and nick represents vg^{ni} (a spontaneous derivative of vg^{BG}) while C.S. denotes Canton-S (a wild-type strain).


Kb
RP-47
1.0
0.64
NICK
BG
83b27
CS
21
3
4R
7
7R
OR^R

possible that this allele is more severe due to structural alterations in the *vg* region DNA flanking the $vg^{21\text{-}3}$ insert (i.e., like $vg^{21\text{-}4}$). Comparison of *vg* transcript levels with the RP49 control indicates that both $vg^{21\text{-}4Rev}$ and $vg^{21\text{-}7Rev}$ have enhanced *vg* transcription compared to vg^{21}, $vg^{21\text{-}3}$ and $vg^{21\text{-}7}$; $vg^{21\text{-}4}$ has transcript levels comparable to vg^{21}, $vg^{21\text{-}3}$ and $vg^{21\text{-}7}$ (data not shown).

Discussion

Hybrid dysgenesis was utilized to induce a number of P-element alleles at the *vg* locus and the molecular lesion associated with each was characterized. These mutations were derived from one P-element induced parent allele (vg^{21}) by either loss of internal P-element sequences, loss of adjacent genomic *vg* sequences, loss of the entire original P-element insert and flanking genomic sequences, or from insertion of additional sequences into the original P-element insert. There are a number of precedents for the first three types of alterations. P-element internal deletions have been observed by many investigators (Daniels et al, 1985; Searles et al. 1986; Tsubota and Schedl, 1986). Searles et al. (1986), based on their sequence data of revertants of a P-element induced RPII215 allele, speculated that internal deletions may preferentially originate in the P-element terminal repeats. The two internal deletion alleles sequenced here are consistent with this theory. The $vg^{21\text{-}4Rev}$ specific deletion begins 13 bp into the 5' terminal repeat, while the deletion associated with $vg^{21\text{-}7}$ starts 14 bp internal to the 3' terminal repeat (Figure III-5), implying similar mechanisms in the generation of these deletions and the ones observed above (Searles et al, 1986). The $vg^{21\text{-}4}$ deletion is similar to these examples, except that the mutant deletes 36 bp of genomic *vg* DNA precisely from the end of the 3' terminal repeat (Figure III-5). This type of deletion may be conceptually similar to the larger deletions reported previously (Daniels et al, 1985; Tsubota and

Schedl, 1986). However, since the previous examples were analyzed only at the restriction map level, we can not be sure of this. These examples demonstrate that the terminal repeats can stimulate both internal and external deletions. Larger deletions similar to those seen with vg^{21-9} and vg^{21-12} are relatively common (Daniels et al, 1985; Engels and Preston, 1981; Tsubota and Schedl, 1986) and represent another consequence of aberrant P-element excision. Thus, these three classes of vg^{21} alleles were derived from predictable classes of putative P-element aberrant excision or transposition events.

The fourth class of vg^{21} derivatives includes those alleles caused by further insertions into extant P-element sequences. Although not yet sequenced, the results of the vg^{21-3} analyses are consistent only with further insertion of P-element sequences into the vg^{21} P-element insert. The restriction map predicts that this insert may have resulted from gene conversion or recombination between a large P-element (the vg^{21-3} element) and the vg^{21} insert. Alternatively, the vg^{21} insert may have been excised and the larger vg^{21-3} insert integrated at the same site (W. Engels, personal communication). These alternatives are currently being tested by sequencing the vg^{21-3} insert. The second example of further insertion within a P-element insert is $vg^{21-7Rev}$. This allele is due to the insertion of approximately 1 kb of P-element DNA sequence into the residual 158 bp of P-element sequence found in vg^{21-7}; by P-element integration at the 3' end (at base 100 of vg^{21-7}) and what appears to be homologous recombination at the 5' end. Conversely, this could have been generated by deletion of the 5' end of

vg$^{21\text{-}7}$ up to base 100 followed by integration of the *vg*$^{21\text{-}7Rev}$ element into the remaining portion of the *vg*$^{21\text{-}7}$ element. Thus, we have observed two separate examples of P-element insertions into pre-existing P-element sequences. Since *vg*$^{21\text{-}6}$ is very similar to *vg*$^{21\text{-}3}$ it appears that 3/9 derivatives of *vg*^{21} described here are due to secondary insertions. Interestingly, one of these insertions was isolated as a revertant allele (*vg*$^{21\text{-}7Rev}$) while the other two were isolated as phenotypically more extreme alleles (*vg*$^{21\text{-}3}$ *and vg*$^{21\text{-}6}$). Secondary insertions of P-elements into the region of existing P-elements have also been observed at the yellow and singed loci (W. Eggleston and W. R. Engels, personal communication) and the Sxl locus (Salz et al, 1987). However, these insertions have not been characterized in detail. The examples reported here may be similar to homologous recombination events reported for *Drosophila* retrotransposons involved in transpositional memory (Mizrokhi et al, 1985), or the unusual double transposons observed with the Ac-Ds controlling elements in maize (Doring et al, 1984). Conversely, they may represent examples of simultaneous excision and insertion of P-elements. The presence of an extra base in the 5' terminal repeat of *vg*^{21} and all its sequenced derivatives argues that homologous recombination may be involved. If the *vg*$^{21\text{-}7}$ 5' end was excised and replaced with another P-element, one would expect that the derivative would not have the extra base in the terminal repeat. Further, if the *vg*$^{21\text{-}7Rev}$ insert was due to an integration event at both 5' and 3' ends an 8 bp duplication should flank the 1.1 kb insert. The observation that the 8 base pairs at the 3' end of

the $vg^{21\text{-}7Rev}$ insert (i.e., 100-107 on Figure 7) are not duplicated at the other end of the element also argues that homologus recombination has occurred at the 5' end of the element. More examples will have to be isolated and sequenced to determine the extent of the roles of homologous recombination and integration in the mechanisms of secondary insertion.

During the initial cloning of the locus, only one insert in two million dysgenic flies was found (Chapter II). Thus, secondary insertions at the vg^{21} insertion site are several hundred-fold more prevalent than the primary insertion event. This is likely to be due to the presence of P-element sequences at the vg^{21} site, since all of the alleles were derived from the same progenitor stock (OR^R), and the same P-cytotype stock ($\pi 2$) was used for both primary and secondary dysgenic crosses. This may indicate that P-elements themselves are hotspots for insertions, perhaps by virtue of their expression in the germ line or their ability to cause chromosome breaks under dysgenic conditions (Engels and Preston, 1981). Further examples will have to be characterized to assay the mechanism and prevalence of secondary insertions. It is interesting that this class of P-element secondary insertion is so prevalent at *vg* but rarely identified at other loci where similar types of analyses have been done (Chia et al, 1986; Searles et al, 1986; Tsubota and Schedl, 1985; Voelker et al, 1984). Coding region insertions are unlikely to be reverted or enhanced by this type of mutation, perhaps explaining why secondary insertions have not been

observed for these types of P-element mutants (i.e., white locus; O'Hare and Rubin, 1983). Experiments similar to those reported here have been conducted on P-element insertion mutants into non-coding sequences at the RPII215 (Searles et al , 1986; Voelker et al, 1984), rudimentary (Tsubota et al, 1985; Tsubota and Schedl, 1986), and yellow (Chia et al, 1986) loci. These alleles were all within the 5' promoter regions of the respective genes while the vg^{21} insert is in the 3' region, at least with respect to adult *vg* transcription. It is possible that secondary insertion mutants are not as detectable within the promotor physical environment, due to potentially different mechanisms by which P-element inserts in promotors disrupt gene function.

The effects of the recessive viable P-element induced alleles on *vg* transcription were determined. These alleles were shown to reduce the level, but not the size, of adult *vg* transcripts. However, we can not be sure that the inserts disrupt *vg* expression the same way in the earlier stages of development when the wing phenotype is determined. Since all of these alleles contain insertions at the same genomic site, and differ from each other only by what internal P-element sequences are present (except $vg^{21\text{-}4}$), these sequenced alleles should provide an excellent opportunity to examine how P-element sequences can interfere with gene function.

The P-element inserts map in the 3' region of the *vg* gene as assayed by the *vg* adult transcription unit (Chapter II, Addendum) and are transcribed anti-parallel to adult *vg* transcription. Since all of the

inserts are at the same genomic site, and do not cause an alteration in transcript size, insertional interruption of the coding sequences is unlikely. Termination of *vg* transcription within the P-element inserts is also unlikely, since a complete revertant ($vg^{21\text{-}7Rev}$) has additional sequences inserted, without removing or altering any of the sequences present in the more phenotypically extreme parent allele ($vg^{21\text{-}7}$). The same rationale makes *vg* splicing or polyadenylation within the P-element inserts unlikely. Furthermore, examination of DNA sequences within the sequenced P-element inserts shows no putative polyadenylation sites in the same orientation as *vg* transcription.

It is possible that P-element products may be involved in determining the severity of the mutants. In this model, the P-element products (i.e., the P-element repressor) would bind to specific P-element sequences and either enhance or suppress *vg* transcript levels by an unknown process. This was tested by crossing males of each allele to a multiply marked *vg* chromosome (in either a P or M cytotype) and observing changes in phenotype over several generations. In fact, four *vg* alleles (vg^{21}, $vg^{21\text{-}3}$, $vg^{21\text{-}6}$, and $vg^{21\text{-}7Rev}$) were shown to be dependent on the absence of repressor producing P-elements for their mutant phenotype to be expressed. However, vg^{21} and its repressor-suppressed derivatives reported in this study were all analyzed in a non-suppressing genetic background, thus their phenotypes were not influenced by the presence of P-element repressor. P-element suppression appears to be a separate phenomenon, and will be discussed elsewhere (Chapter IV).

Parkhurst and Corces (1985) proposed a transcriptional interference model to explain the action of gypsy insertions at the forked locus. They speculated that enhancer elements in the gypsy LTR can act at a distance and suppress transcription from the forked promotor. The fact that none of the P-element derived *vg* alleles discussed here cause aberrant *vg* transcripts is consistent with their model. However, the absence of a well-defined P-element enhancer (Laski et al, 1986) and the distance of the P-element inserts from the adult *vg* promotor (> 6 kb) makes this explanation unlikely for the vg^{21} derivatives. Also, the $vg^{21\text{-}7Rev}$ results are contrary to this model, since the revertant has additional sequences rather than fewer.

A model which is consistent with the results of our analysis has been proposed by Bingham and Chapman (1986). They suggested that a transposon transcribed in the opposite direction to the white gene in which it is inserted may cause a decrease in white gene transcription by collision of transcription complexes. This model, when applied to P-elements, would implicate two regions of P-element sequences in the disruption of the target gene function. The presence of a P-element promotor would cause a more extreme *vg* phenotype due to P-element transcription, while the presence of a transcription terminator would lead to a less extreme *vg* phenotype since the P-element transcripts would be shorter and less likely to interfere with *vg* transcription. In this model, the vg^{21} allele which has promotor and terminator sequences present (Laski et al, 1986) produces short P-element transcripts in adult flies which interfere with productive *vg*

transcription causing a cryptic phenotype. Since the truncated *vg* transcripts would probably not be polyadenylated, it is likely they would be unstable and thus not be detected by Northern analyses. The $vg^{21\text{-}7}$ allele produces a more extreme phenotype due to longer P-element transcripts since the P-element terminator sequences have been deleted (Laski et al, 1986). These longer transcripts would be more likely to interfere with *vg* transcription. The $vg^{21\text{-}4}$ allele would have the same cryptic phenotypic effect as vg^{21} since it contains the same P-element sequences, but it is a more extreme mutant due to the loss of some genomic *vg* sequences. The $vg^{21\text{-}4Rev}$ allele would be reverted since the P-element promotor region has been deleted (Laski et al, 1986). However, $vg^{21\text{-}4Rev}$ is not a complete reversion since it still has the 36 bp deletion of genomic *vg* DNA which made $vg^{21\text{-}4}$ more extreme than vg^{21}. This model can also account for the revertant phenotype of $vg^{21\text{-}7Rev}$ as due to the restoration of the P-element terminator sequences by the secondary insertion. The model does not explain why *vg21-7Rev* is less extreme than *vg21*. As well, there is no direct evidence which establishes that the identified adult transcripts are the functional vg transcript. If this is established, careful quantification of the levels of *vg* transcription for each allele is also required, to determine whether the reduction of transcription is rigorously correlated with the severity of *vg* phenotypes. As well, *vg* expression during larval stages will have to be analyzed to establish if the changes in RNA levels seen in adults reflects the effects seen earlier in development when the wing phenotype is formed. However, the

results presented here provide evidence for models in which transcription per se, within a transposable element, can interfere with target gene transcription.

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Chapter IV: Suppressible P-element alleles of the vestigial locus in *Drosophila melanogaster*[1]

Introduction:

Many *Drosophila* mutations, at several loci, have been isolated by P-M hybrid dysgenesis and found to be caused by the insertion of P-element sequences into the respective genes. Hybrid dysgenesis is induced in the progeny of crosses between P-cytotype males and M-cytotype females, but not in the reciprocal cross (reviewed in Engels, 1983). This is presumed to be due to the presence of a maternally inherited repressor of P-element mobilization in P-cytotype stocks. This repressor is absent in M-cytotype stocks. M-strains lack functional P-elements, while P-strains contain functional P-elements (P-factors) and a family of smaller P-elements derived from the P-factors by internal deletions (O'Hare and Rubin, 1983). The P-factor is 2.9 kb long and codes for a transposase (Laski et al, 1986). Only the ends of a P-factor are required for its own transposition or excision, so the smaller P-elements can be mobilized by transposase supplied by an intact P-factor (O'Hare and Rubin, 1983). Since M-strains lack transposase and P-strains have repressed transposition, P-element insertion alleles are stable in either a P or M cytotype. Alleles isolated to facilitate gene cloning by transposon tagging are often stabilized in

1. A version of this Chapter has been published. Williams, J., S. S. Pappu, and J. B. Bell. 1988. Mol. Gen. Genet. 212: 370-374.

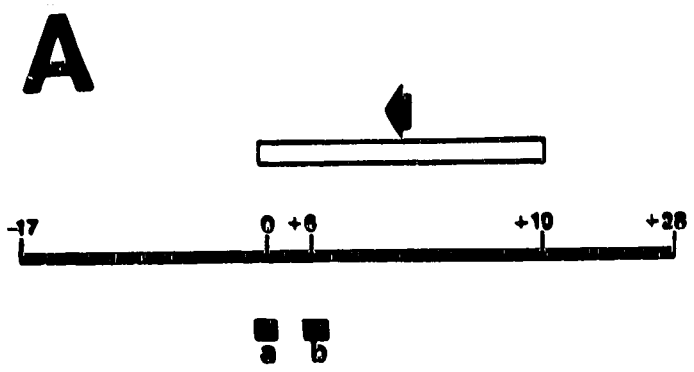
the M-cytotype, since the elimination of extraneous P-elements simplifies cloning. Alleles which are generated in a mutant hunt are often stabilized in the easier to establish P-cytotype. It is important to stabilize P-element alleles, since unrepressed transposase induces a variety of secondary alterations caused by imprecise excision of the P-element inserts (Daniels et al., 1985; Searles et al, 1986; Tsubota and Schedl, 1986; Chapter III). However, little emphasis has been placed on carefully monitoring the effect of the P-element genetic background on stabilized P-element alleles.

