An *in Vivo* Analysis of the *vestigial* Gene in *Drosophila melanogaster* Defines the Domains Required for Vg Function

Julie O. MacKay,*† Kelly H. Soanes,*† Ajay Srivastava,* Andrew Simmonds,‡ William J. Brook† and John B. Bell*†

*Department of Biological Sciences, University of Alberta, Edmonton, Alberta T6G 2E9, Canada, †C. H. Best Institute, University of Toronto, Toronto, Ontario M5G1L6, Canada and ‡Department of Biochemistry and Molecular Biology, University of Calgary, Calgary, Alberta T2N 4N1, Canada

Manuscript received October 16, 2002
Accepted for publication January 3, 2003

**ABSTRACT**

Considerable evidence indicates an obligate partnership of the *Drosophila melanogaster* Vestigial (VG) and Scalloped (SD) proteins within the context of wing development. These two proteins interact physically and a 56-amino-acid motif within VG is necessary and sufficient for this binding. While the importance of this SD-binding domain has been clearly demonstrated both *in vitro* and *in vivo*, the remaining portions of VG have not been examined for *in vivo* function. Herein, additional regions within VG were tested for possible *in vivo* functions. The results identify two additional domains that must be present for optimal VG function as measured by the loss of ability to rescue vg mutants, to induce ectopic sd expression, and to perform other normal VG functions when they are deleted. An *in vivo* study such as this one is fundamentally important because it identifies domains of VG that are necessary in the cellular context in which wing development actually occurs. The results also indicate that an additional large portion of VG, outside of these two domains and the SD-binding domain, is dispensable in the execution of these normal VG functions.

Proper development of the wing in *Drosophila melanogaster* involves the coordinate action of several genes. One of the central genes involved in this process is *vestigial* (*vg*). The *vg* gene is a target of both the *decapentaplegic* (*dpp*) and *Notch* (*N*)/wingless (*wg*) pathways and patterned *vg* expression occurs throughout the entire wing field (Williams et al. 1991, 1993, 1994; Kim et al. 1996). In the embryo, *vg* is expressed transiently in some neural and muscle precursor cells, but the majority of expression occurs within the wing and haltere primordia (Williams et al. 1991). As expected, the pattern and amount of *vg* found in the wing disc varies between *vg* mutants (Williams et al. 1991; Simmonds et al. 1997), and both of these factors determine the severity of the mutant wing phenotype in the adults. For example, the *vg*<sup>83b27</sup> mutant phenotype is due to loss of the *vg* boundary enhancer (Williams et al. 1991, 1993). In this case, the normal elevation of *vg* expression does not occur along the dorsal-ventral boundary of the wing disc and wings are absent. The *vg<sup>1</sup>* mutant is caused by an insertion of a 412-transposon element, which allows more wing tissue to be formed than in *vg<sup>83b27</sup>* flies (Williams and Bell 1988). The *vg<sup>927P</sup>* mutant was derived from the *vg<sup>83b27</sup>* allele by gamma-ray mutagenesis (Williams et al. 1990) and behaves as a *vg* null because the 3′ exons are deleted. Expression assays using *vg* transgenes (Kim et al. 1996; Halder et al. 1998; Simmonds et al. 1998) have shown that *vg* exhibits selector gene characteristics in that it is able to reprogram cells to adopt a wing-like fate when expressed ectopically. Thus, *vg* is involved specifically with, and required for, wing development. However, the molecular function of the VG protein within the nucleus was only recently elucidated (Halder et al. 1998; Paumard-Rigal et al. 1998; Simmonds et al. 1998).

