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Truncated products of the *vestigial* proliferation gene induce apoptosis

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Abstract

The *vestigial (vg)* gene in *D. melanogaster*, whose mutant phenotype is characterized by wing atrophy, encodes a novel nuclear protein involved in cell proliferation. The original *vg* mutant (vg^{BG}) displays massive apoptosis in the wing imaginal disc. Here we tested the hypothesis that the *vg* mutant phenotype could be due: (i) to lack of cell proliferation in null mutants due to the absence of the Vg product and, (ii) to apoptosis in vg^{BG} and other mutants due to the presence of a major Vg truncated product. In agreement with our hypothesis no cell death was observed in null *vg* mutants, and the anticell death baculovirus P35 product is unable to rescue the mutant phenotype caused by absence of the Vg product. In addition, expression of the antiproliferative gene *dacapo*, the homolog of *p21*, induces a mutant wing phenotype without inducing cell death. In contrast the wing phenotype of the original *vg* mutant could be reproduced by the ectopic expression of the *reaper* cell death gene when expressed by *vg* regulatory sequences. In agreement with the hypothesis, the classic *vg* mutant spontaneously displays an increase in *reaper* expression in the wing disc and its phenotype can be partially rescued by the P35 product. Finally, we showed that ectopic expression of a truncated Vg product is able on its own to induce ectopic cell death and *reaper* expression. Our results shed new light on the function of the *vg* gene, in particular, they suggest that the normal and truncated products affect *vg* target genes in different ways.

Keywords: Drosophila; *vestigial* mutant; truncated product; apoptosis

Abbreviations: LTR, long terminal repeat; CDK, cyclin dependent kinase

Introduction

The first *vestigial (vg)* mutant, vg^{BG}, discovered by Morgan in 1911, displays a strong atrophy of the wing. Since then, different *vg* mutants have been isolated with phenotypes ranging from complete absence of wing (vg^{83b27}) to mere nicks at the tip (vg^{ni}), or even a complete wild-type phenotype (vg^{21}).

Controversy exists as to the origin of the vg phenotype Goldschmidt[1] hypothesized that the wing tissue forms normally in the *vg* mutant and then degenerates, whereas Waddington[2] postulated that the vg phenotype is due to the absence of differentiation of the wing tissue.

The work of Fristrom,[3] and O'Brochta and Bryant[4] clearly demonstrates the existence of cell death due to apoptosis in the wing pouch region of the disc, associated with the vg^{BG} and vg^{U} mutants, which could account for the wing phenotype. A significant reduction in mitosis, however, was also observed during the third instar,[5] indicating a reduction in the rate of cell proliferation.

The original vg^{BG} mutation is due to the insertion of a 412 transposable element in the third intron of the *vg* gene. Flies homozygote for the vg^{BG} allele express a minor wild-type size 3.8 kb transcript and a major truncated transcript of 3 kb that ends in the 5′LTR of the 412 element, is polyadenylated, and potentially encodes a Vg truncated product.[6] This raised the possibility that the major truncated product in vg^{BG} mutant flies plays an active part in the induction of the vg^{BG} phenotype. The results of Green[7] were already in agreement with the possibility that vg^{BG} possesses an antimorphic effect. Indeed, using a strain which allows the use of three doses of the *vg* gene, he observed significantly more nicks in the wings of $vg^{+}/vg^{BG}/vg^{BG}$ flies than in vg^{+}/vg^{BG} heterozygotes.

Other *vg* mutants exist: (i) a new vg^{null} mutant from which all *vg* coding sequences were deleted and which is viable in its homozygous state, (ii) the vg^{83b27} mutant which contains a deletion of the 'boundary enhancer' that allows *vg* expression at the level of the dorso-ventral boundary at mid second instar.[8] No *vg* expression was observed in the wing disc of this mutant.

We know now that ectopic expression of *vg cDNA* leads to cell proliferation[9] and, therefore, it is possible that the absence of the gene leads merely to an absence of cell proliferation without necessarily inducing cell death. In this report we explore the possibility that both absence of proliferation and cell death result in a comparable wing phenotype.

In particular we tested the hypothesis that there are two classes of *vg* mutants: (i) one displaying a major truncated Vg product involved in apoptosis and responsible for the phenotype of the vg^{BG} and vg^{U} mutants, and another (ii) characterized by the absence of expression of Vg and resulting in the absence of cell proliferation, which leads to the wing phenotype of the vg^{83b27} and vg^{null} mutants.