A P-element insertion allele (vg^{21}) stabilized in the M-cytotype was used to clone the *vg* locus (Chapter II). Mutant *vg* alleles cause extensive cell death in third instar larval imaginal wing discs which result in absence or loss of parts of the wing margin in adult flies (Fristrom, 1969). Strong *vg* alleles cause complete loss of all wing margin structures, while weak alleles may result in only mild nicking of the wings. The vg^{21} allele is unusual, since all attempts to stabilize it in the P-cytotype resulted in suppression of the *vg* mutant phenotype. This is interesting, since it implies that the P-element genetic background may influence the level of target gene expression. In this study, we investigated the basis of this phenomenon and implicate the P-element repressor as a possible mediator of suppression.

Results and Discussion:

The *vg* gene has been cloned (Chapter II), and the organization of the locus is shown in Figure IV-1A. A series of hybrid dysgenesis induced derivatives of the vg^{21} allele were isolated (Chapter III) and the lesions associated with each are shown in Figure IV-1B. The derivatives resulted from a wide variety of alterations to the vg^{21} allele, including P-element internal deletions and novel recruitment of additional P-element sequences (see Chapter III). Since all of these alleles have alterations at a single, apparently intronic site in the 3' region of the *vg* gene (with respect to the adult transcript; Chapter II Addendum and Chapter III), and all are derived from the same parent allele, they serve as a controlled series of alleles to investigate the basis of the suppression observed initially with vg^{21}. Table IV-1 shows the homozygous phenotypes of each of the alleles, and their phenotypes when heterozygous over vg^{BG} (*BG* denotes Bowling Green, the source of this strong *vg* allele) in both P and M cytotypes. The phenotypic rankings are based on scoring a minimum of 200 flies for each category. We observed virtually no overlap between strains with respect to our phenotypic categories, due to the high penetrance and reproducible expressivity of these *vg* alleles. The vg^{21}, $vg^{21\text{-}3}$, $vg^{21\text{-}6}$, and $vg^{21\text{-}7Rev}$ heterozygotes are completely suppressed when in the P-cytotype. Since none of the other alleles derived from vg^{21} are suppressed when associated with a P-cytotype, (i.e., when the genetic crossing scheme of Table IV-I is used) the effect cannot be *vg* gene-specific, but rather is due to the specific nature of the vg^{21},

Figure IV-1: Molecular organization of the *vg* region and the molecular lesions associated with some P-element derived *vg* alleles. A. Map of 45 kb of DNA from the *vg* region. The open bar indicates the section essential for *vg* function. The arrow indicates the direction of *vg* adult transcription. The data are from Chapter II. The probes used for the Southern and Northern hybridization in Figure IV-2 are designated as "a" and "b", respectively. B. The molecular lesions associated with the vg^{21} allele and its derivatives are shown. The cross hatched bars indicate P-element DNA, while black bars denote genomic DNA from the *vg* region. The open bar denotes deleted DNA. The vg^{21-8} allele is similar to vg^{21-7} while vg^{21-6} and vg^{21-3} are similar. Restriction enzyme designations are: R = EcoR1, P = Pst1, H = HindIII, S = Sal1. X = Xho1 and T = Sst1. The isolation and characterization of these alleles is described in Chapter III.


A
-17
O
+6
+10
+28
a
b

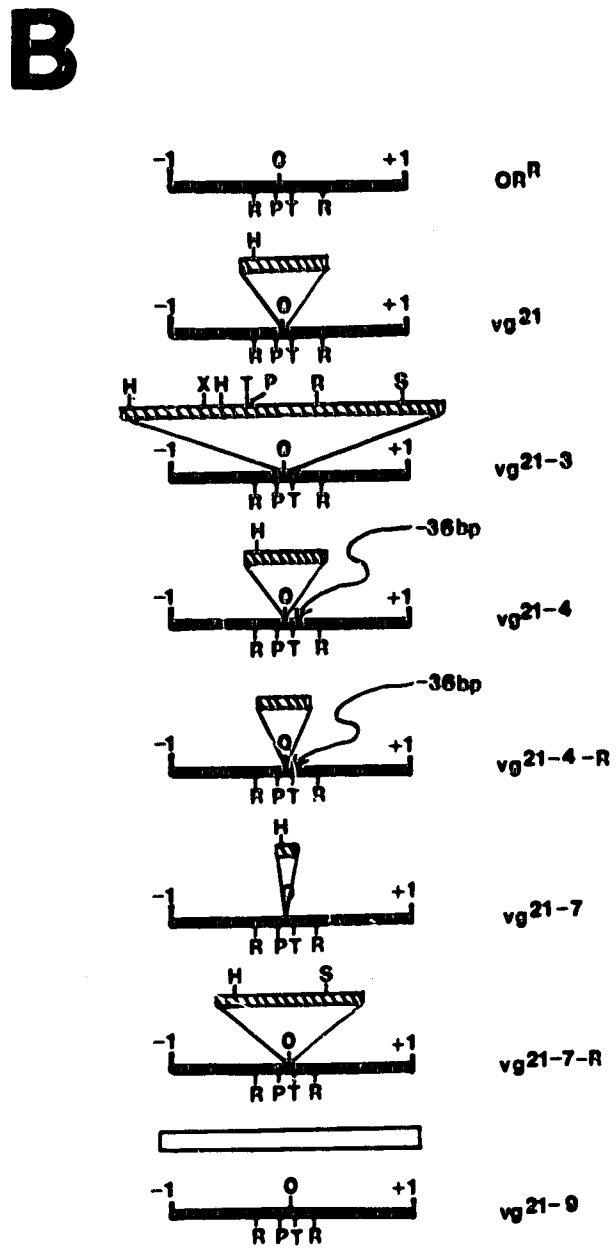

B
-1
O
+1
R P T R
OR^R
H
vg^21
H
X H
T
P
R
S
vg^21-3
-36bp
vg^21-4
vg^21-4-R
vg^21-7
H
S
vg^21-7-R
vg^21-9

Table IV-I: Effects of Cytotype on the phenotypic severity of various *vg* alleles[a]

Allele	Homozygous[b] Phenotype	M-Cytotype[c] Phenotype (*vg* allele/MAP)	P-Cytotype[d] Phenotype (*vg* allele/MAP)
vg^{21}	1	3	1
vg^{21-3}	5	5	1
vg^{21-4}	4	5	5
$vg^{21-4Rev}$	3	5	5
vg^{21-6}	5	5	1
vg^{21-7}	4	5	5
$vg^{21-7Rev}$	1	2	1
vg^{21-8}	4	5	5
vg^{21-9}	L	5	5
vg^{BG}	5	5	5

a. All tests were performed on synthetic medium (Nash and Bell, 1968) at ambient laboratory temperature; about 24° C.

b. The *vg* wing phenotype is ranked on a scale of 1 to 6 where 1 = wild type, 6= an extreme *vg* or null allele phenotype and L denotes homozygous lethality (see Figure I-I). The homozygous phenotypes scored are those seen in the M cytotypes for vg^{21-3} and vg^{21-6} while the remainder are P cytotype phenotypes.

c. The phenotypes in this column are those seen when the *vg* alleles are heterozygous with a mapping chromosome (*al dp b cn* vg^{BG} *c a px mr sp*) in the M cytotype. Repeated backcrossing to MAP/Sm5,Cy shows no change in phenotype over several generations. Mutant allele designations are described in Lindsley and Grell, 1968.

d. As in c., but the alleles are in a P-cytotype, derived from multiple passages of the MAP chromosome through a $\pi 2$ (P-cytotype) genetic background.

vg^{21-3}, vg^{21-6} and $vg^{21-7Rev}$ alleles. The vg^{21} and $vg21-3$ alleles are also suppressed when crossed to other P-cytotype *vg* stocks (i.e., vg^{21-7}, vg^{21-4} and to a deficiency of the locus, Df(2R)vg^{B}) indicating that the vg^{BG} allele is not required for suppression. Homozygous vg^{21} and vg^{21-3} lines were established in the P-cytotype. Both of these lines have a wild-type phenotype. Since vg^{21-3} is wild type in the P-cytotype (i.e. suppressed) this further indicates that suppression requires neither the vg^{BG} allele, nor that the suppressible allele be in a heterozygous state.

It is unlikely that cytotype *per se* suppresses the alleles, since cytotype is only defined in the germ line, while *vg* affects somatic tissue. Thus, some component(s) within P-cytotype stocks which is somatically expressed is the effector of the suppression phenomenon. To test if suppression is maternally inherited the crosses outlined in Table IV-II were performed utilizing the vg^{21} and vg^{21-3} P-cytotype stocks. The results indicate that (at least for the two *vg* alleles tested) the suppression is not maternally inherited, but is observed whenever P-strain chromosomes are present. This strengthens the notion that cytotype *per se* does not cause suppression. Figure IV-2A shows the results of a Southern blot of DNA from P and M cytotype stocks of suppressible or non-suppressible *vg* alleles hybridized to a *vg* region probe which identifies the altered EcoR1 fragments generated by the inserts (see Chapter III). No alterations of the inserts are seen in any of the lanes except vg^{21-3}(P) which, in addition to the insert bands, contains a band the same size as wild type. It is likely that this band is

Figure IV-2: Southern and Northern hybridization analyses of suppressible alleles. Genotypic designations are as: OR^R = Oregon-R; 21 = vg^{21}; 3 = vg^{21-3} et cetera, and the cytotypes are designated as m or p. A. 5ug of adult genomic DNA from adult flies of the indicated genotypes was restricted with EcoR1, electrophoresed on a 1% agarose gel and blotted to nitrocellulose by standard methodology (Maniatis et al., 1982). The DNA was prepared according to Ish-Horowicz et al. (1979). The blot was hybridized to probe "a" (Figure IV-1) which was oligolabeled as in Feinberg and Vogelstein (1983). Hybridization conditions and washes were performed according to Klessig and Berry (1983). B. Poly A^+ RNA was extracted from adults of the indicated genotypes, oligo-dT purified, electrophoresed on a 1.5% formaldehyde agarose gel and blotted onto Genescreen Plus (DuPont) as in Gietz and Hodgetts (1985). [^{32}P] labeled RNA transcripts of probe b (Figure IV-1) were made using the Bluescribe (Promega) cloning and transcription plasmid. Probe preparation, Northern hybridization, and washing were as in Melton et al. (1984). The blot was rehybridized with a ribosomal protein probe (RP-49, O'Connell and Rosbach, 1984) and these results are indicated at the top of the figure. The ribosomal protein DNA probe was prepared and hybridized as above, except that washing was done according to the Genescreen Plus protocol. Size markers (not shown) were *Drosophila* rRNA and mouse B-globin mRNA.

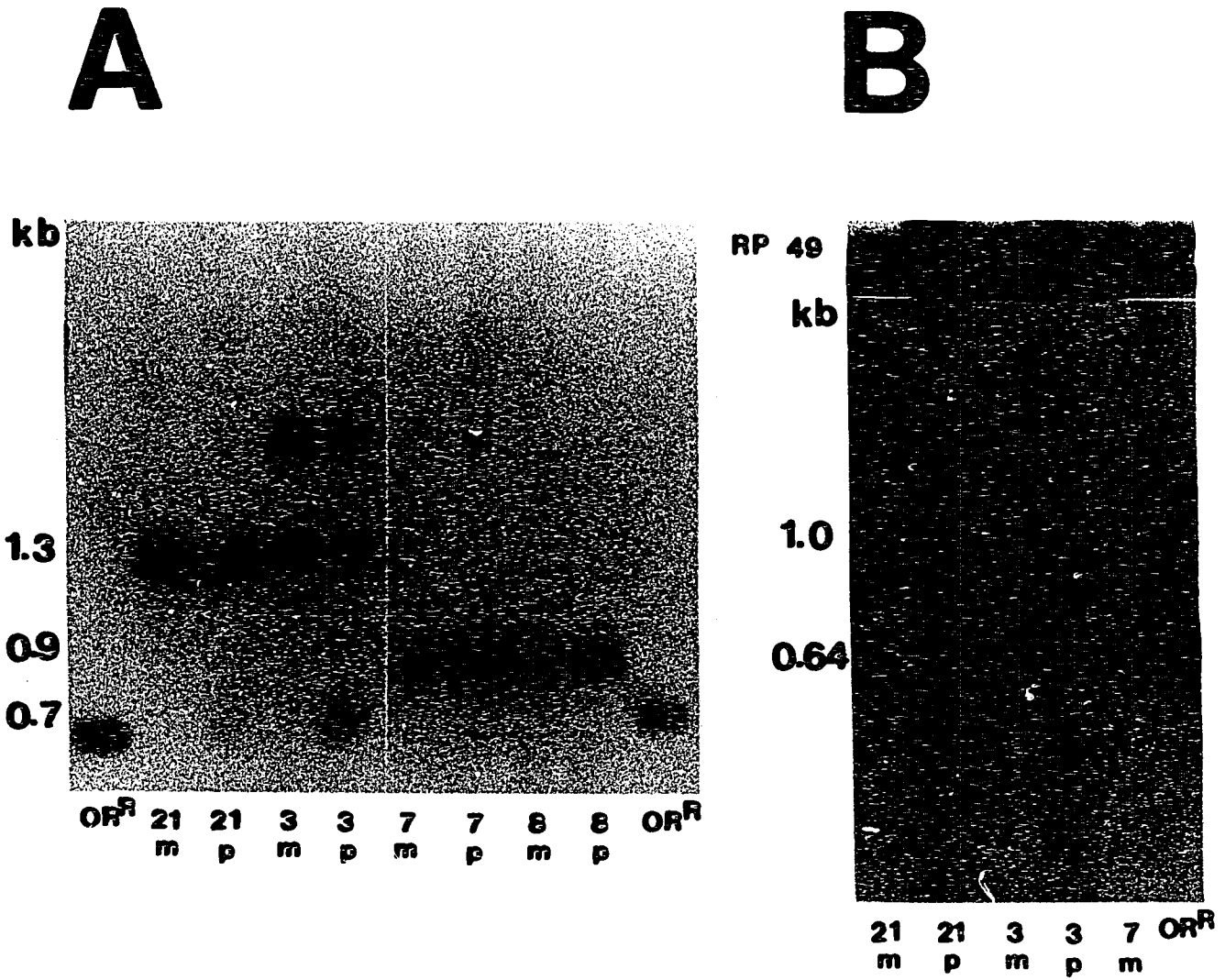

A
B
kb
1.3
0.9
0.7
ORR
21 m
21 p
3 m
3 p
7 m
7 p
8 m
8 p
ORR
RP 49
kb
1.0
0.64
21 m
21 p
3 m
3 p
7 m
ORR

Table IV-II: Cytotype Independence of P-factor Suppression

Cross[a]	F_1 Maternal Cytotype	F_1 *vg* phenotype[b]	Cross	F_1 Maternal Cytotype	F_1 *vg* phenotype
BG(M)♀ x 21(M)♂	M	3	BG(M)♀ x 3(M) ♂	M	5
21(M)♀ x BG(M)♂	M	3	3(M) ♀ x BG(M)♂	M	5
BG(P)♀ x 21(M)♂	P	S	BG(P)♀ x 3(M) ♂	P	S
21(M)♀ x BG(P)♂	M	S	3(M) ♀ x BG(P)♂	M	S
BG(M)♀ x 21(P)♂	M	S	BG(M)♀ x 3(P) ♂	M	S
21(P)♀ x BG(M)♂	P	S	3(P) ♀ x BG(M)♂	P	S
BG(P)♀ x 21(P)♂	P	S	BG(P)♀ x 3(P) ♂	P	S
21(P)♀ x BG(P)♂	P	S	3(P) ♀ x BG(P)♂	P	S

a. Genotypic abbreviations used are: 21 = vg^{21}; 3 = $vg^{21\text{-}3}$; BG = vg^{BG}. The respective cytotypes are designated as M or P.

b. The F_1 *vg* phenotype is either expressed (not suppressed) and is designated as in Table I, or is not expressed (suppressed) and is designated S. The suppressed F_1 (S) flies usually show a wild type phenotype (1) but a small number of flies (<10%) show only partial or no suppression. We feel that this indicates that the chromosomes of the P-strains have different abilities to suppress, probably due to the distribution of P-elements.

due to a low level of spontaneous excision of the vg^{21-3} insert in the vg^{21-3} stock, since these excisions would not be phenotypically detectable in the suppressed line. When the vg^{21} and vg^{21-3} P-cytotype lines are backcrossed twice into a vg^{BG} (M-strain) background the suppression is relieved (i.e., the phenotypes revert to those of the M-cytotype background of Table IV-I). These results indicate that no irreversible alterations of the inserts had occurred to the two suppressed alleles. Northern hybridization, using RNA from P-cytotype and M-cytotype vg^{21} and vg^{21-3} strains, utilizing a probe which recognizes adult *vg* transcripts is shown in Figure IV-2B. The same blot was reprobed with a ribosomal protein probe to quantify the amount of poly A^+ mRNA is present in each lane (see Figure IV-2B). The interference of *vg* transcription associated with the P-element *vg* alleles in the M-cytotype is considerably relieved when these alleles are in the P-cytotype. Thus, the suppression phenomenon may be mediated by increased *vg* gene transcription. This conclusion is supported by our previous observations that various *vg* alleles have reduced levels of *vg* transcripts detected in adult flies (Figure IV-2B - vg^{21-7} lane, and Chapter III). However, since we have not yet detected *vg* transcripts in larvae, we can not be sure that the effects of the inserts at earlier stages of development (i.e., when the wing phenotype is formed) are the same. As well, the biological significance (with respect to the *vg* phenotype) of the adult transcripts has not been demonstrated.