Genetically, *vg* is known to interact with *scalloped* (*sd*), another important wing-patterning gene. Indeed, *vg* must act in partnership with *sd* to manifest selector gene ability (Halder et al. 1998; Simmonds et al. 1998; Guss et al. 2001; Halder and Carroll 2001). The expression pattern of *sd* in the wing imaginal disc (Campbell et al. 1991, 1992; Williams et al. 1991, 1993) is almost identical to that of *vg* (Williams et al. 1993). However, *sd* is more widely expressed in other developing tissues compared with the restriction of *vg* expression to the wing disc. Accordingly, some *sd* mutants result in phenotypes similar to *vg* mutants (Campbell et al. 1992) while other *sd* mutants are lethal as homozygotes. *In vitro* binding experiments have demonstrated a protein-protein interaction between SD and VG, effected via a 56-amino-acid region in the VG protein (Simmonds et al. 1998). The overlapping expression patterns of *sd* and *vg* in wing discs, the nuclear localization of both proteins, and this protein-protein interaction support the idea that VG and SD act in partnership during wing development.
development. Halder et al. (1998) used ectopic expression of \( \text{vg} \) to show that the \( sd \) gene is upregulated wherever \( \text{vg} \) is able to successfully induce ectopic wing structures. In \( sd \) mutants, the ability of \( \text{vg} \) to ectopically induce wing tissue is greatly reduced or nonexistent (Simmonds et al. 1998). As well, when \( \text{vg} \) is expressed in the absence of SD or when it is deleted for the SD-binding domain, VG is detected primarily in the cytoplasm. In contrast, when SD is present, VG is found primarily within the nucleus. Recently, it has been shown that expression of only the \( sd \) TEA domain fused to a full-length \( \text{vg} \) is sufficient to accomplish the nuclear localization of VG (Srivastava et al. 2002). It has also been shown that the association of VG with SD is essential for the activation of target genes, including \( sd \) itself (Halder et al. 1998; Simmonds et al. 1998). Thus, it is evident that SD and VG act together as a transcriptional complex during wing formation, wherein SD provides the DNA-binding activity (Campbell et al. 1992; Srivastava et al. 2002) and nuclear localization signal (Srivastava et al. 2002), while VG provides the activation function. Further, other evidence suggests that this complex provides a qualitatively distinct function required to generate a wing-specific response to globally important signaling pathways (Guss et al. 2001) and the presence of VG complexed with SD changes the DNA-binding specificity of SD (Halder and Carroll 2001).

While the importance of the SD-binding domain of VG has been demonstrated clearly both in vitro and in vivo (Halder et al. 1998; Simmonds et al. 1998), the remaining portions of VG have not been tested for in vivo function. Herein, additional regions within VG have been tested in vivo to assess their respective ability to rescue three \( \text{vg} \) mutations (\( \text{vg}^{9327} \), \( \text{vg}^{9327R} \), and \( \text{vg}^{1} \)), to ectopically induce \( sd \) expression, and also to perform other functions associated with full-length VG. The identification of vertebrate homologs of \( \text{vg} \) \( \text{tondu} \) (Vaudin et al. 1999), and \( \text{fondue} \) (Halder and Carroll 2001) suggested the presence of potential activation domains in VG. Using one-hybrid assays in yeast, potential activation domains were found in two regions, one at the N-terminal and another at the C-terminal region of the protein (Vaudin et al. 1999). Furthermore, in vitro DNA-binding experiments of the VG/SD complex to native and synthetic targets also identify important regions of VG outside the SD-binding domain, which are necessary for efficient and accurate binding of the complex and thus for normal VG function (Halder and Carroll 2001). However, in vitro assays can identify regions that do not function in vivo (Vidal and LeGrair 1999). Thus, we sought to test the functional properties of various VG regions in vivo. The present dissection of the \( \text{vg} \) open reading frame (ORF) also identifies important functional domains in addition to the SD-binding domain. The results corroborate the presence of these essential domains in Drosophila VG and demonstrate an in vivo role for these regions during VG-mediated wing patterning, including induction of the endogenous \( sd \) gene.

### MATERIALS AND METHODS

**Drosophila stocks:** All stocks and crosses were raised at 25°. The \( \text{vg}^{9327} \), \( \text{vg}^{9327R} \), and \( \text{vg}^{1} \) alleles were from our local collection. The \( \text{p}^{\text{Gal4}} \) and \( \text{sd-lacZ} \) strains were a gift from S. Carroll.

**Construction of \( \Delta \text{vg} \) deletions:** All deletions were constructed using inverse PCR in either \( pUC19 \) (Vieira and Messing 1982) or pET16b (Novagen). The deletions tested were those from Simmonds et al. (1998), with necessary modifications for in vivo expression of the encoded VG protein. Primer combinations were chosen so as to dissect the \( \text{vg} \) ORF into various fragments, which were then used in the assays discussed below. The \( \Delta \text{vg} \) constructs were cloned into \( pUAST \) (Brand and Perrimon 1993), using BamHI and BamHII and EcoRI restrictions sites in microinjections. Each construct had a SpeI linker at the deletion junction. Thus each final product encoded not only the desired retained portion of the \( \text{vg} \) ORF, but also two consistent amino acids at the boundaries of each deletion.