In order to test the hypothesis we asked the following questions: (i) is apoptosis observed in both classes of *vg*

mutants? (ii) does ectopic expression of *reaper*, a cell death inducing gene[10] phenocopy the vg^{BG} phenotype? This was achieved constructing a *UAS-reaper* transgene and cell death was induced in those regions of the wing pouch where *vg* is expressed using the UAS-GAL4 system of Brand and Perrimon.[11] (iii) can the anti-cell death baculovirus P35 product save the *vg* phenotype when ectopically expressed at the level of the wing margin? (iv) does absence of proliferation in the *vg* regulation sequences produced by ectopic expression of the *dacapo* gene, the Drosophila homolog of the p21, induce a wing phenotype? (v) finally does the truncated Vg product involved in apoptosis? This was tested by ectopically expressing a truncated *vg cDNA*.

Results

Cell death is observed only in mutants displaying truncated transcripts

It has been reported that massive cell death is observed in vg^{BG} and vg^{U} mutants at the beginning of the third instar[3,4] (Figure 1b). Phenotypes and molecular alterations are given in Table 1. In the vg^{BG} mutant a major truncated transcript potentially encoding a truncated product has been observed. The vg^{U} mutant is due to an inversion that fuses the *mastermind* and *vestigial* genes and induces lethality at the homozygote state.[12] Northern analysis showed that vg^{U} also displays a truncated transcript (I. Frouin, personal communication).

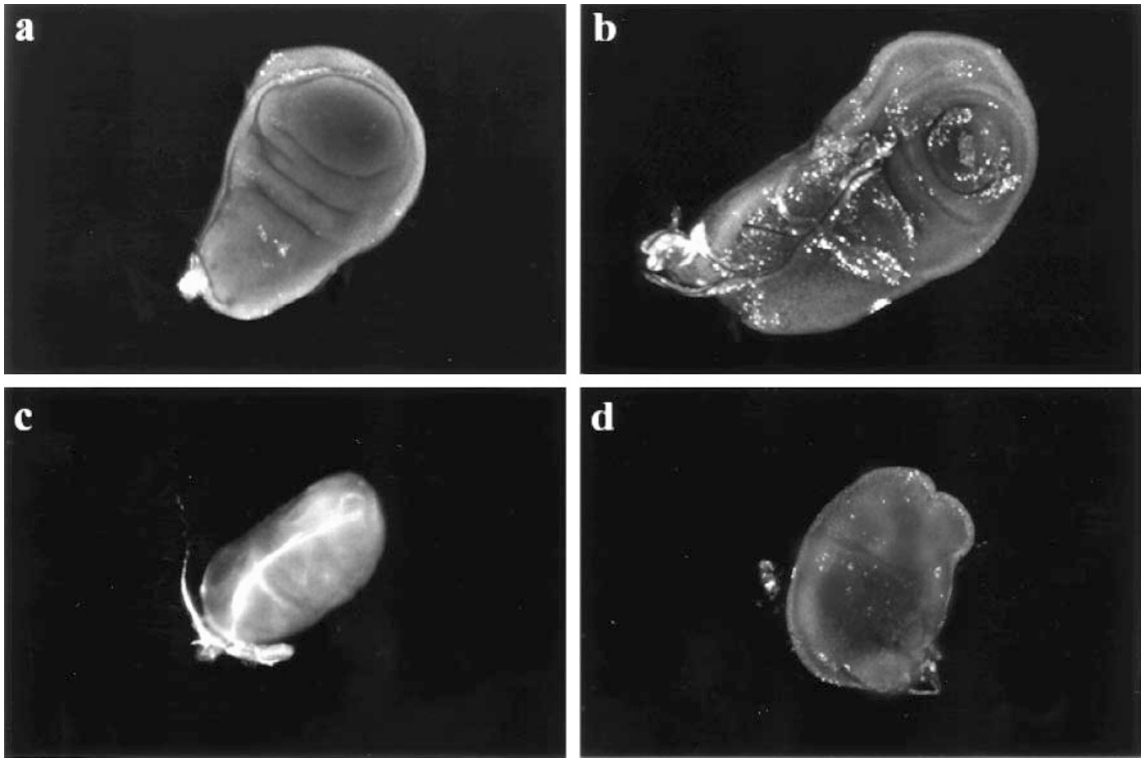

Figure 1 Confocal images of acridine orange staining. Third instar wing disc of: (**a**) wild-type, (**b**) vg^{BG}, (**c**) vg^{83b27}, (**d**) vg^{null} strains. Cell death is observed only in the vg^{BG} mutant

Table 1 Description of the different *vg* mutants

Strain	Phenotype	Molecular data
vg^{+}/vg^{+}	Normal.	3.8 kb transcript.
vg^{BG}/vg^{BG}	Strong reduction of the wing.	A minor 3.8 kb transcript and a major 3 kb transcript that could be polyadenylated and result in a major truncated product[6].
vg^{U}/vg^{+}	Strong reduction of the wing. Dominant phenotype. Lethal homozygote.	Displays for the mutant allele a complex pattern and truncated transcript could be visualised.
vg^{83b27}/vg^{83b27}	Strong reduction of the wing.	Deletion of the 'boundary enhancer'. No *vg* transcript in the wing disc. Normal *vg* expression in the other tissue.
vg^{null}/vg^{null}	Complete absence of wings.	Deletion of the *vg* 8 exons.