The vg^{21} (M-cytotype) strain was used to clone the *vg* locus. *In situ* hybridizations to salivary gland chromosomes, Southern filter

hybridizations, and library cloning data (Chapter II), indicated that the vg^{21} strain had several defective P-elements present throughout the genome. This observation indicates that the P-strain mediated suppression observed above is not due to the presence of P-element sequences *per se*, but is probably due to the presence of specific P-factors. Since only four of the P-element induced *vg* alleles are suppressible, comparison of these with the non-suppressible alleles may help define what P-element sequences are required for suppression. The vg^{BG}, $vg^{21\text{-}4}$, $vg^{21\text{-}4Rev}$, $vg^{21\text{-}7}$, $vg^{21\text{-}8}$ and $vg^{21\text{-}9}$ alleles did not react (i.e., are nonsuppressible) to the presence of P-factors. Of these, the $vg^{21\text{-}4}$ and $vg^{21\text{-}4Rev}$ alleles have a 36 bp deletion of *vg* genomic DNA (see Chapter III) which may account for their insensitivity to P-factor suppression. The non-suppressible $vg^{21\text{-}7}$ allele is due to a deletion entirely within the P-element insert of the parent vg^{21} allele (Chapter III) which removes one end of the P-element. While the four suppressible alleles (vg^{21}, $vg^{21\text{-}3}$, $vg^{21\text{-}6}$ and $vg^{21\text{-}7Rev}$) have varying amounts of P-element insert DNA, the three examined extensively (vg^{21}, $vg^{21\text{-}3}$, $vg^{21\text{-}7Rev}$) all contain at least 200 bp of DNA from each end of a P-factor in their respective insert (Chapter III). These results indicate that both ends of the P-factor may be required for the suppression phenomenon. This is consistent with a model in which the effector of suppression binds to the same P-element sites as the transposase. The P-element transposase is normally present only in the germ line due to a germline specific splice between the third and fourth P-element exons (Laski et al., 1986). Since the *vg* gene is

somatically expressed (i.e., affects wing development), it is unlikely that the transposase is responsible for suppression. However, P-element repression is produced in somatic tissues, since repressor producing P-elements repress transpositions when an *in vitro* modified construct is present which produces somatically active transposase (H. Robertson and W. Engels, personal communication) Thus, it is possible that it is P-element repressor that is responsible for the suppression phenomenon.

The basis of the repressor function has not been determined but genetic data indicate that complete P-factors are not necessarily required (Hagiwara et. al., 1987). In fact, one repressor producing P-element identified in a natural population has only the first three exons of the P-element intact (Nitasaka et al., 1987). An *in vitro* modified P-element consisting of just the first three P-element exons also acts as a repressor of transposition (H. Robertson and W. Engels, personal communication). When W. Engels introduced this repression-active but defective P-element into the genome of a $vg^{21\text{-}3}$(M) strain the *vg* phenotype was suppressed (W. Benz and W. Engels, personal communication). This supports a model in which suppression of $vg^{21\text{-}3}$ and probably the other suppressible *vg* alleles, is mediated by the P-element repressor. This model suggests that Engels' repressor-producing construct is similar to the repressor produced in natural P-strains, since $vg^{21\text{-}3}$ is suppressed by both Engels' construct and the π2 strain. The observation that suppression is cytotype

independent implies either that repressor levels in somatic tissues are not influenced by the maternal cytotype, or that the suppressible alleles are very sensitive to even low levels of repressor that may be present in the M-cytotype. The observations reported here would not eliminate the possibility that other forms of P-element regulation exist. For instance, Simmons and Bucholz (1985) proposed that extrachromosomal P-elements may inhibit transposition by competitive titration of available transposase. Although the somatic effects we observe would not be predicted by this model, we can not eliminate the possibility that this type of regulation also exists. It is also possible that other P-element products that are produced by P-strains and Engels' construct are the mediators of suppression. The first P-element exon has sequence homology to the Tn3 resolvase coding region (Satta et al., 1985a, b). Since resolvase is a DNA binding protein, it is possible that defective P-elements that produce proteins with an intact first exon will suppress *vg*, as these products may bind to the ends of P-element inserts (Hagiwara et al., 1987). Perhaps only a subset of these products would also be repressors of transposition. It would also be of interest to determine if the KP elements which repress P-induced hybrid dysgenesis (Black et al, 1987) suppress $vg^{21\text{-}3}$. The introduction of a variety of defective P-elements into the $vg^{21\text{-}3}$(M) genome should help define exactly what P-element products effect suppression, and whether or not these products may act as repressors of transposition as well. The generation of more vg^{21} or $vg^{21\text{-}3}$ derivatives which have lost their suppressibility will help define which P-element sequences

are required for this phenomenon to occur. It would be of interest to determine if, indeed, these sequences are the same as those required for transposition.

If the effector of suppression binds to the ends of the P-element inserts as predicted, then it is probable that this mediates suppression. In Chapter III, it was suggested that in the M-cytotype (i.e., no repressor), transcription originating within the P-element inserts might cause the *vg* phenotypes of the P-element derived alleles. However, Karess and Rubin (1984) demonstrated that P-element transcription does not appear to be reduced in P-cytotype stocks. This would imply that P-element suppression is not mediated by reducing the expression of inserts, so it is probably a separate phenomenon. It is not known what this phenomenon is, but altered chromatin structure mediated by repressor binding is one possibility.

P-factor mediated suppression of P-element insertion mutants has also been observed at the singed locus (H. Robertson and W. Engels personal communication). The bristle phenotype of certain P-insertion singed alleles is suppressed by P-cytotype while the female sterility phenotype is enhanced. The phenomenon may be similar to the suppressor function of the maize Spm mobile elements. This function is mediated by complete Spm elements (McClintock, 1954) and requires that the suppressed Spm insertions have intact Spm ends (Schiefelbein et al., 1985). The mutations are usually enhanced rather than suppressed (McClintock, 1954), so more *Drosophila* examples are needed to decide whether the analogy with the results described here

is valid. It may be that P-element suppression is relatively common, and will be observed when tested for in other P-element insertion mutants. The effect may be gene specific, or may depend on the position of the insert within the gene. Since the *vg* P-element inserts described here are apparently within the 3' region of the gene (with respect to the adult transcript), there may be a different effect from that associated with insertions in promotor regions. Putative protein binding to P-element sequences in the promotor region may disrupt the establishment or maintenance of transcription complexes thus causing a more severe phenotype (enhancement), while inserts in the other regions of the gene (i.e., as in *vg*) may be suppressed. Several more rigorously characterized examples of P-element mediated alterations in target gene expression will be needed to determine this. However, our results indicate that comparison of the effects of different P-element alleles on target gene expression may be confusing if the genetic background of each allele is not carefully monitored.

Acknowledgements

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Chapter V: The functional complexity of the vestigial locus in *Drosophila melanogaster*[1]

Introduction

A wild type vestigial (*vg*) gene is required for normal wing imaginal disc development, since the absence of a vg^+ gene product results in extensive cell death in this disc (Fristrom, 1969). This results in concomitant complete loss of adult wing margin structures in strains containing null alleles, while hypomorphic alleles have less severe wing margin loss. Thus, the phenotypes produced by *vg* alleles range from those which are homozygous wild type through nicked, notched, or strap wing phenotypes to the classical more extreme alleles (described in Lindsley and Grell, 1968). In addition to wing margin loss, the extreme *vg* alleles also exhibit haltere reduction, erect postscutellar bristles, female sterility and other less well defined phenotypes (i.e., extended duration of first and third larval instars, pupal lethality, leg and abdominal abnormalities; Erk and Podraza, 1986; Bownes and Roberts, 1981a; Borot and Goux, 1981). All but two extant extreme *vg* alleles are completely recessive and define a single complementation group in that they are non-complementing in trans and affect all four phenotypes associated with such *vg* alleles. Of course, hypomorphic weaker *vg* alleles do not show each of these four phenotypes, so

1. A version of this Chapter has been submitted for publication. Williams, J., A. L. Atkin, and J. B. Bell. 1988. EMBO J.

complementation of these phenotypes in trans (i.e., female sterility) is possible (Lindsley and Grell, 1968).

Three *vg* alleles exist which differ from those described above. Two of these behave as strong dominants (vg^U and vg^W). Both dominant alleles show more severe wing and haltere deficiencies when heterozygous with other *vg* alleles, indicating that they likely produce an antimorphic product; at least with respect to the wing and haltere phenotypes. The third exceptional *vg* allele (vg^{83b27}) is unusual in that it defines a second *vg* complementation unit. The vg^{83b27} allele when homozygous produces an extreme wing phenotype, but complete or nearly complete complementation occurs when it is in trans with all recessive fertile and viable *vg* alleles (Alexandrov and Alexandrova, 1988). However, the recessive sterile or lethal *vg* alleles are only weakly complemented or not complemented at all. Moreover, homozygous vg^{83b27} flies affect only the wing and haltere phenotypes, and have normal female fertility and postscutellar bristles. Thus, vg^{83b27} appears to have a different genetic basis from all other previously described *vg* alleles (Alexandrov and Alexandrova, 1988 and our unpublished results).

In two previous Chapters (Williams and Bell, 1988; Williams et al., 1988; Chapters II and III respectively), we reported the cloning of the *vg* locus and the physical mapping of the lesions associated with various *vg* alleles, including vg^{83b27}. The relevant respective physical lesions affecting *vg* function were localized to a 19 kb stretch of DNA within the cloned region. In the present study, cDNAs corresponding to a 3.8 kb

vg transcript were isolated and located on the physical map within the 19 kb sequence of DNA previously shown to be involved in *vg* function. An exon map was established for the cDNA and correlated with respect to the lesions associated with the classical *vg* viable and lethal alleles previously placed on the physical map. The results indicate that the 3.8 kb transcript is the functional *vg* transcript affected by classical *vg* alleles. However, analysis of the vg^{83b27} allele indicates that it has no effect on this transcription unit and defines further sequences, within a major (4.5 kb) *vg* intron, which are required for normal wing development. Several small adult vg transcripts were identified in chapter II (Addendum). The probe which identified these transcripts is entirely contained within both the vg^{83b27} deletion and the 4.5 kb intron. A model is presented, in which this adult transcription unit corresponds to the second complementation unit defined by vg^{83b27}.

Materials and Methods

D. melanogaster culturing: *D. melanogaster* stocks were grown at 24°C and maintained on a synthetic medium (Nash and Bell, 1968). The phenotypes and origins of the alleles used in this study are described in Chapters II and III.

Materials: Restriction enzymes and other DNA modifying enzymes were obtained from BRL or Pharmacia and used according to the manufacturer's instructions. All radioisotopically labelled compounds were purchased from either New England Nuclear or ICN. Nick translated and oligolabelled probes were made with [α^{32}P]-dCTP (3000 Ci/mmole), RNA probes were labelled with [α^{32}P]-UTP (3000 Ci/mmole), and DNA sequences utilized [α^{35}S]dATP (500 Ci/mmol). The third instar larval disc cDNA library was kindly provided by Dr. G. Rubin. The RP49 clone was a gift from Dr. M. Rosbach.

DNA manipulations: All DNA isolations and manipulations were performed as previously described (Chapter II).

Genomic libraries: The *vg*^{79d5} and *vg*^{83b27} libraries were constructed in λGT10. Genomic DNA was digested with EcoR1, size selected on 1% agarose gels, and electroeluted onto a dialysis

membrane. Purification, ligation and packaging were done as previously described (Chapter III). These genomic libraries, as well as the cDNA library, were plated on C600 Hfr, transferred to biodyne membranes (Pall) and prepared for hybridization by standard methodologies (Maniatis et al., 1982).

Southern and Northern filter hybridizations: All gels for Southern or Northern hybridization analyses were blotted onto Genescreen Plus membranes using the capillary blot protocol recommended by the manufacturer (DuPont). RNA for Figure V-6 was extracted, purified by oligo(dT) chromatography, and run on 1.5% formaldehyde agarose gels as in Gietz and Hodgetts (1985). All other RNA samples were extracted by the guanidinium thiocyanate/CsCl method (Berger and Kimmel, 1987) and analyzed on 1% formaldehyde agarose gels as above. Hybridization conditions for all plaque lifts, genomic Southerns, and DNA-probed Northerns were as in Klessig and Berry (1983). Preparation of oligolabelled DNA probes and washing of filters was done as previously described (Chapter II). RNA probes for Northern hybridization analyses were prepared from restriction fragments cloned into Bluescribe (Vector Cloning Systems) and using the transcription protocol of Melton et al. (1984). Hybridizations and washing of RNA-probed Northerns were as described in Chapter III. Southern hybridization blots of recombinant λ phage or plasmid DNAs were hybridized to nick-translated probes as in Maniatis et al. (1982).

DNA sequencing: All sequencing was performed by either the dideoxy method (Sanger et al., 1977) from inserts in M13mp18 and M13mp19 or by double-stranded DNA sequencing (Chen and Seeburg, 1985) of inserts cloned into Bluescribe.