**Inverse PCR:** The following TaqPhu (20.1) PCR protocol with appropriate primer combinations was used: 94° for 5 min (hot start), followed by 35 cycles of 94° for 30 sec, 68° for 7 min, and holds at 72° for 3 min and then 4°. The total reaction mix was 50 μl, and the full-length \( \text{vg} \) ORF (in pET16b or pUC19) was used as template.

**Micro-injections:** Micro-injections were performed as described in Rubin and Spradling (1983), using \( \Delta \text{vg} \) deletions in \( pUAST \) and helper \( \Delta \text{2-3} \). Two or more independent transgenic lines of each deletion construct were obtained and used in subsequent assays.

**Ectopic expression assay:** The UAS-Gal4 system was utilized (Brand and Perrimon 1993). To express the UAS\( \Delta \text{vg} \) constructs ectopically, each was crossed to \( sd-lacZ; \text{p}^{\text{Gal4}} \) flies (driven by \( \text{p}^{\text{Gal4}} \)). The resulting larvae were stained with X-gal to assess the ability of ectopic \( \text{vg} \) to induce ectopic \( sd \) expression (assayed with a \( sd \) \( \text{lacZ} \) reporter). X-Gal staining was performed for 1.5 hr as described in Bellens et al. (1989).

**Flip-out clones:** Flip-out clones (Ito et al. 1997; Papavannopoulos et al. 1998) were induced in flies trans-heterozygous for an actin \( > + > \) \( \text{GAL4(AyGAL4)}\)\( /\text{UAS-FP} \) chromosome (Y. Hiromi) and the relevant \( \text{vg} \) deletion-bearing UAS constructs, according to the protocol described in Liu et al. (2000). Respective larvae with the above genotype also carried a hs\(-\text{FLP122} \) construct (Struhl et al. 1993). Briefly, the clones were induced by heat shock and then allowed to grow for 48–72 hr at 25° after induction.

**Antibody staining:** Antibody staining was performed as described in Williams et al. (1991). VG antibody was obtained from S. Carroll and used at 1:400 dilution. WG antibody was obtained from Developmental Studies Hybridoma Bank and was used at a dilution of 1:10.

**Rescue assays:** To assess the ability of each \( \Delta \text{vg} \) deletion construct to rescue each of three \( \text{vg} \) mutants (\( \text{vg}^{9327} \), \( \text{vg}^{9327R} \), or \( \text{vg}^{1} \); Williams et al. 1990), the UAS-Gal4 system was used again. All UAS\( \Delta \text{vg} \) lines were made homozygous and the corresponding chromosomal location of each insert was determined (data not shown). From these transgenic lines, UAS\( \Delta \text{vg} \) \( \text{vg} \) lines were generated and crossed to a \( \text{vg}^{Gal4} \) \( \text{vg} \) line. Pupae from this cross were scored for the extent of wing tissue restoration. Since the parental lines were homozygous for either \( \text{vg}^{9327} \) or \( \text{vg}^{9327R} \) or heterozygous for \( \text{vg}^{3278} \)/\( \text{Balancer} \), any increase in the amount of wing tissue in the homozygous
### RESULTS

The deletion constructs used herein are labeled according to the primer combinations that were used in their construction along with a Δ symbol to designate that each is a partial deletion of the vg ORF. A schematic of the deletions made within the vg ORF and the corresponding deleted amino acids is shown in Figure 1. The shaded bars indicate regions that are still present while open bars indicate the regions that are deleted. The previously identified SD-binding motif of the VG protein (amino acids 281–335) is also indicated in Figure 1, and several of the constructs include this motif within the deleted portion.