We assayed cell death in the vg^{null} and vg^{83b27} mutants in which a complete absence of *vg* transcript is observed in wing imaginal discs. The vg^{83b27} mutant, which is associated with the absence of the 'boundary enhancer', generates a wild-type Vg product during embryogenesis as shown by antibody staining,[13] then expression disappears. Neither mutant, displays significant cell death in wing imaginal disc as assayed by acridine orange staining at third instar (Figure 1c and d). No cell death in the imaginal discs of these mutants was observed during late second instar (data not shown). We could not, however, discount the possibility that cell death occurs in these mutants before.

We tested by semiquantitative RT–PCR the expression of the *reaper* gene in the wing disc. *reaper* is a gene that is responsible for cell death during embryogenesis.[10] Results show (Figure 2) that *reaper* expression is higher in the vg^{BG} mutant than in either the wild-type strain or null mutants. This indicates that cell death indicators like *reaper* are more highly expressed in the vg^{BG} mutant where a truncated Vg product is potentially expressed than in null mutants and suggests that the truncated product of vg^{BG} could act by inducing directly or indirectly *reaper*.

Ectopic expression of *reaper* mimics the vg phenotype

In the wing disc, *vg* expression is dependent on two enhancers which are sequentially activated. The first called 'the boundary enhancer' is under the control, at mid second instar, of *Notch*, by the binding of *Su(H)*[8] (Figure 3a). The second, 'the quadrant enhancer', is indirectly regulated during the third instar by *dpp*.[14]

In order to determine whether cell death alone can account for the vg phenotype, *UAS-reaper* strains were constructed. Using a driver in which sequences of the yeast Gal4 transcription factor were placed under the control of the 'boundary enhancer' (*vg-Gal4* driver), we expressed several transgenic *reaper* lines in wild-type and vg^{BG} backgrounds. The *reaper* gene induces cell death according to the 'boundary enhancer' (data not shown) and a complete absence of wings was observed even in a

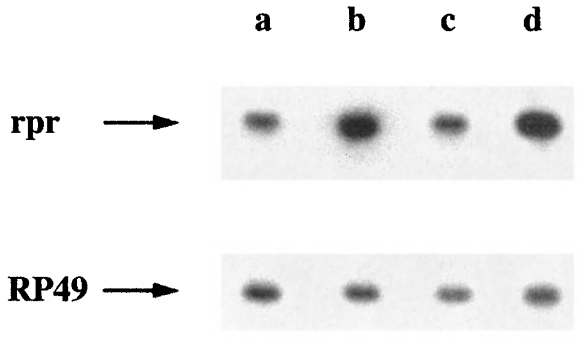

Figure 2 RT–PCR analysis of *reaper* transcripts in wing discs in various genetic background (**a**) wild-type, (**b**) the vg^{BG} mutant, (**c**) the vg^{null} mutant, (**d**) progeny of the *UAStr* by *ptch Gal4* in a vg^{null} background. RNA were isolated from wing discs if third instar larvae and analyzed by RT–PCR for *reaper* and *RP49* transcripts. Aliquots of each RT–PCR products were electrophoresed in agarose gel