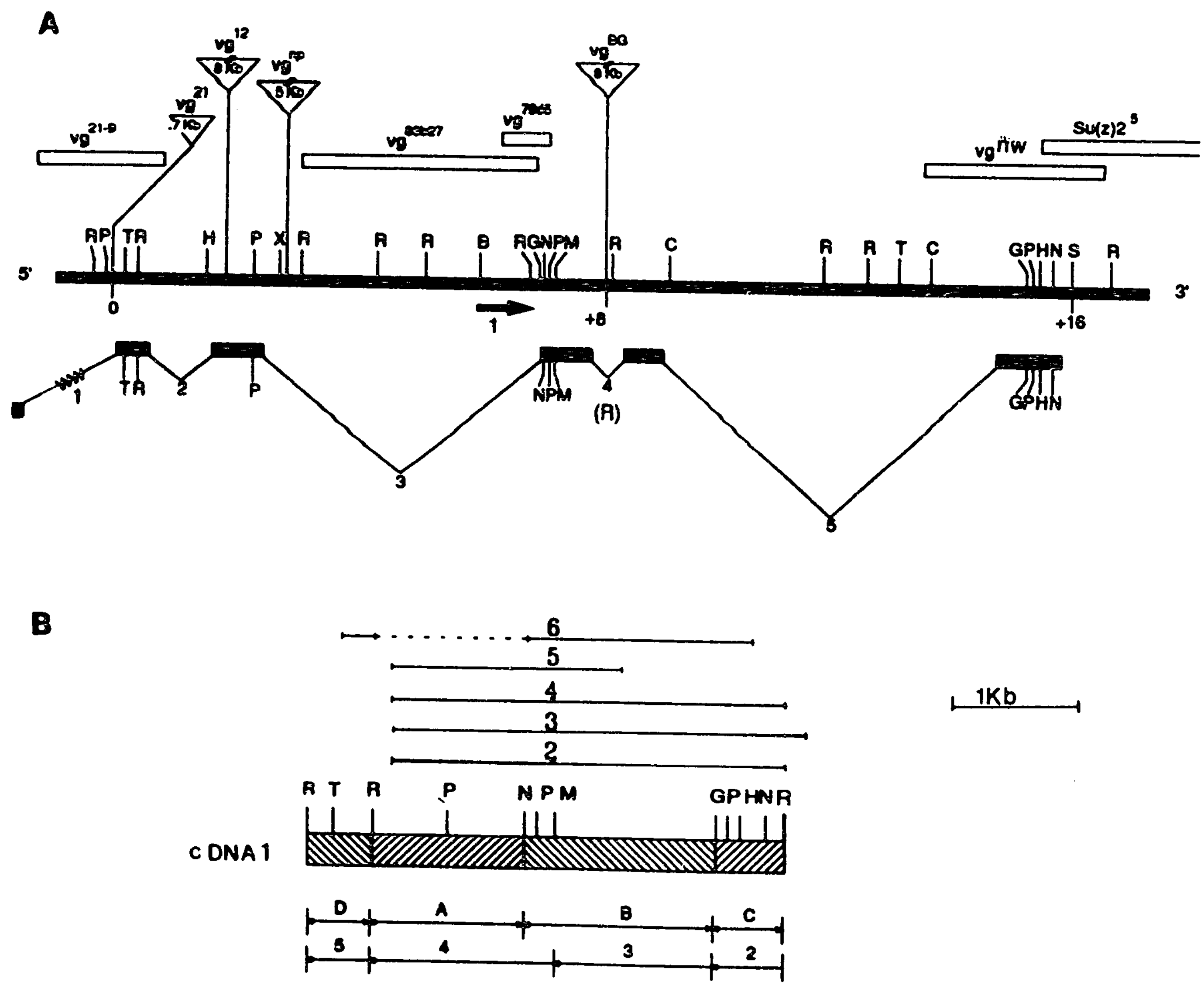
Results

cDNA isolation:

A restriction map of the *vg* region with the physical locations and the nature of lesions associated with various *vg* alleles is shown in Figure V-1. These mutations define the previously characterized region comprising approximately 19 kb of DNA which is required for *vg* function (Chapter II). Several DNA probes from within the 0 to +16 interval (Figure V-1A) identified a low abundance 3.8 kb transcript, present in post 8-12 hour embryos, but undetected in larval stages. An imaginal disc cDNA library derived from third instar larvae was then screened and six *vg* cDNAs whose restriction patterns indicated independent origins were isolated. All six cDNAs hybridized to EcoR1 DNA restriction fragments scattered throughout the 0 to +16 interval on the physical map of genomic *vg* DNA. The hybridization patterns of cDNAs 1-4 were nearly identical except that cDNA1 hybridized to an additional proximal EcoR1 fragment (Figure V-1B). This is consistent with the observation that cDNA1 is the longest cDNA, encompassing two EcoR1 fragments of 3.2 kb and 0.5 kb in size. Subcloning, restriction mapping and hybridization of cDNA1 restriction fragments to cloned genomic restriction digests of DNA from the *vg* region allowed orientation of the cDNA exons with respect to the genomic physical map. This resulted in defining six exons, with all but the most 5' exon assigned to definitive positions. This alignment is presented in Figure V-1A, and a restriction map of cDNA1 is shown in Figure V-1B

Figure V-1. The vestigial locus of *D. melanogaster*. Panel A shows a partial restriction map of the locus. Pertinent restriction sites are indicated as R=EcoR1, S=Sal1, T=Sst1, P=Pst1, B=BamH1, G=BglII, H=HindIII, X=Xho1, C=Cla1, M=Sma1, and N=HincII. The open bars above the restriction map designate deletions associated with specific *vg* alleles, while triangles designate insertion alleles. Coordinate "0" is designated as the site of the original P-element insert (vg^{21}) used to clone the locus, and +16 is 16 kb distal to "0". The data are from Chapters II and III. The bars below the restriction map denote the exons of cDNA1, with some pertinent restriction sites indicated. The five mapped introns are labelled 1 to 5. The location of intron 3 has been conclusively determined by DNA sequencing of genomic and cDNA clones spanning this intron. Similar sequencing from the genomic EcoR1 site (at coordinate +.4) distally demonstrated that the cDNA internal EcoR1 site is genomic and the 5' splice site of intron 2 is located approximately 150 bp more distally. The localization of intron 4 is inferred by the absence of an EcoR1 site (R). This site is not polymorphic in any *Drosophila* stock we have examined. One cannot yet discount the possibility of minor exons in introns 4 and 5 or of minor introns within the major exons. The splice junctions indicate that transcription is from left to right. The black arrow denotes the orientation and extent of the antisense RNA probe used in Figure V-6. B. A restriction map of cDNA1 is shown. The terminal EcoR1 sites are linkers utilized in the cDNA cloning protocol. The extent of cDNAs 2-6 is indicated as numbered lines above this map, and these lengths were

determined by homology to restriction fragments and partial restriction mapping. Segments A-D (below cDNA1) denote the location and extent of DNA probes and 2-5 (also below cDNA) denote the extent and polarity of the RNA probes used in this study. A 1 kb scale bar indicates the relative sizes of the cDNA's.



A
B
5'
3'
0
+8
+16
cDNA 1
1Kb

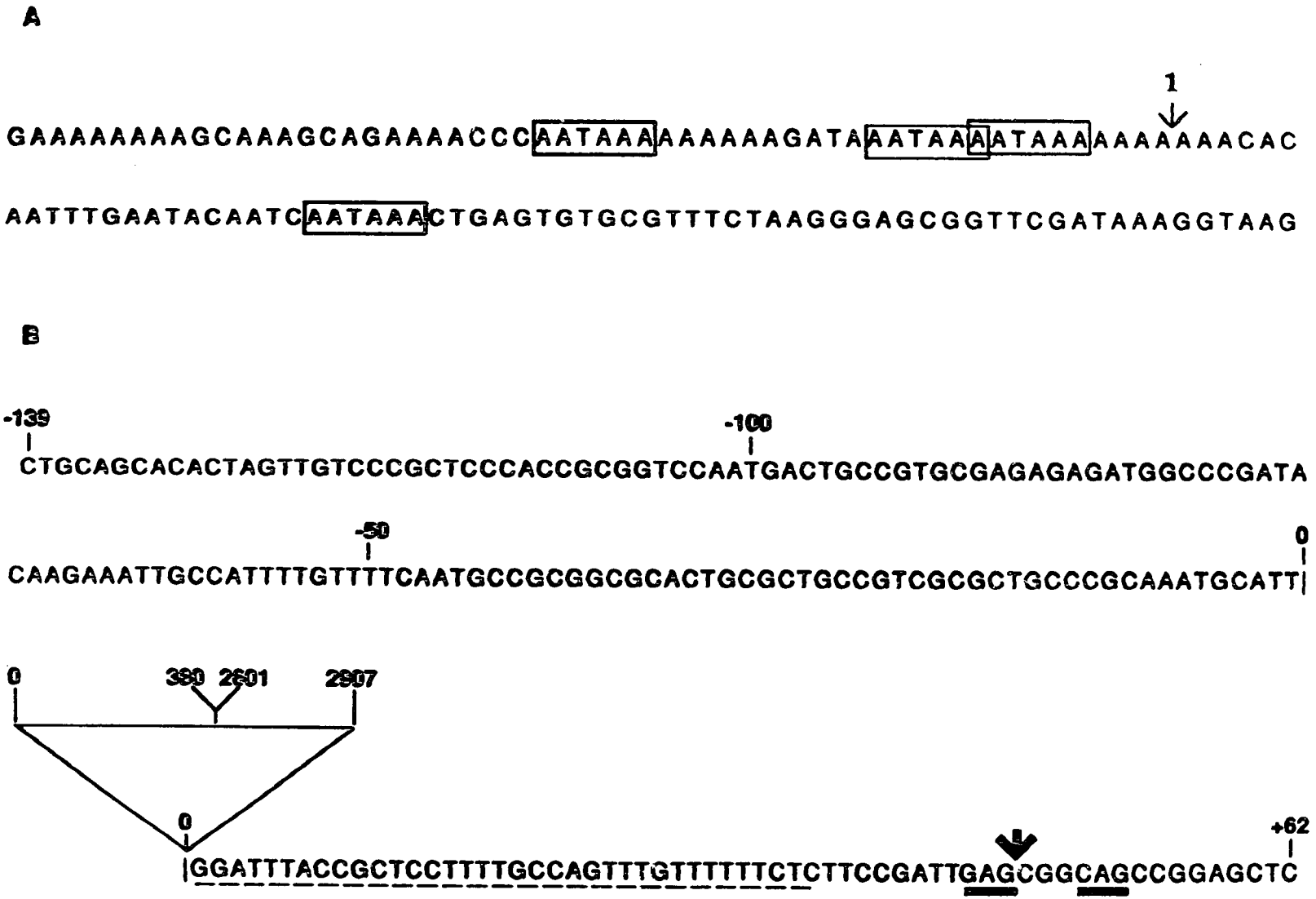
with the extent of cDNAs 2-6 also indicated. cDNA6 is unusual in that it has homology to the 5' and 3' exons, but not to one internal exon (see Figure V-1B). Whether this represents a functionally significant splicing product or merely an aberrant event is unknown. Hybridization of radiolabelled cDNA subclones to Northern blots of RNA obtained throughout ontogeny identified a 3.8 kb transcript expressed in post 8-12 hour embryos (see below). Thus, it appears that the isolated cDNAs correspond to the 3.8 kb transcript identified previously. Since cDNA1 is 3.7 kb long and cDNA3 extends a further 100 bp distally (Figure V-1B), it is likely that the cDNAs represent essentially the full length of the 3.8 kb *vg* transcription unit. Hybridization of single stranded RNA probes to Northern blots indicates that transcription is from proximal to distal in relation to the physical map shown in Figure V-1A.

DNA sequencing of the putative 3' end of cDNA1 identified two overlapping poly-A addition sites preceeded by a third poly-A site. DNA sequencing of M13 clones of genomic *vg* DNA from the +16 Sal1 site indicates that these poly-A addition sites are located approximately 150 bp proximal to the Sal1 site, and thus map the 3' end of this transcription unit to this region (Figure V-2A). This is consistent with the distal limits of the locus as described above.

Correlating the locations of exons with the lesions of vg alleles:

Essentially, all recessive viable and recessive lethal *vg* alleles are non-complementing and thus define a single complementation group.

Figure V-2. DNA sequence analysis at the 3' and 5' ends of the 3.8 kb transcription unit. A. The genomic DNA sequence of the distal (3') region of the transcription unit is illustrated, and this was determined by single stranded sequencing of an OR^R-derived restriction fragment from the +16 Sal1 site (Figure V-1) located approximately 100 bp 3' to the sequence shown. Open boxes designate polyadenylation signals, and the arrow indicates the 3' end of cDNA1. cDNA3 extends approximately 100bp 3' to cDNA1 (Figure V-1B), but preliminary evidence indicates that this is likely due to a long poly A tail in this cDNA. B. The DNA sequence in the vicinity of the vg^{21} P-element insert (near the 5' end of the transcription unit), is shown. The open triangle denotes the 687 bp insert of an internally deleted P-element which is the physical lesion associated with vg^{21}. Numbers within this insert indicate which P-element sequences are present (O'Hare and Rubin, 1983). The genomic Pst1 and Sst1 sites which flank the vg^{21} insert are at coordinates -139 bp and +62 bp, respectively. The data are from Chapter III. The arrow indicates where the cDNA and genomic sequences diverge, while underlined bases show the putative splice junctions. The resulting intron extends proximal to the Pst1 site, since the remaining cDNA sequences do not match the sequence shown. DNA sequence analyses of cDNA1 and genomic clones distal to the Sst1 site indicate that the Sst1 site in the cDNA corresponds to the genomic site (data not shown). The dashed line indicates the extent of the vg^{21-4} deletion (Chapter III).


A
1
GAAAAAAAAGCAAAGCAGAAAACCCAATAAAAAAAAAAGATAAATAAAATAAAAAAAAAACAC
AATTTGAATACAATCAATAAACTGAGTGTGCGTTTCTAAGGGAGCGGTTCGATAAAGGTAAG
B
-139
-100
CTGCAGCACACTAGTTGTCCCGCTCCCACCGCGGTCCAATGACTGCCGTGCGAGAGAGATGGCCCGATA
-50
0
CAAGAAATTGCCATTTTGTTTTCAATGCCGCGGCGCACTGCGCTGCCGTCGCGCTGCCCGCAAATGCATT
0
380
2601
2907
0
+62
GGATTTACCGCTCCTTTTGCCAGTTTGTTTTTTCTCTTCCGATTGAGCGGCAGCCGGAGCTC

Since there are now two *vg* transcription units identified (the 3.8kb and the adult transcripts defined in Chapter II Addendum), previously characterized classical alleles (Chapters II and III) were examined with respect to the exons of the 3.8 kb transcription unit to predict how these alleles might affect the 3.8 kb transcription unit. The results, summarized below, indicate that the 3.8 kb transcription unit is the functional *vg* transcript. A previously characterized classical vg allele, Df(2R)vg^{56}, was shown to delete DNA near to the centromere distal limit of the sequences required for *vg* function (Chapter II). The very weak phenotype of Df(2R)vg^{56} (i.e., Df(2R)vg^{56}/vg^{BG} shows only a strap-wing phenotype) is likely due to a position effect of the vg^{56} breakpoint with respect to the boundaries of the 3.8 kb transcription unit, since the centromere proximal deletion endpoint of vg^{56} is now known to be ~3 kb from the 3' end of the transcription unit. *Su(z)2*^{5} exhibits an intermediate *vg* phenotype, stronger than Df(2R)vg^{56} but weaker than vg^{BG}. Interestingly, the deletion endpoint of *Su(z)2*^{5} is within the 3.8 kb transcription unit but only ~150 bp from the 3' end (Figure V-1). Thus, the *Su(z)2*^{5} breakpoint likely affects 3' end maturation but probably does not affect the product drastically, thus resulting in only an intermediate *vg* phenotype. The deletion alleles vg^{nw} and $vg^{21\text{-}9}$ (at the 3' and 5' end of the transcription unit, respectively) are null alleles, consistent with the fact that both deletions remove large exonic regions of the locus. The null allele, vg^{12}, has a large insertion into an exonic region, while the hypomorphic mutants vg^{BG} and vg^{np} are insertions into regions that appear to be intronic

(see Figure V-1). Sequence analysis of the insertion sites of these alleles will be required to determine the exact relationship of the respective insertions to the *vg* exons. The vg^{79d5} strain is homozygous viable with a strap-wing phenotype, and the physical lesion associated with it is an ~500 bp deletion (Figure V-1). Cloning of the altered region and DNA sequence analysis indicates that the distal endpoint of the vg^{79d5} deletion removes 28 bp of a *vg* exon (see below). The use of more distal alternate splice sites would generate an internally truncated transcript which may retain partial function. A P-element insertion allele (vg^{21}) is located near the 3' end of the *vg* transcription unit (Figure V-1). The genomic DNA sequence of the region surrounding the vg^{21} insertion site is presented in Figure V-2B. These sequence data and those from cDNA1 indicate that vg^{21} is positioned within an intron of unknown size, since the 5' exon of cDNA1 has not yet been mapped with respect to genomic DNA. However, since only cDNA1 extends proximal to this splice site (Figure V-2B), more 5' cDNAs will be needed to confirm this result.