The ability of each UASvg transgene to induce sd was assessed by X-gal staining for the product of a sd-lacZ reporter in third instar larvae containing this reporter, the UASvg transgene, and a ptcGal4 driver. A sd-lacZ reporter has been shown previously to be activated by the presence of ectopic transgenic VG (Simonds et al. 1998). This was used (Figure 2A) as a positive control for each of the deletion constructs. A negative control, where only the pUAST vector was transformed into flies, was also used (Figure 2B). To ensure that adequate levels of VG were being produced in the wing discs in these ectopic expression assays using the ptc-GAL4 driver, VG antibody staining was performed on larvae from UASvg X sd-lacZ; ptcGal4 crosses. While some constructs were deleted for the anti-VG epitope (Δ4-10, Δ4-8, and Δ2-8), all constructs that encoded proteins that were amenable to antibody detection (full-length vg, Δ5'-5, Δ5'-6, Δ5'-7, Δ1-2, Δ1-4, and Δ7-8) showed ectopic expression of the vg transgene. A representative wing disc from this group (Δ1-4) was utilized to detect VG with antibody staining and is shown in Figure 2C. N-terminal deletion constructs Δ5'-5, Δ5'-6, and Δ5'-7 (which delete amino acids 2–65, 2–170, and 2–278, respectively) are still able to induce sd expression, but this ability is very weak (Figure 2D) compared to the level of sd-lacZ staining induced by expression of full-length VG (Figure 2A). C-terminal deletion constructs able to induce ectopic expression activity included Δ1-2 and Δ1-4 (amino acids 335–426 and 356–

### Figure 1

Deletions made within the vg ORF. All deletions are shown with respect to the full-length construct (453 amino acids). Solid bars represent intact regions and open bars designate areas that have been deleted. The corresponding deleted amino acids are indicated to the right of the bars and the construct names are indicated on the left. Construct names refer to the primer sets used in preparation of each construct, and F refers to a full-length vg construct. The known SD-binding domain (amino acids 281–335) is also shown (vertical shaded bar). The results of the ectopic sd expression assays as well as the wing phenotype rescue assays for vg83b27 and vg are summarized in the two columns on the right. A minus sign (−) indicates a negative result with respect to both assays, while plus (+) signs indicate positive results presented in a graded fashion, wherein the most positive response (+++) is that produced by wild-type vg constructs in the respective assays. The positions of the functional domains identified herein are indicated by solid bars above the protein map.
453 deleted, respectively). Wing imaginal discs from flies expressing these constructs (Figure 2E) show a somewhat stronger X-gal stain than that of the N-terminal deletion constructs (Figure 2D), but much less than that produced by full-length UASvg. The one internal deletion construct that could induce ectopic expression of sd-lacZ was Δ7-8 (amino acids 187–278 deleted). Interestingly, in this case, the level of sd-lacZ activation (Figure 2F) appears comparable to that induced by the full-length vg construct. None of the remaining deletion constructs caused activation of sd-lacZ (data not shown) and thus were similar to the negative control staining produced by the pUAST vector alone. The discs presented in Figure 2, A–F, were selected for staining in the wing blade portion only and the variability in the endogenous pattern in the notum is not reproducible.

The ability of the various deletion constructs to ectopically induce sd is summarized in Figure 1. Multiple transgenic lines (from 2-6) were assayed for each deletion construct and no significant differences were observed between different transgenic lines for the same construct. As expected, all of the UAS-vg constructs that were deleted for the SD-binding domain had no activity in the ectopic sd expression assay (see Figure 1) and these constructs were also negative with respect to showing ectopic production of VG as detected with anti-VG antibody. Thus, the ability to bind SD is a dominant prerequisite in assessing any other aspect of VG function in vivo.