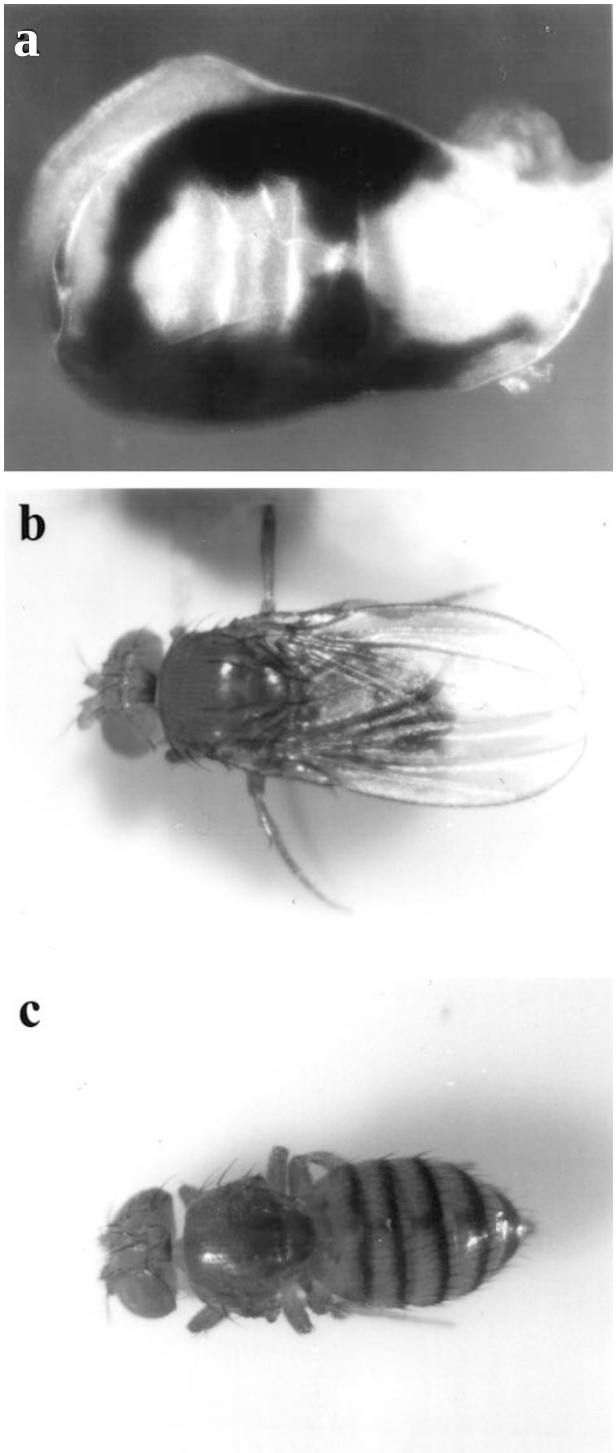

Figure 3 The 'boundary enhancer' sequences permit expression of *vg* at the level of the wing margin and in notum structures. (**a**) This can be visualized by the cross of the UAS *Lac Z* strain by the *vg* Gal4 strain. (**b**) The phenotype of a wild-type fly. In (**c**) the reaper product is expressed in *vg* regulatory sequences determined by the 'boundary enhancer'. A dramatic shift in the wing phenotype is observed (*cf.* **b**)

wild-type background (Figure 3c). Therefore inducing cell death in cells of the wing imaginal disc where *vg* is expressed phenocopies the *vg* mutant phenotype.

The P35 baculovirus product partially suppresses the vg^{BG} phenotype

Cell death induced by the *reaper* gene can be prevented by expressing the baculovirus P35 protein.[15] Using a *UAS-P35* strain, we ectopically expressed the transgene in a vg^{BG} and vg^{83b27} mutant background using the *vg-Gal4* driver. In order to visualize more clearly the effects, a strain with two *UAS-P35* transgenes was constructed.

Ectopic *P35* expression partially suppresses the vg^{BG} phenotype (Figure 4b and c). The fact that a complete wild-type phenotype is not observed is normal since vg^{BG} mutant cells that are rescued from death are presumably still not able to proliferate to give rise to normal wing structures. The effect with the P35 product is not observed with the vg^{83b27} strain (Figure 4d). These results are in