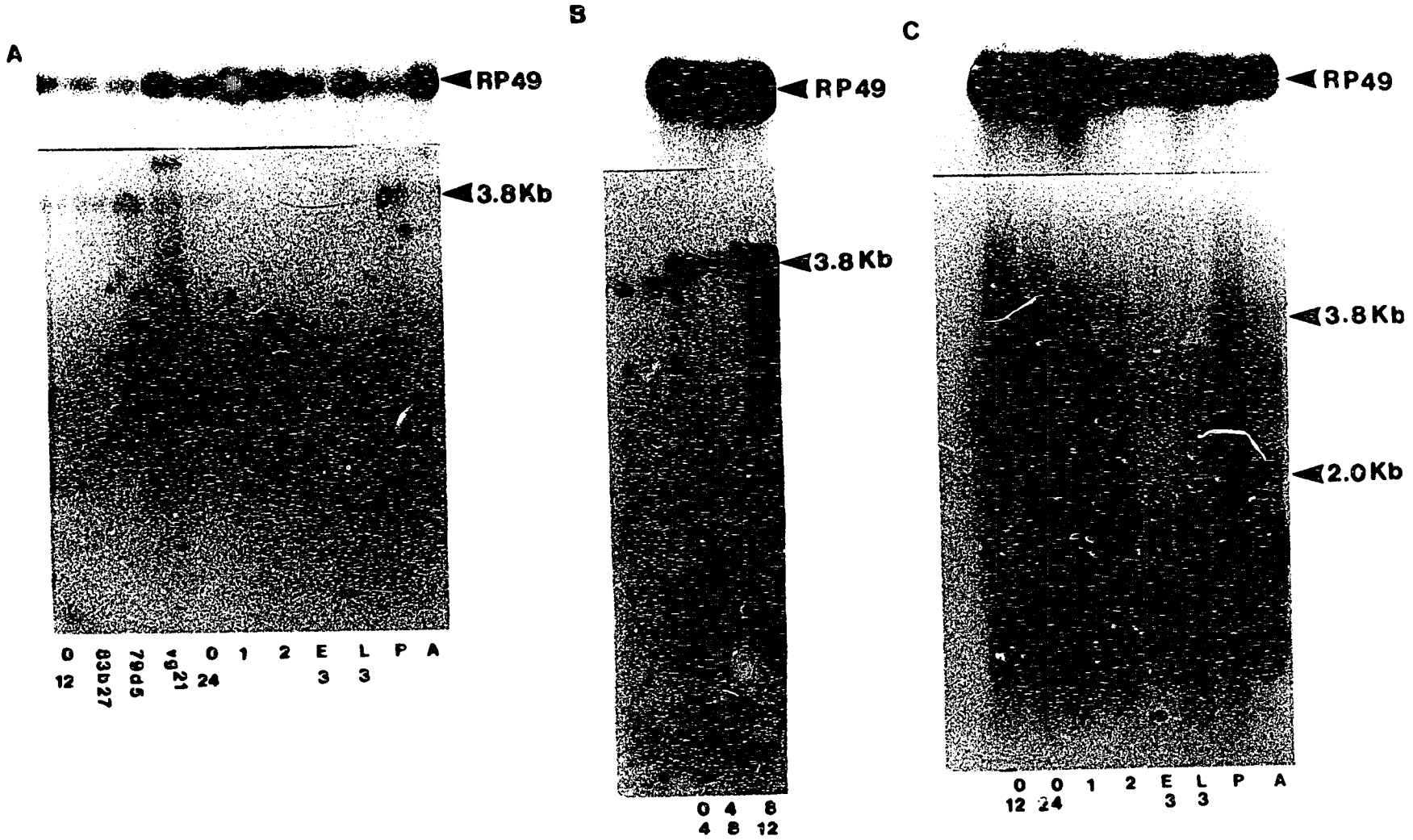
Secondary derivatives of vg^{21} show varying *vg* phenotypes associated with changes in the amount of P-element DNA at this site (Chapter III), consistent with the notion that vg^{21} is an intronic insertion. The vg^{21-4} allele, a homozygous viable phenotypically extreme derivative of vg^{21}, is associated with a 36 bp deletion of genomic DNA immediately adjacent to the vg^{21} insert (Figure V-2B). This deletion removes the branch site of the putative 3' splice junction. This is consistent with the hypomorphic nature of vg^{21-4}, since

characterized branch site deletions appear to reduce the efficiency of splicing rather than abolish it (reviewed in Padgett et al., 1986). Thus, all of the non-complementing *vg* alleles examined in detail appear to have explainable *vg* phenotypes based on their predicted effects on the 3.8 kb transcription unit. These data, in addition to the fact that the 3.8 kb transcript is the only *vg* transcript detected in the imaginal disc cDNA library, provide compelling evidence that the 3.8 kb transcript is the functional *vg* transcript. Thus, the significance of the previously defined adult transcripts is unclear.

Northern Analysis:

Northern hybridization analysis of RNAs collected throughout ontogeny was used to determine the temporal profile of *vg* transcription. Figure V-3A shows such a Northern blot probed with cDNA probe A (see Figure V-1B). Vestigial is expressed at maximal levels in embryos and pupae, and at a lower level in adults. It is interesting that *vg* is expressed in pupae, since pupal lethality is common with many of the strong *vg* alleles. Although we have not yet detected *vg* transcription in larvae, the transcript must be present since the *vg* cDNAs were isolated from a larval imaginal disc cDNA library. This may indicate that the transcript is spatially localized in larvae (i.e., only in imaginal tissues), or may simply reflect technical difficulty in detecting very much reduced levels of transcription. The onset of embryonic expression was determined and is shown in Figure V-3B. The transcript is expressed at very low levels in 0-4 and 4-8 hr

Figure V-3: Northern hybridization analysis of *vg* transcription throughout ontogeny. A. The indicated Northern hybridization blot was hybridized to DNA probe A (Figure V-1B). Size markers are shown in kilobases, with the arrow indicating the 3.8 kb *vg* transcript. The lane designations are: 21=vg^{21}, 79d5=vg^{79d5}, 83b27=vg^{83b27}, and the RNA was collected from 0-24 hr embryos. The remaining lanes contain OR^R RNA from the following stages: 0-12 and 0-24 hr embryos, 1=first instar larvae, 2=second instar larvae, E3=early third instar larvae, L3=late third instar larvae, P=brown pupae, and A=adult. The size markers were a BRL RNA ladder B. This panel shows a Northern hybridization blot of OR^R embryonic RNA samples harvested from samples of the indicated embryonic ages (raised at 23°C). The probe and arrow designations are as in panel A. C. This panel shows a Northern hybridization blot probed with RNA probe 3 (Figure V-1B). The arrows indicate the 3.8 kb *vg* transcript and the prominent 2 kb transcript also detected by cDNA probe B. Ontogenic stage designations are as in panel A. All three blots were reprobed with a ribosomal protein probe (RP49; O'Connell and Rosbach, 1984) to standardize the amount of RNA loaded per lane. The RP49 hybridizations are shown at the top of each panel. All of the above Northern hybridizations were from RNA loaded onto 1% formaldehyde-agarose gels.


A
RP49
3.8Kb
0 12
83b27
79d5
vg21
0 24
1
2
E 3
L 3
P
A
B
RP49
3.8Kb
0 4
4 8
8 12
C
RP49
3.8Kb
2.0Kb
0 12
0 24
1
2
E 3
L 3
P
A

embryos, but is expressed at much higher levels in 8-12 hr embryos. The 0-8 hr expression may be due to minor contamination of these samples with later stage embryos. The *vg* transcription unit remains expressed through the remainder of embryogenesis (i.e., 12-16 hr and 20-24 hr embryos) before decreasing to the limit of detection in first instar larvae (data not shown).

The cDNA probes A, C, and D (see Figure V-1B) recognize only the 3.8 kb transcript. However, probe B also recognizes a 2 kb transcript, and several other transcripts very weakly (data not shown). Duplicate filters of the disc cDNA library were screened with probes B and C to isolate B-specific cDNAs. However, the only cDNA isolated from this screen was a partial cDNA of the 3.8 kb transcript (cDNA5, Figure V-1B). Also, since probes A and D do not recognize the 2 kb transcript, it is unlikely that this transcript represents the cDNA6 splicing product. Antisense RNA probes of A and D recognize the 3.8 kb transcript, while an antisense RNA probe of B recognizes the 3.8 kb and 2.0 kb transcripts, as well as several other transcripts expressed throughout development (Figure V-3C). The multiple transcripts are recognized by antisense RNA probes both proximal and distal to the SmaI site at +7 (Figure V-1). Since these transcripts are not detected with proximal or distal cDNA clones, are not represented in the disc library, and the RNA probes wash off filters at stringencies which do not melt off the RNA probe bound to the 3.8 kb transcript (data not shown), Thus, it appears that this exonic region may encode a protein domain which is present in other non-*vg* RNAs as well (as seen in Figure V-3C).

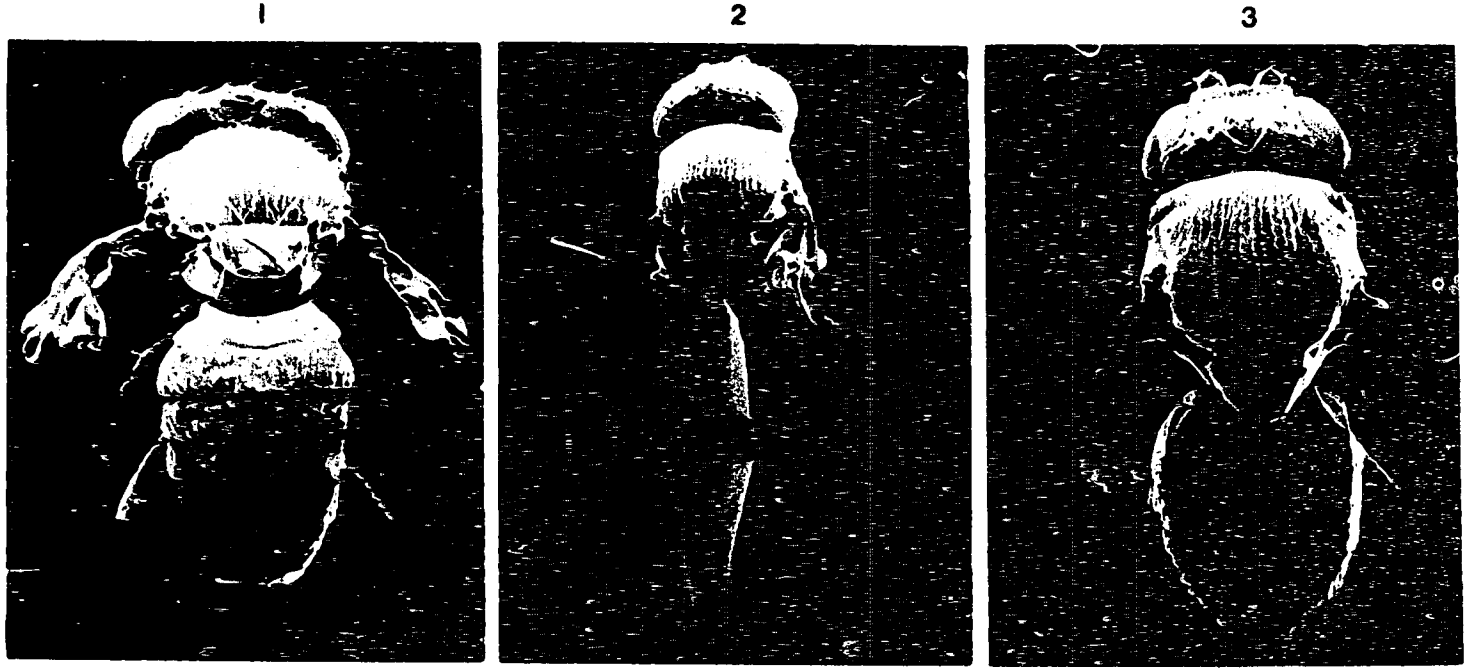
This is not necessarily unusual, since RNA probes have been demonstrated to detect small regions of cross homology which remain undetected with DNA probes (Cavener et al., 1986). This exonic region was sequenced distally from the Sma1 site at coordinate +7 (Figure V-1). This region comprises 285 bp, is GC rich, and has only one open reading frame through it (data not shown). If translated, this region would produce a 95 amino acid domain which contains serine, alanine and glycine rich stretches. Neither this protein motif nor the DNA sequence is strongly homologous to any known cloned *Drosophila* gene (Bionet, 1987). However, weak homologies exist between the *vg* polyserine and polyalanine stretches and the comparable regions seen encoded in the engrailed gene (Poole et al., 1985). However, since these stretches are in the opposite polarity in the respective proteins, the meaning of the homology, if any, is obscure.

vg^{83b27} analysis:

The *vg*^{83b27} allele shows a different pattern of complementation from other vg alleles. Homozygous *vg*^{83b27} flies show extreme *vg* wing and haltere phenotypes, but are wild type with respect to the postscutellar bristle and female fertility phenotypes. As well, *vg*^{83b27} completely, or nearly completely, complements all recessive viable *vg* alleles (Alexandrov and Alexandrova, 1988, and our unpublished results). Thus, *vg*^{83b27} identifies a second *vg* complementation unit. Figure V-4A shows an example of this complementation ability with *vg*^{BG}. Most of the hybrid heteroallelic flies are wild type, but rare

Figure V-4: Complementation behaviour of vg^{83b27} and DNA sequence analysis of vg^{83b27} and vg^{79d5}. A. Whole flies of vg^{BG}(1), vg^{BG}/vg^{83b27}(2), and vg^{83b27}(3) genotypes are shown. The vg^{83b27} homozygotes show strong wing and haltere reductions which differ from the corresponding reductions seen in vg^{BG} or other classical *vg*-allele homozygotes. In addition, vg^{83b27} flies have normal postscutellar bristles. B. This panel shows the DNA sequence of the 135 bp BglII/Pst1 region affected by the vg^{83b27} and vg^{79d5} alleles. The 3' splice acceptor site of cDNA1 is indicated with intronic bases italicized. Labelled arrows denote the 3' deletion endpoints associated with the vg^{83b27} and vg^{79d5} lesions.