The ectopic expression of vg provides another assay of VG function in vivo. It has been established that expressing VG in tissues where it is not normally present, but where SD is present, results in the production of ectopic wing tissue (Kim et al. 1996). Thus, loss of the ability to produce this ectopic tissue indicates the loss of VG function. An eyelessGal4driver was used to express various UASvg constructs. A representative from each vg deletion group that was able to induce at least some ectopic sd expression was compared to a full-length UASvg construct with respect to being able to produce ectopic wing tissue in the eyes. As expected, full-length vg did produce ectopic tissue and UASΔvg7-8 also exhibited this ability at a similar level (results not shown). However, the loss of either the 5’ putative activation motif (using UASΔvg5-5) or the 3’ activation motif (using UASΔvg1-4) abolished the ability to produce the ectopic outgrowths in the eye, thus indicating the importance of having both of these regions for normal function. Normal VG function may also be visualized in mitotic clones produced using Flp-out technology (Ito et al. 1997). Mitotic clones expressing wild-type VG or a mutant truncated version of VG were marked by coexpression of green fluorescent protein (GFP) in the same clones. Clones expressing only a marker GFP served as a negative control (not shown) and exactly resembled the clones in Figure 3, A and B, which express GFP and a nonfunctional VG. Further, ectopic expression of wild-type vg is also known to induce ectopic wg (Liu et al. 2000). Thus, when the various Δvg constructs are used to induce ectopic vg expression, loss of either the 3’ (using UASΔvg1-4) or the 5’ putative activation domain (using UASΔvg5-6) results in the inability to induce ectopic vg (Figure 3, A–F). However, the UASΔvg7-8 construct behaves like full-length vg in retaining this ability.

Figure 2.—Ectopic expression assay results. (A) sd-lacZ expression in a UASvg ptcGal4;sd-lacZ background. Superimposed on the normal sd-lacZ pattern is the vertical stripe (arrow) indicating ectopic sd-lacZ expression. The UASvg is a full-length vg, which serves as a positive control for the activity of the various vg deletion constructs. (B) No ectopic sd-lacZ expression is present in a pUAST; ptcGal4; sd-lacZ background. In this case only the normal endogenous sd pattern is seen, and this serves as a negative control for the activity of the vg deletion constructs. (C) VG is expressed in the UASΔvg constructs and can be detected with an anti-VG antibody. The white arrow identifies the ectopic VG vertical stripe in a UASΔvg1-4; ptcGal4 background while the yellow arrow identifies endogenous VG expression. For the ectopic sd assays (D–F), only representative results from those constructs able to promote ectopic sd expression (black arrows) are presented. (D) Δ5-5 (amino acids 2–65 deleted). (E) Δ1-2 (amino acids 335–426 deleted). (F) Δ7-8 (amino acids 187–278 deleted).
Figure 3.—The putative activation domains are required for adhesive properties of the cells in which VG is expressed and for the induction of wg in the hinge as judged by Flp-out clonal analysis. Red channel: Wing discs from late third instar larvae allowed to grow for between 48 and 72 hr after clone induction and stained for WG. (B, E, H, and K) The VG-expressing clones are marked by the coexpression of GFP from a UAS-GFP construct and (C, F, J, and L) the overlay of VG and WG expression is shown. (A–C) Flp-out clones from the construct deleting the 3' putative activation domain of vg (UAS\(\Delta vg1-4\)). These clones resemble control GFP clones with characteristic irregular shape, small size, and inability to induce WG in the hinge region. (D–F) Flp-out clones from the construct deleting the 5' functional domain of vg (UAS\(\Delta vg5-6\)). These clones also resemble control GFP clones as in A–C. (G–I) Clones expressing a UAS\(\Delta vg7-8\) construct retain both putative activation domains. Note that the clones are larger, circular in shape with smooth edges, and now are able to induce WG non-cell autonomously in some clones that overlap the hinge region of the disc (demonstrating the existence of adhesive differences within the clones), and are unable to induce wg bordering the clones that overlap the hinge region (Figure 3, A–F). The opposite behavior is exhibited by clones from a UAS\(\Delta vg7-8\) or a UASvg full-length genetic constitution (Figure 3, G–L). Not only are the clones larger with smoother borders, but also functional VG-expressing clones can induce wg expression non-cell autonomously in some clones that overlap the hinge region of the disc (see arrowheads in Figure 3, H, I, K, and L). This assay again emphasizes the functional importance of the two putative activation domains in vivo in the fulfillment of normal VG functions.
While the ectopic sd expression assay or ectopic vg assay provides a quick method to map potential activation domains within VG, the ability of these deleted proteins to substitute for normal VG function needed to be examined as well. Thus, each of the deleted transgenes was also tested for the ability to rescue the phenotypes of vg83b27 and vgR homyzygous flies. The vg83b27 mutant stems from a deletion within intron 2 that includes the vg boundary enhancer (vgBE), and flies homozygous for this allele have virtually no wings. The vgR mutant phenotype is less severe and is caused by the insertion of a 412 transposon into intron 3. However, since these two vg alleles retain some vg function, the vg83b27R null allele was also included in the rescue assay. The ability of each deletion construct to rescue each of these three vg mutants was assessed by comparing the progeny from a cross of UASvg; vg × vgGal4; vg to the phenotypes of flies homozygous for the respective vg tester allele. In the case of vg83b27R, the homozygous vg progeny were selected from a cross of vg83b27R vgGAL4/Cy × vg83b27R UASvg/Cy. The rescue of vg83b27R by the full-length UASvg construct is shown in Figure 4A and that of vgR in Figure 5A, and these results are also summarized in Figure 1. The same negative control (pUAST construct alone) was used in these rescue experiments as in the ectopic sd assay. No wing tissue restoration was observed in the control flies (results not shown). In each case, a correlation was observed between the ability to induce ectopic sd expression and the ability to rescue each vg mutant phenotype. For vg83b27R, deletion constructs Δ5′-5, Δ5′-6, and Δ5′-7 were able to produce only a partial rescue, as shown in Figure 4B for Δ5′-5. The rescue ability of deletion constructs Δ1-2 and Δ1-4 was also only partial (Figure 4C for Δ1-4) while that of Δ7-8 was virtually complete (Figure 4D). The vgR mutant phenotype was not rescued as completely as vg83b27R, but the level is still substantial. Moreover, in each case, the relative ability of the deletion constructs to rescue vgR correlates with that observed for vg83b27R (Figure 5, B-D vs. Figure 4, B-D), so all rescue results are summarized in Figure 1. Again, there were no notable differences in the degree of rescue when comparing multiple transgenic lines for a particular deletion construct. Selected rescue data for the vg83b27R mutant are portrayed in Figure 6, and these data agree with those for the two leaky vg alleles. Only the constructs representing regions that exhibited partial rescue activity for the other two alleles are portrayed for vg83b27R since all other constructs were completely negative. As expected, even the constructs that gave partial rescue of vgRC and vgS32 did not rescue vg83b27R as well (Figure 6, A-C vs. D-I). The UASvg7-8 was unable to produce a full rescue of vg83b27R (Figure
1371 Drosophila Vg Functional Domains in Vivo