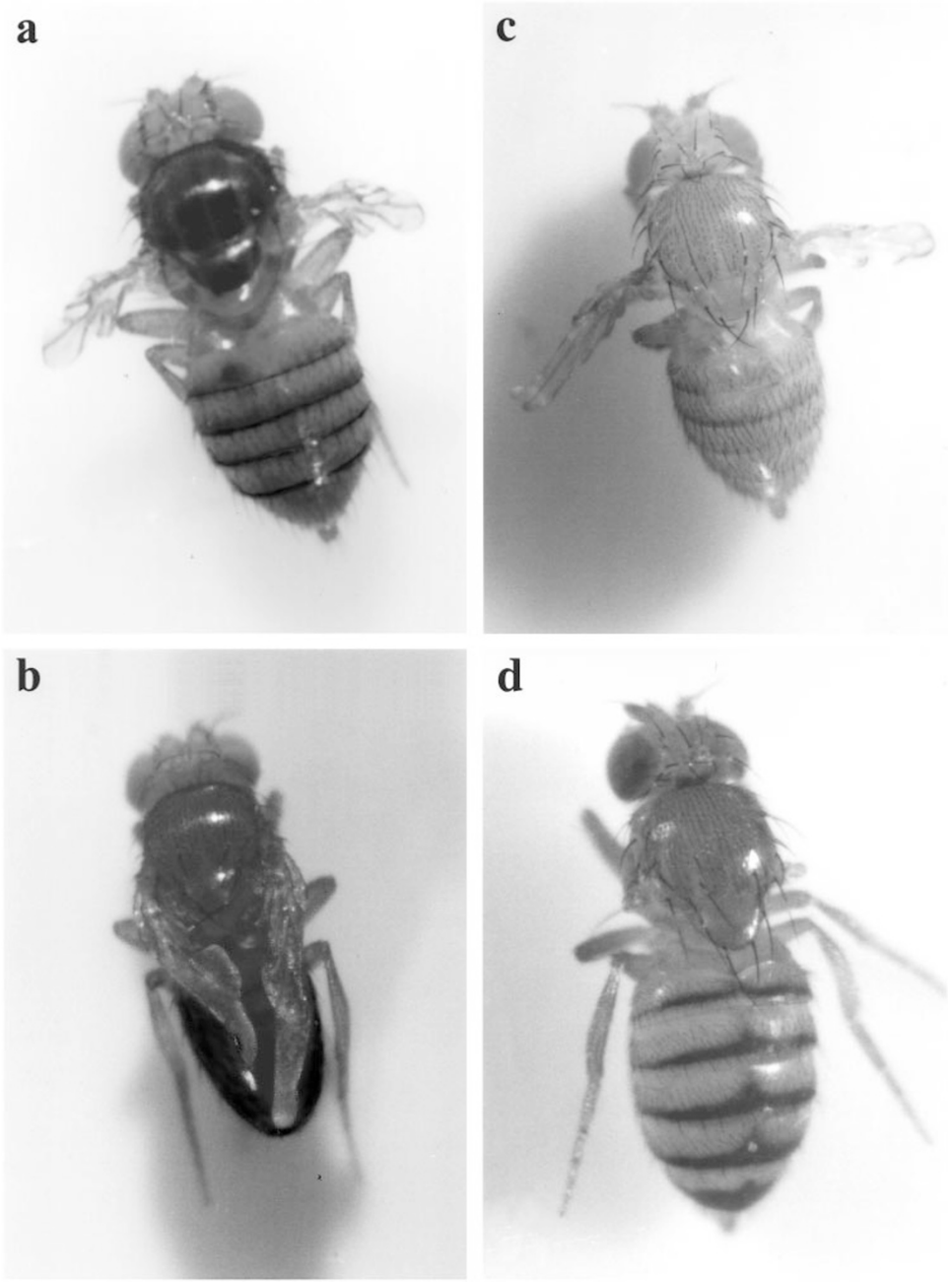

Figure 4 (**a**) the vg^{BG} mutant. (**b** and **c**) The vg^{BG} mutant where the baculovirus product P35 is expressed under the control of the boundary enhancer regulatory sequences. A significant increase in wing phenotype can be observed. (**d**) The same experience with vg^{83b27} mutant. No effect on wing phenotype is observed