A


1
2
3

B


83b27
SPLICE
AGATCTTGAAATGTAATCCAACAAGTAAATTAATAATTTGGTACATTTTCAGTTCAACCAGGATTTGTA
Bgl II
79d5
CTCCTCCTCGGGCGTCAACTTGGCCGCCTCATCGAGTGCCTCGGGCAGCTCCCACTCGACCTGCAG
Hinc II
Pst

heterozygotes fail to exhibit complete complementation. The wing phenotype of these latter flies is unusual, showing bubble wings and unequal wing deficiencies uncharacteristic of classical *vg* wing deficiencies (Figure V-4; results not shown). Recombination analysis was utilized to demonstrate that both the complementation behaviour and the wing phenotype of vg^{83b27} are closely linked to other *vg* alleles. Female $vg^{83b27}/b\ cn\ vg^{nw}$ heterozygotes were mated to homozygous vg^{83b27} males and wild type recombinants were selected. Two phenotypically wild type flies were isolated from 1000 flies scored, but neither were recombinant. Rather, they were due to the weak (or occasional) complementation between vg^{83b27} and vg^{nw}. These results indicate that the mutant phenotype of vg^{83b27} is very closely linked to a classical *vg* allele (vg^{nw}). Heterozygous females of vg^{83b27}/vg^{79d5} and vg^{83b27}/vg^{21} genotypes (which are complementing combinations) were mated to homozygous vg^{79d5} and vg^{21} males respectively, to select recombinants that had lost the ability to complement the above alleles. No genetic recombinants were isolated from among the 5000 F_2 flies scored for each cross. This provides evidence that both phenotypes are *vg*-specific, and not due to a strong suppressor of classical *vg* alleles linked to vg^{83b27} which is itself unaffected by this hypothetical suppressor. Previous analyses of vg^{83b27} (Chapter II) detected a single 4.0 kb deletion that physically maps within the locus (Figure V-1). This deletion removes most of the 4.5 kb intron of the *vg* transcription unit. This is surprising since homozygous vg^{83b27} flies

have an extreme vg phenotype more typical of that expected for null alleles that perturb exons. The 5' end of this deletion clearly does not overlap a *vg* exon since it maps at least 500 bp from the 3' end of the upstream exon. However, the 3' end of the vg^{83b27} deletion appears to map quite close to the 5' end of the downstream exon, as does the 3' endpoint of the vg^{79d5} deletion(Figure V-1).

Genomic Southern hybridization analysis of vg^{83b27} and vg^{79d5} indicated that both vg^{83b27} and vg^{79d5} break within a 70 bp HincII/BglII fragment. The vg^{83b27} deletion appears to break closer to the BglII site while the vg^{79d5} deletion endpoint is closer to the HincII site (data not shown). The cDNA exon/intron junction in question is also within this small DNA restriction fragment. Genomic libraries of vg^{79d5} and vg^{83b27} were constructed and clones of the relevant region were isolated (see Methods). The results of DNA sequence analysis of genomic vg^{83b27}, vg^{79d5} and vg^{+} cloned DNA from this region are shown in Figure V-4B. The cDNA exon/intron junction is indicated, and the genomic sequence indicates that the junction is a consensus 3' splice junction. The vg^{79d5} deletion includes 28 bp of the downstream exon. Northern blot analysis of RNA isolated from vg^{79d5} embryos indicates that this allele has normal levels of a transcript approximately 100 bp smaller than the wild type 3.8 kb transcript (Figure V-3A). This indicates that vg^{79d5} uses an alternate splice site ~ 100 bp within the exon affected by the deletion. A number of putative splice junctions are within this region, in all three frames (data not shown). Since vg^{79d5} is only an intermediate allele, it is

likely that the splice is in the correct reading frame. However, isolation and sequencing of cDNAs from vg^{79d5} will be required to confirm this. The vg^{83b27} deletion removes only the proximal two bases of the Bgl II site. Thus, the 3' deletion endpoint of vg^{83b27} is about 50 bp upstream from the splice site and should not affect splicing. This is consistent with Northern hybridization analyses which indicate that the 3.8 kb transcript is unaltered in both size and amount in vg^{83b27} embryos (Figure V-3A). Finally, the vg^{83b27} deletion does not remove a mini exon within the 4.5 kb intron, since sequence analysis of genomic and cDNA clones has conclusively mapped the ends of this intron (Figure V-1, legend).

Analysis of the vg^{83b27} complementation phenotype:

If the vg^{83b27} lesion, indeed, has no effect on the 3.8 kb *vg* transcript, then perhaps the complementing behaviour of the vg^{83b27} allele is simply due to the presence of this functional *vg* transcriptional unit. This was tested by selecting derivatives of vg^{83b27} that had lost the ability to complement other *vg* alleles. Males of vg^{83b27} genotype were treated with 4000 rads gamma irradiation (Co^{60}), mated to vg^{21-7} flies (i.e., a strap-wing vg^{21} derivative that is normally complemented in combination with vg^{83b27}, Chapter III) and non-complementing progeny selected. One such fly was isolated from 1.2×10^4 screened and it had a phenotype typical of that produced by a *vg* null allele. It differed from vg^{83b27} in that it had lost the complementation ability, and also displayed erect postscutellar bristles

and homozygous female sterility. Genomic Southern hybridization analysis (Figure V-5) indicates that this allele has the original vg^{83b27} deletion as well as a second deletion similar in size and location as that of vg^{nw} (see Figure V-1). Since this second deletion removes the 3' exon of the 3.8 kb transcript, by inference it appears that the complementation ability of vg^{83b27} is likely due to the ability to make an intact 3.8 kb transcript. Thus, the puzzling feature of this allele is not its complementation ability, but its homozygous extreme *vg* wing phenotype. Since the phenotype of vg^{79d5} homozygotes is much weaker than vg^{83b27} homozygotes and the vg^{79d5} effect can be attributed to alterations of the downstream exon, then the more extreme homozygous phenotype of vg^{83b27} must be due to the deletion of DNA unique to the vg^{83b27} allele and thus proximal to the vg^{79d5} deletion. Since this DNA is entirely intronic with respect to the 3.8 kb transcript, this implies that there are DNA sequences within the intron which are required for normal wing and haltere development. Thus, the second *vg* functional unit defined by vg^{83b27} resides at least partially within this intron.

The adult *vg* transcripts defined previously are good candidates for the second functional unit, since the RNA probe used to identify these transcripts maps entirely within the vg^{83b27} deletion. The polarity of the RNA probe (see Figure V-1A) indicates that the polarity of the *vg*-specific adult transcripts are opposite to that of the 3.8 kb transcript. Figure V-6 shows a Northern hybridization blot probed with the above strand-specific RNA probe. The adult transcripts are

Figure V-5: Genomic Southern hybridization analysis of *vg*$^{83b27\text{-}R}$. An autoradiogram of a genomic Southern hybridization blot of EcoR1(R), Pst1(P) and Xho1(X) digested DNA from Canton-S (C.S.), *vg*$^{83b27\text{-}R}$, and *vg*^{83b27} strains is shown. The probe was a 1.9 kb Sal1 DNA fragment from coordinates +16 to +18 (Figure V-1A). The arrows indicate the novel deletion fragments in EcoR1 and Xho1 digested *vg*^{83b27} DNA. The *vg*$^{83b27\text{-}R}$ lesion removes a Pst1 site at approximately +15, creating a fusion fragment the same size as that seen from Pst1 digested Canton-S DNA (6.5 kb). Subsequent stripping and reprobing of the blot with other *vg*-region clones indicates that *vg*$^{83b27\text{-}R}$ also contains the expected 4 kb deletion diagnostic of *vg*^{83b27}, but no other detectable alterations (data not shown).

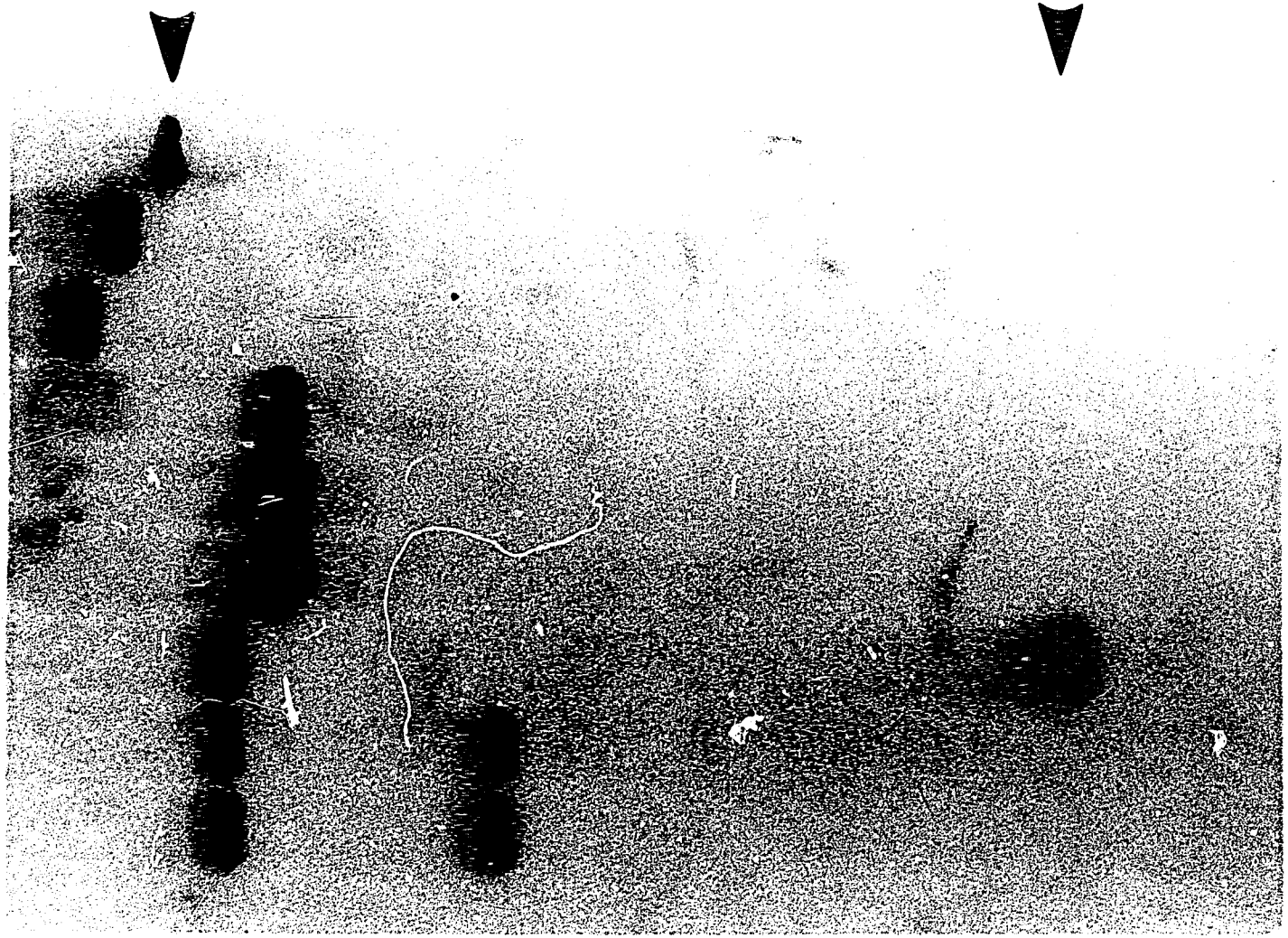
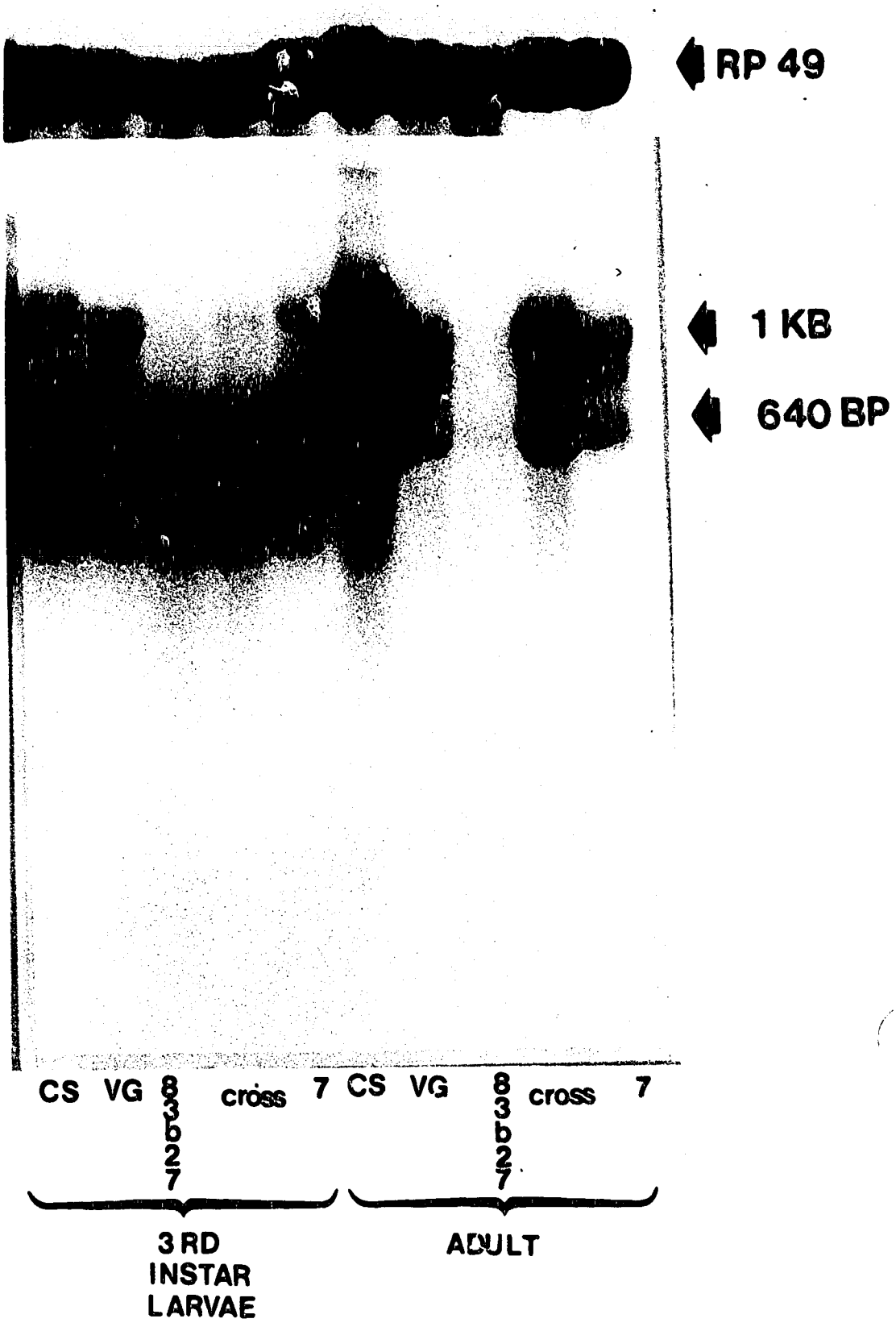

83b27R x
83b27 x
C.S. x
83b27R P
83b27 P
C.S. P
83b27R R
83b27 R
C.S. R

Figure V-6: Northern analysis of adult *vg* transcription. RNA was purified from third instar larvae and adult flies, electrophoresed on 1.5% formaldehyde-agarose gels and probed with the RNA probe indicated in Figure V-1A. The strain designations are CS=Canton-S, vg=vg^{BG}, 83b27=vg^{83b27}, 7=vg^{21-7} and cross=$vg^{823b27/vg21-7}$ heterozygotes. A subsequent reprobing of the blot with a ribosomal protein probe (RP49) allows standardization of the amount of RNA per lane. The size markers were *Drosophila* rRNA and mouse B-globin mRNA.