Figure 6.—Wing rescue assay for vg\(^{\Delta 7-8}\). (A–C) Wild-type wings. (D–F) Wings from flies with the genotype UAS\(\Delta vg7-8\)/+; \(vg^{\Delta 7-8}/vg^{\Delta 7-8}\) vgGAL4. (G–I) Wings from flies with the genotype UAS\(\Delta vg1-4\) \(vg^{\Delta 7-8}/vg^{\Delta 7-8}\) \(vg^{\Delta 7-8}/vg^{\Delta 7-8}\) \(vg^{\Delta 7-8}/vg^{\Delta 7-8}\). (J–L) Wings from flies with genotype UAS\(\Delta vg5-5\) \(vg^{\Delta 7-8}/vg^{\Delta 7-8}\) \(vg^{\Delta 7-8}/vg^{\Delta 7-8}\). (B, E, H, and K) Higher magnification of the anterior wing margins from the panels showing the full wings. (C, F, I, and L) Higher magnification of the posterior wing margins of these wings. A, D, G, and J are all at the same magnification. Anterior is up.

6, J and K) but a full-length vestigial construct is also unable to rescue this allele fully (results not shown). Since \(vg^{\Delta 7-8}\) and wild type still behave similarly, this partial rescue is likely a reflection of the relative strength of the vgGAL4 driver. Note that there is considerable rescue of the anterior bristles using \(vg^{\Delta 7-8}\), which is not as apparent with \(vg^{\Delta 1-4}\) and not observed with \(vg^{\Delta 5-5}\) (Figure 6, E vs. H and K).