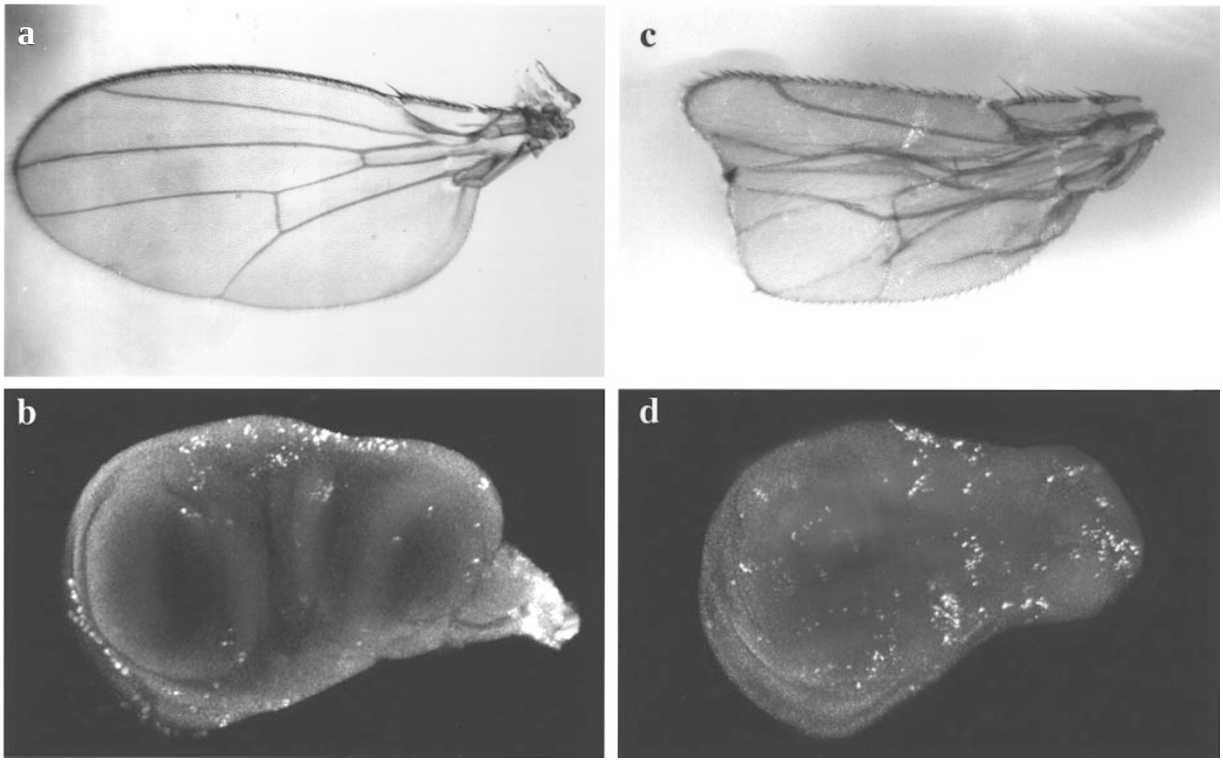

Figure 5 (**a**) F1 of the cross UAS*vg*; vg^{83b27} × *vg* Gal4; vg^{83b27}. When expressed by *vg* regulatory sequences, the Vg normal product can rescue the vg^{83b27} phenotype. (**b**) Cell death observed by acridine staining of the wind disc of the F1 of the same cross. (**c**) The same experiment in a vg^{BG} background, where the rescue is incomplete. (**d**) Cell death observed by acridine staining of the F1 of the cross in (**c**)

favour of the hypothesis that the vg^{BG} phenotype but not that of vg^{83b27} is due to cell death.

Ectopic *vestigial* can rescue the vg^{83b27} phenotype but not that of vg^{BG}

In order to test the role of the 'boundary enhancer' in the vg phenotype, *UAS-vg* strains were constructed[6] and expressed using the *vgGal4* driver. No effect was observed in a wild-type background, whereas in a vg^{83b27} background the extreme *vg* mutant phenotype was completely rescued (Figure 5a). Acridine orange staining showed that cell death is not observed in a vg^{83b27} background (Figure 5b). This confirms that lack of wild-type Vg product in the region defined by the 'boundary enhancer' accounts for most of the vg^{83b27} phenotype. This is not the case in a vg^{BG} background where nicks at wings tips were observed (Figure 5c). Moreover, cell death is observed in the wing disc of the latter according to the driver expression, especially in the notum (Figure 5d). From these results we can infer that the amount of normal vg product ectopically produced by the transgene in a vg^{BG} mutant background could not fully counteract the amount of the endogenous vg^{BG} truncated product.

The antiproliferative gene *decapo* induces a mutant phenotype

The *decapo* gene encodes a product sharing homologies with the P21 protein, which is regulated by the P53.[16,17] P21 can inhibit proliferation by functioning as a general inhibitor of almost all cyclin-CDK complexes. In *Drosophila* ectopic expression of *dacapo* has been used to inhibit myoblast proliferation in the wing imaginal disc. We used this system to ascertain whether lack of proliferation could induce a similar phenotype to that observed in vg^{83b27} and vg^{null}. *dacapo* was induced in the 'boundary region' using the *vgGal4* driver. No effect was observed in a vg^{+} background. However, in a vg^{+}/vg^{null} background, which normally displays a wild-type phenotype, a wing mutant phenotype was observed (Figure 6a), indicating that lack of proliferation can also lead to phenotypes similar to those induced by *reaper*. We established by acridine staining that no significative cell death was observed in the F1 of this cross (*UAS dacapo* × vg^{null}; *vgGal4*).

The Vg truncated product induces apoptosis and *reaper* expression

In order to test the possibility that the Vg truncated product is responsible for the vg^{BG} phenotype by inducing cell death, transgenic flies with a truncated *vg cDNA* (called *UAS-vgtr*) were constructed (see Materials and Methods). We have previously shown that *vg* is able to induce its own expression and that of the *scalloped (sd)* gene. Moreover, Sd and Vg interact in a two hybrid system.[19] The truncated *cDNA* lacks the domain of dimerization with the Sd product[20,21] and, unlike the