RP 49
1 KB
640 BP
CS
VG
8
3
b
2
7
cross
7
CS
VG
8
3
b
2
7
cross
7
3 RD
INSTAR
LARVAE
ADULT

vg-specific since they are completely absent in vg^{83b27}, and have decreased levels in vg^{BG} and vg^{21-7} (see also Figure III-8). Interestingly, the vg^{83b27}/vg^{21-7} heterozygote has more of this transcript. Since vg^{83b27} does not produce RNA that can hybridize to the probe, this result must indicate enhanced transcription from the vg^{21-7} chromosome in the heterozygote. The 0.6 kb transcript identified in third instar larvae is not *vg*-specific since it is also present in vg^{83b27} larvae. Although it is possible that the 1 kb RNA in larvae is *vg*-specific and analogous to that in adults, the clear non-*vg* nature of the larval .6 kb transcripts precludes definitive statements about any larval transcript. Again, detection of cross hybridizing transcripts is not unusual using RNA probes (Cavener et al., 1986). Since the adult transcripts are at least partially encoded within the vg^{83b27} deletion, they are good candidates for the second functional unit, implying that the *vg* region contains two separate transcription units both implicated as essential for wing development.

Discussion

In this study we report the isolation of cDNAs corresponding to a 3.8 kb *vg* transcript. Mutational lesions affecting this transcript appear to be responsible for the phenotype of the classical non-complementing recessive viable and recessive lethal *vg* alleles for several reasons. The transcript is expressed in third instar larval imaginal discs (i.e., *vg* cDNAs were isolated from an imaginal disc cDNA library), the tissue which undergoes cell death in *vg* mutants. As well, the cDNA exons are spread throughout the exact region previously defined by deficiency and mutant analyses (Chapter II) as essential for *vg* function. The respective mutant phenotypes of all classical *vg* alleles examined are explainable by alterations to this transcription unit. In the cases of $vg^{21\text{-}4}$ and vg^{79d5}, sequence data indicate that the mutant lesions alter splicing. Indeed, the small exonic deletion predicted with vg^{79d5} sequencing data is observed on Northern hybridization blots. An intronic P-element insertion allele (vg^{21}) is associated with an aberrant larger sized *vg* transcript (Figure V-3A). Finally, the severity of the *vg* phenotype of an allele is correlated with how the data predict the respective lesions would affect the 3.8 kb transcript. The evidence argues strongly that classicial *vg* alleles are the result of alterations to, or influences on, the 3.8 kb transcription unit. Null alleles which destroy the integrity and thus the biological activity of this transcript (ie. vg^{nw}, $vg^{21\text{-}9}$) show extreme wing and haltere loss, erect postscutellar bristles and female sterility, as well as other poorly defined phenotypes including

developmental delay, pupal lethality, and leg or abdominal abnormalities. Hypomorphic alleles which likely only partially remove the biological activity of this transcript (ie. vg^{79d5}, $Su(z)2^{5}$, vg^{21}, $vg^{21\text{-}4}$) will show less severe defects.

An exceptional *vg* allele exists (vg^{83b27}) which displays a complex complementation pattern. Homozygous vg^{83b27} flies have severe wing and haltere reduction, characteristic of extreme *vg* alleles.. However, vg^{83b27} complements all recessive viable *vg* alleles, and weakly complements some recessive lethal *vg* alleles (i.e., vg^{nw}, vg^{12}). Thus, vg^{83b27} defines a second *vg* complementation group. Interestingly, the molecular lesion associated with vg^{83b27} does not appear to affect the 3.8 kb transcription unit, but instead deletes most of a 4.5 kb intron. Adult *vg*-specific transcripts have been identified within this intron (Chapter II, Addendum). These transcripts are transcribed in the opposite direction to the 3.8 kb transcript, and are quantitatively altered in various *vg* allelic backgrounds (Figure III-8 and Figure V-6). I propose that these adult transcripts represent the second *vg* complementation group which is defined by vg^{83b27}. Mutations which remove this transcript's biological activity would be associated with extreme wing and haltere reduction and developmental delay, but would not affect viability, female fertility or postscuttellar bristle development. This model implies that the adult transcripts are also expressed at earlier stages of development since that is when the wing phenotype is determined. This is difficult to assess, since the RNA probe also detects non *vg*-specific transcripts in earlier

developmental stages (Figure V-6, and Chapter II, Addendum). Since *vg*^{83b27} has no effect on postscutellar bristles nor female fertility, this second complementation group is somehow functionally different from the classicial *vg* complementation group.

The identification of a *Drosophila* transcription unit within an intron is not novel (Henikoff et al., 1986). The unusual feature of this *vg* example is the implication that both transcription units are involved in wing development. Since these transcripts are produced from opposite DNA strands, they are unlikely to code for related products. This differs from the numerous characterized examples of genes with more than one product derived from alternate processing of a primary transcript (Bermingham and Scott, 1988; Kuziora and McGinnis, 1988; O'Conner et al., 1988; Schwartz et al., 1988). However, other interpretations of the data can also explain the extreme *vg* phenotype of *vg*^{83b27}. For example, an exon which is required in some developmental stage or tissue but not others may be present within the relevant intron. In this model the adult transcripts also encoded in the intron would not be related to *vg* function at all. I have only matched genomic DNA sequences with cDNA sequences for the cDNAs that have presently been identified. However, since none of the disc-isolated cDNAs contain this putative exon, and no candidate transcripts have been identified on Northern hybridizations, I feel that the alternate exon explanation is unlikely. As well, it is difficult to explain the partial complementation that *vg*^{83b27} exhibits with *vg*^{nw} and *vg*^{12} in a model which implies differential splicing or processing of the same primary transcript, since

vg^{nw} and vg^{12} grossly disrupt two different exons of the primary transcript. Another model implies that vg^{83b27} deletes a binding site or regulatory sequence which is essential for normal regulation of *vg* expression. The observation that vg^{83b27} embryos appear to have approximately normal levels of the 3.8 kb transcript argues against this model. However, tissue dependant alterations could again be invoked. Isolation of a cDNA representing the adult transcription unit, and the generation and analysis of more *vg* alleles with properties like vg^{83b27} will help determine whether the second transcription unit is, indeed, the basis of the second complementation group defined by vg^{83b27}.

There are some puzzling questions that arise from the data. For example, levels of the adult transcript are decreased by mutations which also affect the 3.8 kb transcript (i.e., vg^{BG} and $vg^{21\text{-}7}$ as well as other P-element derived alleles; Chapter III). This implies either that the adult transcription unit requires sequences from throughout the *vg* region, or that expression of the adult transcription unit requires previous expression of the 3.8 kb transcript. The intriguing observation that adult *vg*-specific transcription from the $vg^{21\text{-}7}$ chromosome is enhanced when this allele is heterozygous with vg^{83b27} (Figure V-6) argues in favour of the latter model. As well, since deficiencies of the locus resemble classical alleles in phenotype, it appears that mutational changes that affect the 3.8 kb transcript are epistatic. That is, a "classical" non-complementing *vg* phenotype is observed with deletion mutants whose molecular lesions affect the 3.8 kb transcript alone, or affect both transcription units. The vg^{83b27} class of mutants would be

rare since they would be expected to affect only the adult transcript. Since most *vg* mutant screens employ vg^{BG} to select F_1 mutants, the vg^{83b27}-like alleles would be missed entirely, due to their complementation activity towards vg^{BG}. A modified mutant screen is currently being utilized to selectively isolate more vg^{83b27}-like alleles for analysis.

If, indeed, *vg* has two functional transcription units, it is of interest to speculate what their respective functions may be. Based on wing disc regeneration results, Bownes and Roberts (1981b) proposed that *vg* has abnormal wing disc positional information. In addition, several *vg* alleles have been shown to have aberrant purine nucleotide pools (Silber and Becker, 1981) as well as aminopterin resistance (Silber, 1980) in adult flies. The observation that aminopterin resistance is not rigidly correlated with the wing phenotype (J. Silber, personal communication) may indicate that the metabolic and positional information phenotypes are separable genetic functions. Determination of the encoded products of the 3.8 kb transcription unit and the adult transcription unit should help resolve this question.

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Chapter VI: General Discussion

This thesis reports the cloning and preliminary characterization of the *vg* locus of *Drosophila melanogaster*. The results are recorded in the preceding chapters, each representing a version of a published or submitted manuscript. Chapter II deals with the cloning of the *vg* region, and mapping of the lesions associated with various *vg* alleles into this region. A *vg* P-element insertion allele (vg^{21}) was generated, and used to isolate *vg* region clones by transposon tagging. A short chromosome walk spanning 46 kb was initiated in both directions from the P-element insert. The centromere-distal limits of the locus were established by mapping the breakpoint of $Df(2R)vg^{56}$, an allele that removes DNA distal to *vg* but is only a cryptic *vg* allele. Several transcription units in the centromere-proximal interval of the chromosome walk were identified. Complementary DNA's were isolated representing the three most centromere-distal (ie. vg-proximal) of these transcription units. Since these transcripts do not appear to be altered quantitatively or qualitatively by *vg* alleles, they likely are centromere-proximal to the physical limit of sequences important for *vg* function. Consistant with this hypothesis is the observation that all extant *vg* alleles with detectable lesions have alterations within the 19 kb interval between the $Df(2R)vg^{56}$ breakpoint and these proximal transcription units. Genomic Southern and/or genomic library cloning analyses were used to map the lesions associated with these extant *vg* alleles. Altogether, the alterations associated with over 20 alleles or

chromosome deficiencies were mapped. Only one allele (vg^{168}) among those examined had no detectable alteration within the region and thus could not be physically mapped. It is likely that vg^{168} is a point mutation in the *vg* region.

Several interesting observations were made from this physical analysis. Firstly, the lesions associated with four recombinationally mapped *vg* alleles (vg^{nw}, vg^{BG}, vg^{np}, vg^{21}) were found to show good correlation with the genetic recombination map of these alleles (Carlson et al., 1980). Since the four lesions are distributed over the entire region defined above, this agrees with the prediction that the aforementioned interval is required for *vg* function. Secondly, a revertant allele (vg^{ni}) of a 412 transposable element insertion allele (vg^{BG}) was surprisingly found to be due to a secondary insertion of a 8 kb roo transposable element into the original 412 insert. This indicates that the *vg* region is not sensitive to extra DNA *per se*, since the nearly wild type vg^{ni} revertant has a total of 17 kb of extra DNA inserted into the locus. It also implies that the vg^{BG} 412 insert is intronic, since the mutant phenotype can be reverted by a secondary insertion of DNA. The inversion breakpoints associated with two dominant *vg* alleles were also mapped within the 19 kb interval. It is difficult to interpret the significance of the observation that the breakpoints were only 2 kb apart until functional data (ie. exon maps) of *vg* are generated. Finally , a complex allele (vg^{83b27}) which complements recessive viable *vg* alleles was found to be associated with a 4 kb deletion within the 19 kb interval. These observations implied complex regulatory mechanisms

for the *vg* locus. In fact, the vg^{83b27} analysis proved invaluable in the later functional analysis of the region.

Although not included in the final published version of the first manuscript (ie. Chapter II), adult *vg* transcripts were identified using an RNA probe from the internal region of the locus. Since these were the only transcripts defined at that time, Northern hybridization characterization of these transcripts constituted the "functional" analysis included in Chapters III and IV. Based on the identification and analysis of the 3.8 kb transcription unit reported in Chapter V, it obviously would have been wise to not include the analyses of the adult transcripts in the published papers. Of course, at the time I had not yet discovered the low level 3.8 kb transcript, the detection of which was probably due to the improved RNA extraction protocols used later or my improved technical skills. Thus, it seemed reasonable to include the adult transcript story in the versions of Chapters III and IV which were ultimately published.

Chapters III and IV report the results of an analysis of secondary and tertiary derivatives of vg^{21}, the original P-element allele used to clone the locus. The analysis was undertaken to generate a series of *vg* alleles, all derived from the same parent chromosome with alterations at one site. These mutants would serve as a controlled series of alleles to facilitate future functional analyses of *vg* expression. However, these analyses led to the two truly novel observations of this thesis; the identification and characterization of secondary insertions of P-elements into existing P-element sequences, as well as the

identification of P-element alleles whose phenotypes are suppressed in P-cytotype stocks.

Chapter III reports the generation of nine vg^{21} derivatives, and the characterization of the lesions associated with each of these. In the case of vg^{21} and four of these derivatives, this included DNA sequencing of the inserts. Three of the nine derivatives were due to secondary insertion of P-element sequences into existing P-elements. The sequence analysis demonstrated the mechanism by which one of the secondary insertions arose ($vg^{21\text{-}7\text{-}R}$). This insertion was generated via a second P-element integrating at one end and recombining at the other end of the existing P-element insert. Although secondary insertions have been detected at other loci (yellow and singed, Eggelson and Engels, personal communication) the *vg* alleles were the first reported examples. Subsequently, a paper similar to Chapter III was published by Geyer et al (1988) characterizing secondary insertions at the yellow locus. Interestingly, their results also supported models in which the secondary insertions arose by gene conversion rather than simple insertion of the second P-element. More examples will have to be characterized to determine the prevalence of P-element secondary insertion, as well as the relative roles of integration versus homologous recombination events in their generation.

The effects of these alleles on adult *vg* transcription were also determined, and a model in which P-element transcription interferes directly with *vg* expression was proposed. Although this model is still strictly possible with respect to the adult transcripts, it is not a valid

model to explain how the inserts affect the 3.8 kb *vg* transcript defined in Chapter V. This is because the 3.8 kb transcript is transcribed in the opposite direction to the adult transcripts, so simple collision of Pol-II complexes is unlikely. Alternative models, in which P-element transcripts spliced to *vg* exons mediate *vg* dysfunction, are possible . In fact, the presense of an extra 4.5 kb transcript in vg^{21} embryos (see Figure V-3A) is consistent with this model, since the fusion product of a P-element promoted transcript and the 3.8 kb transcript (linking the P-element and the downstream 3.8 kb transcript exons) would be approximately 4.5 kb (since the vg^{21} insert is .7 kb). Since the *vg* promoter has not yet been mapped to genomic DNA (See Chapter V) it is also possible that the 4.5 kb transcript is due to *vg* promoted transcripts which have not spliced out the P-element within the first intron. Of course, this model would only be possible if the exon 5' to the P-element is found to map quite close to the P-element insert. More data have to be generated before such models can be evaluated. These data would include accurate mapping of the 5' end of the 3.8 kb transcription unit, Northern analysis of the vg^{21} derivatives and the generation and analysis of cDNA's representing the aberrant 4.5 kb transcript observed in vg^{21} embryos (to establish if this transcript is P-element or *vg* promoted).

Chapter IV reports the analysis of cytotype-dependant P-element alleles. Various genetic and molecular experiments were conducted and the results indicated that somatic P-element repressor molecules can suppress the *vg* phenotypes of certain P-element alleles (eg $vg^{21\text{-}3}$).