In these assays, the SD-binding domain (amino acids 281–335) as well as N-terminal amino acids 1–65 (called domain 1) and C-terminal amino acids 335–453 (called domain 2) are required for optimal VG activity. The general location of the these domains identified by in vivo experiments correlates well with the domains located by in vitro experiments (Vaudin et al. 1999) and with those implicated as important in the DNA-binding ability of the VG/SD complex (Halder and Carroll 2001). Although the deletion construct that left both domains 1 and 2 intact (\(\Delta 7-8\)) exhibited virtually the same levels of ectopic sd expression and rescue ability of the leaky vg alleles as those of full-length vg, neither it nor wild-type could fully rescue \(vg^{\Delta 7-8}\). Further, since the ectopic behavior of \(vg^{\Delta 7-8}\) is also concordant with other activities associated with ectopic expression of wild-type vg, such as wing outgrowths in eye tissue or induction of \(vg\), the failure to rescue \(vg^{\Delta 7-8}\) is not likely due to the nature of the \(vg^{\Delta 7-8}\) deletion. When full-length VG was compared to the protein produced by \(vg^{\Delta 7-8}\) with respect to folding ability using a computer-based analysis (Rost and Sander 1993, 1994; Rost 1996), the result was that this 91-amino-acid portion was not predicted to possess any significant secondary structure. Thus its deletion would not impair proper folding of the remainder of the protein. Computer analysis of structural motifs (Bairoch et al. 1997) predicts a putative nuclear localization sequence within the region deleted in \(vg^{\Delta 7-8}\). However, since the protein encoded by this deletion construct behaves with virtual wild-type activity, one can infer that this motif is either nonfunctional or not required for full-length VG function. Furthermore, it is likely that VG enters the nucleus via its ability to bind to SD and utilizes the nuclear localization signal therein (Srivastava et al. 2002).

DISCUSSION

The results presented herein are consistent with an in vivo role of VG acting as a transcriptional activator in Drosophila cells in conjunction with binding to SD. It was already known that ectopic expression of vg induces the ectopic expression of sd in the same pattern. Further, it was also known that an expressed vg construct lacking the ability to bind SD was also unable to induce ectopic sd expression (Simmonds et al. 1998). However, it was not known which portions of the vg ORF were necessary to carry out these functions of VG in vivo. The data presented herein are necessary and novel in that they identify functional roles for regions of the VG pro-
tein in the cellular context within which wing development actually occurs. Previous biochemical studies, although useful, gained their conclusions from artificial situations. We demonstrate for the first time that three regions of VG are necessary for full physiological function. VAudin et al. (1999) identified the same N-terminal and C-terminal domains as herein by using a yeast one-hybrid assay capable of providing an activation function. However, these results were merely suggestive until confirmed by in vivo results. Halder and Carroll (2001) showed that these domains are necessary for binding a VG/SD complex to a consensus SD-binding site in vitro, but a direct extrapolation to an in vivo situation is difficult. Both sets of in vitro data leave open the possibility of other important functional motifs in vivo. A more definitive way to assay for functional motifs for the VG protein is by an in vivo analysis such as presented herein.