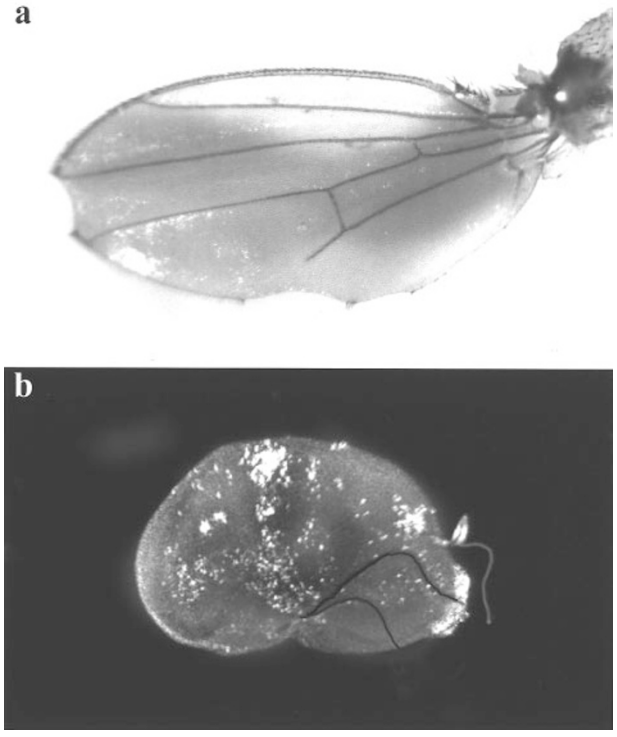

Figure 6 (**a**) The *dacapo* gene inhibits proliferation without inducing cell death. In a UAS *dacapo*; $vg^{+} \times vg$ gal4; vg^{null} offspring display a mutant phenotype. (**b**) The Vg truncated product is able to induce cell death. In the F1 of the cross UAS *vgtr*; $vg^{null} \times$ *patched* Gal4; vg^{null}, cell death is observed according to the expression of *patched* regulatory sequences (*cf.* Figure 1d

transgene with the complete *vg cDNA*, is unable to induce *scalloped* expression, when ectopically expressed (data not shown). It has been shown that a transgene lacking only the domain of dimerization with Sd produces a Vg product localised primarily in the cytoplasm.[20] This indicates that dimerization is necessary for translocation to the nucleus.

No effect on cell death was observed when the Vg truncated product was ectopically expressed using the *patched-Gal 4* driver in a wild-type background. In a vg^{null} background, however, when the same driver was used, ectopically expressed truncated Vg product is able to induce cell death according to the driver (Figure 6b). The absence of results in a vg^{+} background is probably due to the fact that the amount of endogenous *vg* product prevents a visible effect of the truncated one.

We tested the possibility that *vg* induces cell death by inducing *reaper* expression. Indeed we had already observed that *reaper* expression is higher in the vg^{BG} mutant than in the wild-type strain and in the mull mutant (Figure 2). We tested the expression of *reaper* in the wing disc in the F1 of the same previous cross (*UAS-vgtr* by *patched-Gal 4*) in a vg^{null} background. We observed a higher expression of *reaper* than in the vg^{null} mutant. Taken together our results indicate that the transgene is active inducing expression of the *reaper* cell death gene and triggering apoptosis.

Discussion

The Vg product plays a key role in cell proliferation of the wing disc integrating developmental cues by its two enhancers, the 'boundary' and the 'quadrant' enhancers. Ectopic expression of *vg* in all imaginal discs leads to ectopic proliferation of wing tissue.[8] All *vg* mutants characterized so far have been considered to be hypomorph with a wing phentoype due to cell death, as a consequence of a lack of normal Vg product.

Here we propose an alternative hypothesis based on the fact that one category of the *vg* mutants (vg^{BG}, vg^{U}) displays both normal and truncated transcripts, these latter possibly leading to Vg truncated products.

We tested the hypothesis that truncated Vg products are responsible for apoptosis, while absence of normal product leads to an absence of cell proliferation. Both situations should result in a wing phenotype for different reasons.

Our results using the *UAS-reaper* transgene clearly show that cell death in the 'boundary enhancer' region is sufficient to produce a vg-like phenotype. The boundary enhancer region is a region 20 cells wide which overlaps *cut* and *wingless* expression at the wing margin.[22] Since most of the wing mutants, such as *Notch*, *sd* and *Serrate*, display abnormal expression of *cut*, there is thus a correlation between the wing phenotype and the integrity of this part of the wing disc. Massive cell death induced in other parts of the disc does not necessarily lead to a wing phenotype (Silber, unpublished data). This region seems to be at third instar under the particular influence of cell cycle genes[23] exhibiting specific arrests in G1 and G2.[23] This could denote the incapacity of this region to regenerate, compared to other parts of the wing pouch.

Three lines of evidence are in favour of the hypothesis that lack of normal Vg product does not result in cell death, even if they are not completely conclusive: (i) no cell death is observed in lack of function mutants (vg^{null} and vg^{83b27}) and the expression of *reaper* is normal. (ii) the P35 product cannot rescue the phenotype of these mutants. (iii) the induction of the *dacapo* gene, the homolog of *P21*, by *vg* regulatory sequences, leads to a lack of proliferation and a mutant phenotype.