Since only those alleles which have both P-element ends intact are suppressed, this suppression may be due to binding of P-element repressor to the ends of the P-element inserts (ie. the same sites the P-element transposase binds). A particularly useful experiment cited in Chapter IV was performed by W. Benz and W. Engels. They crossed $vg^{21\text{-}3}$ into a genetic background containing a single repressor producing P-element and found that this resulted in suppression of $vg^{21\text{-}3}$. Although not yet published, W. Benz and W. Engels have extended this analysis (poster at 1988 Toronto Drosophila Research Conference). Despite having unpublished evidence that several singed alleles are either suppressed or enhanced by P-element repressor (H. Robertson and W. Engels, pers. comm.), they have chosen to use $vg^{21\text{-}3}$ as an assay for P-element repressor. This is probably due to a wide range of possible phenotypes between wild-type wings and the extreme wing phenotype of $vg^{21\text{-}3}$ M-cytotype flies. In fact, they have shown that $vg^{21\text{-}3}$ is partially suppressed by intermediate repressor levels. Thus, $vg^{21\text{-}3}$ seems to have good general utility for studies of the P-element repressor; presumably due to the sensitivity displayed by "suppressible" *vg* alleles in their response to the mediator of suppression..

It was also demonstrated in Chapter IV that adult *vg* transcripts are enhanced when the *vg* phenotype is suppressed. However, since the functional significance of these transcripts is unclear, the molecular basis of suppression can not be ascertained from this observation. Northern analysis of the 3.8 kb transcript in M or P cytotype $vg^{21\text{-}3}$ stocks may provide valuable insight into this phenomenom. One possible

model is that binding of P-element repressor to the ends of the P-element inserts mediates suppression. If both ends of the P-element are present, an interaction between repressor molecules at opposite ends may occur. In some cases this interaction will result in suppression of the inserts effect on target gene expression (ie. *vg*$^{21-3}$ and some singed alleles) while in other cases the inserts effect on target gene expression is enhanced (ie. other singed alleles). Enhancement versus suppression would depend on the location of the insert within the target gene. P-element inserts without both ends cannot undergo this interaction and will not respond to P-element repressor molecules. Thus, in this model, the response of various P-element singed alleles to repressor would depend on their location within the singed transcription unit. Of course, the expression pattern and organizational complexity of a target gene may also influence the response of the insert to P-element repressor molecules. I stress that this is only a model, and the characterization of more examples of cytotype dependant alleles will be required to assess it. As well, the molecular basis of the putative interaction between the P-element ends is entirely obscure. It will be of interest to see if P-element cytotype suppression and enhancement turn out to be functionally similar to the suppressor function of the Suppressor/Mutator family of Maize transposable elements (McClintock, 1954; Schiefelbein et al, 1985).

Chapter V reports Northern and cDNA analyses of the 19 kb region previously defined as essential for *vg* function. This study was facilitated by the acquisition of a cDNA library from Dr. G. Rubin,

constructed from larval imaginal disc poly A+ RNA. Since the *vg* wing and haltere phenotypes arise due to disc cell death this library should be enriched for functional *vg* cDNAs. Indeed, all the *vg* cDNAs analysed in Chapter V were isolated from this library, despite the fact that several other libraries (either my own or ones provided by Dr. T. Kornberg) were screened. When characterized, all the disc cDNAs were found to represent a low abundance 3.8 kb transcript, present in embryos and pupae and transcribed with opposite polarity compared to the previously defined adult transcripts. The low level of transcription could account for the inability to detect this transcript in the Northern analysis of Chapter II (Addendum). As well, examination of the RNA probes used to screen the region for transcripts (Chapter II addendum) revealed that none of these RNA probes are in the correct orientation or location to detect an exon of this 3.8 kb transcription unit. Obviously, the presence of two *vg* transcription units was surprising. Therefore, the 3.8 kb transcription unit was analysed with respect to existing *vg* lesions, to establish if this transcript is the functional *vg* transcript. Exons of the 3.8 kb transcription unit are distributed throughout the essential 19 kb interval, and the lesions associated with various *vg* alleles are predicted to affect these exons. Two null *vg* alleles (vg^{nw} and vg^{12}) were associated with gross alterations to *vg* exons. A deletion mutant with an intermediate *vg* phenotype ($Su(z)2^{5}$) was shown to delete the 3' end of the transcription unit, including the poly-adenylation signals but only 100-200 bp of exonic DNA. Two intermediate *vg* alleles (vg^{79d5} and $vg^{21\text{-}4}$) were demonstated to alter

splice donor sites of different exons. As well since the cDNAs were isolated from a disc library the transcription unit is expressed in imaginal discs (the tissue in which cell death occurs) and the transcript size is altered in both vg^{79d5} and vg^{21}. Thus, the data from this analysis provided compelling evidence that the 3.8 kb transcription unit is in fact the functional unit affected by classical *vg* alleles.

Although not reported in this thesis, several experiments have been conducted to attempt to elucidate the basis of the dominant *vg* alleles (vg^{W} and vg^{U}). The analysis was primarily limited to vg^{W}, since vg^{W} is also a homeotic mutant associated with dominant haltere to wing transformations and posterior wing duplications (Bownes and Roberts 1981a). The location of the inversion breakpoints of both vg^{W} and vg^{U} were reported in Chapter II. Comparison of these breakpoints with the exon map presented in Figure V-1 indicates that both inversions break within different *vg* introns. Thus, the dominant *vg* phenotypes are probably associated with an aberrant *vg* product encoded by either the proximal *vg* region, the distal *vg* region, or both. Simple deletion of the *vg* promoter is unlikely to mediate dominance, since the recessive lethal allele $vg^{21\text{-}9}$ deletes the promimal end of the locus (Figure V-1). As well, hybrid dysgenesis was used to screen for dominant vg^{21} derivatives; none were found in 20,000 chromosomes. Since hybrid dysgenesis induces chromosome deletions and inversions at a high frequency, this indicates that simple inversions may not be enough to cause dominant *vg* alleles. This is consistent with the fact that only two dominant *vg* alleles have been reported.

As reported in Chapter II and Bownes and Roberts (1981a), the proximal end of the vg^W inversion is cytologically inseparable from engrailed (*en*). This is intriguing, since *en* is an homeotic gene which is associated with wing duplications. A 35kb chromosome walk was initiated in the *en* region, utilizing the fusion lambda clone which spans the vg^W breakpoint (see Chapter II). Genomic Southern analysis indicated that the vg^W breakpoint was within *Df(2R)SFX-31*, a 180 kb deletion including *en*. Comparison of the published restriction map of the entire deficiency (Kuner et al, 1985) with the restriction map of the 35kb chromosome walk indicated that the vg^W breakpoint was approximately 8kb upstream of the promoter of the invected gene. Since invected cDNAs have been cloned and sequenced (Coleman et al,1987) a restriction fragment from the chromosome walk which should recognize the 5' invected exon was used to screen the Rubin disc cDNA library. Indeed, cDNAs were isolated and sequence analysis of one of these indicated that it was an invected cDNA. Thus, the vg^W breakpoint has split the *vg* gene and fused the distal half of the locus to the 5' end of the invected gene. The proximal end of the *vg* locus is fused to DNA from the invected region, distal to both the engrailed gene and the invected gene. This is intriguing, since invected is a homeobox containing gene, expressed in similar spatial and temporal patterns to the engrailed gene, but for which no function has been assigned (Colman et al, 1987). It is possible that the disruption of the invected gene by the vg^W breakpoint mediates the dominant wing duplications seen with vg^W. To test this, γ-ray mutagenesis was utilized to select

revertants of vg^W which had lost the dominant *vg* phenotype. Four vg^W revertants were isolated, and all four behaved genetically as *vg* null alleles. This is not surprising, since the vg^W inversion splits the *vg* locus. All four revertants also had reverted the homeotic effects of vg^W. This implies that the homeotic phenotype of vg^W and the dominant *vg* phenotype may have the same genetic basis. Genomic Southern analysis indicated that all four revertants retained the vg^W inversion, but had secondary deletions in the invected gene . These results imply that the *vg* dominance is mediated by the fusion of the distal portion of the *vg* gene (the 3' end of the 3.8 kb transcription unit) to sequences from the invected region. It is unknown if this involves a gene fusion or a more complex interaction. As well, the results indicate that the homeotic effects are also mediated by this fusion. Since invected is related to engrailed, it is possible that the homeotic effects are due to inappropriate expression of the invected gene, or perhaps a *vg*-invected gene fusion product or other more complex alterations (McGill et al, 1988). Novel homeotic phenotypes have been shown to result from gene fusion of the *Ubx* and *Abd-A* genes (Casanova et al, 1988; Rowe and Akam, 1988). Clearly more work needs to be done to understand the basis of *vg* dominance and the homeotic effects of the vg^W inversion.

The identification of the 3.8 kb *vg* transcription unit leaves one to explain the significance, if any , of the adult transcription unit. Fortunately, analysis of the vg^{83b27} allele, which defined a second *vg* complementation group, implied that its complementation ability was

due to the production of an intact 3.8 kb transcript in vg^{83b27} flies, and that deletion of intronic sequences was responsible for the wing and haltere phenotype of vg^{83b27}. Since the adult transcripts are at least partially encoded within this intron, a model was proposed in which this second transcription unit is associated with the second functional unit defined by vg^{83b27}. Lesions which remove the 3.8 kb transcription unit or both transcription units cause classical *vg* allele phenotypes which show strong wing and haltere reduction, erect postscutellar bristles and female sterility. Alleles which remove only the second transcription unit (ie. the adult transcription unit) would result in strong wing and haltere reduction as well as developmental delay. The wing phenotype of vg^{83b27} is different from the wing phenotype of classical *vg* alleles (Figure V-4). As well, vg^{83b27} has no effect on postscutellar bristles or female sterility. Thus this second complementation group is somehow functionally different from the classical *vg* complementation group. It would be interesting to examine cell death patterns in vg^{83b27} wing discs, to determine if the wing phenotype is caused by a similar cell death process as that seen in classical vg alleles (ie. vg^{BG}). Mutations of the 3.8 kb transcription unit exhibit epistasis over mutations of the second complementation group since the *vg* phenotype of whole locus deletion alleles (eg. vg^{B}) is the same as mutations which should affect only the 3.8 kb transcription unit (eg. $vg^{21\text{-}4}$, vg^{79d5}). This may account for the observation that the adult transcript is expressed at reduced levels in vg^{BG} and a variety of P-element alleles which affect the 3.8kb transcription unit. If previous

expression of the 3.8 kb transcription unit is necessary for subsequent adult transcript expression, then the model presented in chapter III to account for how the P-element alleles affect levels of the adult transcripts is invalid. In this case, only the effects on the 3.8 kb transcript would be significant; the reduction of adult transcription would merely be a consequence of these effects. However, this model remains untested; perhaps analysis of the predicted products of cDNAs from both transcription units will help explain this phenomenon.

There are presently no data demonstrating a link between the exons of the adult transcripts and the functional sequences deleted in *vg*^{83b27}. It cannot be ruled out that future investigations will fail to establish this link, and that a correct alternative interpretation of the data will be found. However, circumstantial evidence does exist which indicates that the adult transcripts are at least functional. This evidence arises from my unpublished investigations to detect sequences in the *vg* region which are evolutionarily conserved. The *Drosophila melanogaster* and *Drosophila virilis* species diverged an estimated 60 million years ago (Beverly and Wilson, 1984). From reported sequence analysis of *D. melanogaster* and their cognate *D. virilis* genes (Kassis et al, 1986; Blackman and Meselson, 1986; Schaeffer and Aquadro, 1987; Bodner and Ashburner, 1984), conserved regions between genes (ie. coding) show 10-15% divergence (10-15°C lower melting point) while completely non-conserved sequences show approximately 50% divergence (50°C lower melting point). The *Drosophila virilis vg* locus was cloned and physically mapped by restriction endonuclease analysis.

Hybridization of exon-containing D. melanogaster restriction fragments to Southern blots of *D. virilis* restriction digests indicated that the *D. virilis* locus is organized similarly to the *D. melanogaster* locus with respect to the total size and relative positions of the regions homologous to *D. melanogaster* exons. Southern hybridization blots of restriction digested *D. melanogaster* subcloned DNA were hybridized sequentially to three nick translated probes. These were: 1. *D. virilis* complete *vg* locus genomic DNA 2. cDNA 1 (represents all characterized exons of the *D. melanogaster* 3.8 kb transcription unit) 3. *D. melanogaster* complete *vg* locus genomic DNA. In each hybridization experiment, the filters were hybridized under reduced stringency (37°C), and the resultant hybrids melted off by sequential washes at increasing stringency until all hybrids had denatured (assayed at each wash by autoradiography). The results indicated that essentially all restriction fragments tested from the *D. melanogaster vg* locus denatured from their *D. virilis* hybrids at approximately 10-20°C below the melting point of the *D. melanogaster*/*D. melanogaster* hybrid.Thus, by this assay, the entire *vg* locus (including introns) is conserved between *D. melanogaster* and *D. virilis* (comparing to the divergence estimates given above). This is perhaps not surprising, since Henikoff and Eghtedarzadeh (1987) showed that short patches of conservation exist between intronic regions of the GART locus for which no functional significance in terms of gene products can be assigned. However, substantial exons should show extensive conservation (if functional), so restriction fragments containing short patches of

homology should be separable from restriction fragments containing exons by examining the intensity of the hybridization signal. Indeed, several strong hybridization signals were detected in the interspecies hybridization experiment, and most of these are restriction fragments which contain exons of the 3.8kb transcript. This indicates that the exons of the 3.8 kb transcript are conserved across species boundaries, and implies that the intensity of signal in interspecific hybrids can be used to distinguish between short patches of homology and the more extensive homology characteristic of functional exons. Two extensive regions of homology which did not correlate with exons of the 3.8 kb transcription unit were also identified. Each major *vg* intron (see Figure V-1) contained one of these regions. When the conserved region in the intron deleted by vg^{83b27} (defined by the .74 kb Eco-R1 fragment at +5 of Figure V-1) was used to probe a Northern blot of wild type *D. melanogaster* adult RNA, a 1 kb transcript was identified. Thus, this provides circumstantial evidence that the 1 kb adult transcript is functional, due to the evolutionary conservation of a restriction fragment containing an exon of it.

Several experiments need to be performed to extend the analyses presented in this thesis. First, cDNA's representing the adult transcript need to be isolated and an accurate exon map generated. It will be of interest to see if an exon of this transcript maps to the conserved region within the second major intron (defined by the 2.0 kb Eco-R1/Cla-1 fragment at +10 of Figure V-1). Sequence analysis of both the exon within the conserved .74 Eco-R1 fragment and the

corresponding region from D. *virilis* will confirm if, in fact, this exon is conserved. Generation and analysis of more vg^{83b27}-like alleles may pinpoint the functional sequences deleted in vg^{83b27}, to test if these sequences are the exons of the second transcription unit. As well, to account for the wing and haltere phenotype of vg^{83b27}, a demonstration that the adult transcripts are expressed at earlier stages of development is required. Finally, P-element mediated DNA transformation may be used, to further define *vg* sequences which can complement vg^{83b27}. These analyses are currently in progress.

The analysis reported in this thesis as well as the experiments proposed above should together provide a good foundation for future functional analysis of the *vg* locus. This would of course involve the sequencing of the 3.8 kb cDNA, and analysis of the predicted product. As well, the second transcription unit should be sequenced, if indeed it is demonstrated to be functional. This analysis may help one to understand the molecular basis of the *vg* cell death phenotype. Previously published results support models in which *vg* cell death is due to either altered purine salvage pathways (Silber and Becker, 1981) or altered disc positional information (Bownes and Roberts, 1981b). Naively, since two transcripts have been identified, it is possible that one transcript is involved in purine metabolism while the other is required for disc positional information. Examination of the products of both transcripts as well as tissue *in-situ* hybridization to determine the spacial organization of the transcripts should help evaluate this model. It will be of interest to see if *vg* proves to be a good model system for

the study of cell death in *Drosophila*.

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