From our results using ectopic sd-lacZ induction, the ability to rescue vg mutations, and the ability to carry out other functions associated with normal vg, it can be discerned that certain portions of the vg ORF, in addition to the SD-binding domain, are necessary to accomplish normal VG function. These appear to be the critical regions, as other portions can be deleted without effect. More specifically, the N-terminal amino acids (approximately the first 65) and C-terminal residues from 335 to 453 seem to play an important role in the induction of sd-lacZ. When the N-terminal deletion Δ5′-5 (deleting amino acids 2–65) is assayed, the ectopic expression ability is reduced markedly compared to that seen with the full-length vg construct, although it is not eliminated completely. Moreover, the larger N-terminal deletions (amino acids 2–170 and 2–278, respectively) do not further lower the ability to express sd. Thus, it seems that the fundamentally important region is already removed with the Δ5′-5 construct. For C-terminal deletions Δ1-4 and Δ1-2 (amino acids 356–453 and 335–426, respectively), the ability to ectopically express sd is much less than that produced by full-length vg but somewhat stronger than that produced when the N-terminal deletion constructs are assayed. Deletions Δ5′-5, Δ5′-6, and Δ5′-7 retain the encoded amino acids missing from Δ1-4 and Δ1-2 and vice versa. Taken together, these data suggest the presence of two important functional domains for VG: one within amino acids 1–65 (domain 1) and the other within amino acids 336–453 (domain 2). See Figure 1 for the position of these domains within the VG protein. Although the precise boundaries of these domains have not yet been determined, domain 1 is very likely within the first 65 amino acids (deleted in vgΔ5′-5) since this is the region most highly conserved between D. melanogaster and the mosquito Aedes aegypti (Halder and Carroll 2001). There is 82% identity over the first 66 amino acids, but over the next 20 amino acids the identity drops to 35% and even further beyond that. In agreement with this notion, the extent of “functional” loss in UASΔvg 5′-6 and 5′-7 is no stronger than that exhibited by UASΔvg 5′-5, which deletes the first 65 amino acids only. The activity of domain 2 appears to be weaker, since domain 1 deletions produce a slightly more drastic impairment of VG function than do domain 2 deletions (amino acids 356–453 or 335–426). However, homology between Drosophila and mosquito VG is also high within the SD-binding domain of VG and, in fact, remains strong to the carboxyl terminus of VG (82% identity from residue 335 to 453; Halder and Carroll 2001). The data herein define the presence of two necessary functional domains for the VG protein in vivo. These domains correlate well with data that predict two activation regions using in vitro experiments, including yeast one-hybrid assays (VAudin et al. 1999). The regions identified herein also complement more recent in vitro data, implicating these regions of VG as necessary for binding of the VG/SD complex to target genes (Halder and Carroll 2001). However, in VAudin et al. (1999) the N-terminal region was not as active as the C-terminal region, while in the present in vivo study the opposite may be the case.

The Δ7-8 construct (deleting amino acids 187–278) behaves similarly to full-length vg in all assays reported herein even though the encoded protein lacks 91 amino acids present in normal VG. Thus, a significant portion of the VG protein can be absent without any obvious impairment of functions associated with this protein. This portion of the protein has no homology to other known proteins, including vertebrate VG homologs, and is not predicted to contain any well-defined structural motifs. Thus, the function of this segment, comprising 20% of normal Vg, remains unknown at this time.

While the ectopic sd-lacZ assay illustrates the ability of each region to activate transcription of sd, the ability to rescue the vg mutants (vgE, vgF, and vgG) was also assessed to confirm that these regions are required for the majority of VG-mediated activation of downstream genes. All deletion constructs that were able to rescue vgE mutants were also able to rescue vgG mutants, and these same constructs were the ones that retained the ability to ectopically induce sd. As expected, the constructs that could accomplish some rescue of the leaky vg alleles were able to rescue vgE to a lesser extent, but this merely strengthens the conclusion that the respective deleted regions are essential for in vivo VG function. Furthermore, the relative ability of the constructs to induce the ectopic expression of sd correlates with their relative ability to rescue the wing phenotypes of the tester vg alleles. The full-length vg construct gives almost complete rescue of vgE but rescues vgI to a lesser extent and vgF to an even lesser extent. The most likely explanation for incomplete rescue of vgG and vgF is the vgGAL4 line that was used in these experiments. It was constructed (S. Morimura and M. Hoffmann, unpublished results) by excising the vg gene intron 2 boundary enhancer region (Williams et al.
1991, 1994) from psub26 and cloning this into pGawB (Brand and Perrimon 1993). Because only the vg intron 2 region was used, it is not surprising that a complete vg expression pattern is not produced by this driver.

The SD-binding domain (residues 278–335) has already been shown to be necessary and sufficient for binding of SD and TEF-1 (Simmonds et al. 1998). Further, the deletion of residues 187–278 (vgA7-8) does not result in any obvious impairment of VG function. Although the removal of either putative activation domain does not completely destroy the ability of VG to execute its functions, these functions are impaired considerably. This could be interpreted as a partial functional redundancy or, more likely, it could be interpreted that both domains are required for full normal function. Future efforts will be directed toward identifying a possible functional role for the portion of the VG protein that appears to be dispensable from the results of the present study. Studies aimed at identifying additional protein-protein interactions with VG may be able to implicate this region in other aspects of VG function.

We thank Shelagh Campbell for suggestions and a critical reading of the manuscript and Sean Carroll for reagents. We also thank Sandra O’Keefe for help in the preparation of the figures. This research was supported by a NSERC Canada grant to J.B.

LITERATURE CITED


Communicating editor: T. Schüpbach