In parallel we provide evidence that truncated Vg product in the vg^{BG} mutant could be involved in apoptosis: (i) the *UAS-vg* transgene rescues the vg^{83b27} phenotype, but not that of vg^{BG}, (ii) the *UAS-vgtr* transgene can induce apoptosis when it is ectopically expressed in a vg^{null} background, (iii) the *UAS-vgtr* transgene can induce *reaper* expression. This implies that this Vg truncated product is biologically active.

The Vg product does not display any known DNA binding domain[13] and needs partners to activate other genes. It has been shown that Vestigial and Scalloped products dimerize and constitute an active transcription factor that could affect cell proliferation.[19,20,21] A transgene lacking the domain of dimerization with sd is exclusively located in the cytoplasm. This indicates that nuclear localization of Vg is dependent on its dimerization with Sd.[20,21] We show that the truncated product, which lacks the dimerization domain, induces apoptosis. We were unable to localise the Vgtr product as available antibodies

do not recognize the NH2 terminal part of the Vg product (data not shown).

Truncated products generated from other mutant genes, have also been shown to display a new apoptotic function. This is the case of BID a member of the Bcl2 family,[24] of Bax,[25] and of the spinal muscular atrophy gene.[26]

Recent data show that *vg* induce cell proliferation by controlling cell cycle transition to G1 to the S phase (Silber *et al*, personal communication). Truncated Vg product could trigger apoptosis either (i) by its cytoplasmic localization that would lead indirectly to the activation of the *reaper* gene or (ii) by disrupting the ratio of Vg and Sd and thus of their dimerization. Variation of this ratio dramatically affects cell proliferation in the wing disc.[20,21] This could possibly deregulate *vg* target genes involved in cell cycle transition in a completely different way than the absence of Vg product.

Our results shed new light on the role of Vg because they imply that two kinds of Vg products (normal and truncated) could act differently by activating or repressing genes implicated in cell proliferation and apoptosis, perhaps affecting cell cycle genes differently. Truncated Vg products could either act abnormally on *vg* target genes or regulate other genes not normally *vg* target genes. We are currently investigating how normal and truncated Vg products affect cell cycles genes.

Materials and Methods

Drosophila strain

The vg^{BG} and the vg^{83b27} strains were kindly provided by J. Bell (Edmonton, Canada). The vg^{83b27} mutant is associated with a deletion of the second intron of the *vg* gene, which removes an enhancer involved in the activation of *vg* at the wing margin. The vg^{null} mutant was isolated in our laboratory by targeted P element mutagenesis. Molecular analysis of this viable homozygote mutant shows that all 8 *vg* exons are deleted. The *UAS-P35* line was generously provided by B. Hay and the *UAS-decapo* line a gift of C. Lehner.

Germ line transformation and ectopic expression

The *reaper* cDNA, kindly provided by J. Abrams, was cloned in the pUAST vector[11] and co-injected with the $\Delta 2-3$ helper plasmid into *Drosophila* embryos.

A 3 kb fragment comprising the complete *vestigial* open reading frame was previously isolated and cloned into the *Eco*R1 site of the pUAST vector.[6] This vector was digested by *Nae*I and *Xho*I, and 1830 bp removed. The new vector encodes a 273aa product that terminates at the beginning of the 4th exon. This UAS-*vg* truncated was co-injected with the $\Delta 2$-3 helper plasmid into *Drosophila* embryo. Ectopic expression was obtained by crossing the UAS lines by various Gal4 drivers.

Escherichia coli β-galactosidase assay

Wing discs were dissected from wandering third instar larvae in PBS 0.1 M and fixed for 30 min in 1% p-formaldehyde, 0.2% glutaraldehyde in PBS 0.1 M. After fixation, discs were rinsed in PBS 0.1 M and incubated overnight in a buffer containing 2 mM X-gal, 4 mM potassium ferricyanide, 4 mM potassium ferrocyanide, 4 mM magnesium chloride in 0.1 M PBS.

Cell death

Cell death was visualized with acridine orange according to Abrams *et al*.[10] Observations were made by confocal laser scanning microscopy using a BioRad MRC-600 confocal imaging system.

RT–PCR

RNA from wing discs were isolated with the Gibco BRL trizol reagent kit, treated with DNAse, reverse transcribed and amplified with the following set of primers: *reaper*: PCR primer GTTGTGTAATTCCGAACGAGG, RtAse/PCR primer GTGTCCTTACTCATAA. RP49: TCCTACCAGCTTCAAGATGAC, RtAse/PCR primer GTGTATTCCGACCACGTTACA. Dnase treatment, reverse transcription, amplification protocols and quantitative analysis of RNA by RT–PCR were carried our according to Zider *et al*.[6] Product of RT–PCR were electrophoresed on a 2% agarose gel and stained with ethidium bromide.

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