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Restricted patterning of *vestigial* expression in *Drosophila* wing imaginal discs requires synergistic activation by both Mad and the Drifter POU domain transcription factor

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SUMMARY

The *Drosophila* Vestigial protein has been shown to play an essential role in the regulation of cell proliferation and differentiation within the developing wing imaginal disc. Cell-specific expression of *vg* is controlled by two separate transcriptional enhancers. The boundary enhancer controls expression in cells near the dorsoventral (DV) boundary and is regulated by the Notch signal transduction pathway, while the quadrant enhancer responds to the Decapentaplegic and Wingless morphogen gradients emanating from cells near the anteroposterior (AP) and DV boundaries, respectively. MAD-dependent activation of the *vestigial* quadrant enhancer results in broad expression throughout the wing pouch but is excluded from cells near the DV boundary. This has previously been thought to be due to direct repression by a signal from the DV boundary; however, we show that this exclusion of quadrant enhancer-dependent expression from the DV boundary is due to the absence of an additional essential activator in those cells. The *Drosophila* POU domain transcriptional regulator, Drifter, is expressed in all cells within the wing pouch expressing a *vgQ-lacZ* transgene and is also excluded from the DV boundary. Viable *drifter* hypomorphic mutations cause defects in cell proliferation and wing vein patterning correlated with decreased quadrant enhancer-dependent expression. Drifter misexpression at the DV boundary using the GAL4/UAS system causes ectopic outgrowths at the distal wing tip due to induction of aberrant Vestigial expression, while a dominant-negative Drifter isoform represses expression of *vgQ-lacZ* and causes severe notching of the adult wing. In addition, we have identified an essential evolutionarily conserved sequence element bound by the Drifter protein with high affinity and located adjacent to the MAD binding site within the quadrant enhancer. Our results demonstrate that Drifter functions along with MAD as a direct activator of Vestigial expression in the wing pouch.

Key words: DPP, MAD, Cell growth, Imaginal disc, Transcription, *Drosophila*

INTRODUCTION

The *Drosophila* wing imaginal disc has become a key model system in which to analyze the response of individual cells to one or more patterning signals. During the growth and patterning of a field of equivalent cells, each cell must decide at some point whether to continue proliferation, become quiescent, or choose to differentiate into a particular cell type. These choices are often made in response to nonautonomous extracellular signals acting as organizers and the ultimate decision depends upon the initiation of a cell-specific pattern of target gene activation or repression. However, target gene expression is often coordinated between multiple pathways acting within the same cell. This process of coordination may occur primarily at the transcriptional level and, although still not well understood, represents the true environment within which most cells must respond.

Patterning in the *Drosophila* wing imaginal disc relies upon signals emanating from both the anteroposterior (AP) and dorsoventral (DV) compartmental boundaries (Morata and Sanchez-Herrero, 1999). Positioning of the AP boundary is established by the progressive expression of Engrailed and Hedgehog (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Tabata and Kornberg, 1994) within the posterior compartment. However, regulation of cell proliferation and differentiation along the AP axis is influenced primarily by the secreted Decapentaplegic (DPP) protein, a *Drosophila* transforming growth factor β (TGFβ)/bone morphogenetic protein (BMP) homologue (Zecca et al., 1995). DPP is thought to diffuse as a morphogen to regulate expression of target genes at varying distances from DPP-expressing cells along the AP boundary. Since DPP diffusion should be essentially symmetrical, this results in target gene expression in nested domains centered over the AP boundary. Identified target genes

include the *spalt* (*sal*) (de Celis et al., 1996; Lecuit et al., 1996; Nellen et al., 1996), *optomotor blind* (*omb*) (Grimm and Pflugfelder, 1996; Lecuit et al., 1996; Nellen et al., 1996) and *vestigial* (*vg*) (Kim et al., 1997, 1996) genes, each with progressively wider boundaries of expression located at distances further away from the source of DPP morphogen.

With its broad expression throughout the wing pouch, the nuclear vestigial (VG) protein has been shown to function as an essential regulator of cell growth and differentiation in the *Drosophila* wing imaginal disc (Kim et al., 1996). This is manifested both by the loss of wing tissue in *vg* mutants (Lindsley and Zimm, 1992) and by the effects of ectopic VG expression in heterologous imaginal discs (Kim et al., 1996). VG misexpression can cause transformation into wing tissue, suggesting that VG serves as a key regulator of a 'wing-specific' transcriptional program (Kim et al., 1996). VG is expressed over the entire wing pouch under the control of two separable enhancers. Expression of VG is first activated by the boundary enhancer (vgB) at the DV boundary in response to Notch signaling (Williams et al., 1994), and is later expanded within the wing pouch under the control of the quadrant enhancer (vgQ) (Kim et al., 1996).

The pattern of vgQ-dependent expression within the wing pouch has been of particular interest as a model for the generation of patterned gene expression in response to multiple regulatory inputs (Kim et al., 1996). A *vgQ-lacZ* transgene is expressed in four symmetrical quadrants within the wing pouch but is sharply excluded from cells near the DV boundary (Kim et al., 1996). The precise nature of patterning signals utilized to achieve this distinctive pattern is not fully understood but previous work has demonstrated that nuclear localized MAD protein, a *Drosophila* Smad1 homologue (Newfeld et al., 1996, 1997; Sekelsky et al., 1995) binds directly to sequence elements within the vgQ enhancer to mediate direct activation by DPP signaling (Kim et al., 1997). In addition, vgQ-dependent expression requires a signal from the DV boundary that is thought to be the secreted WG protein (Kim et al., 1996; Zecca et al., 1996). Published reports show that signaling through the *Drosophila* EGF receptor may also be required for normal vgQ-dependent expression (Nagaraj et al., 1999), and a number of studies have demonstrated an autoregulatory function for VG either alone or as a VG/Scalloped (SD) protein complex bound to vgQ (Halder et al., 1998; Klein and Arias, 1999; Paumard-Rigal et al., 1998; Simmonds et al., 1998). Thus, the vgQ enhancer appears to respond to a variety of signals, ultimately resulting in its distinctive expression pattern relative to the AP and DV compartment boundaries.

None of these known input signals, however, can explain why vgQ expression is excluded from cells flanking the DV boundary even though secreted DPP protein, and therefore activated MAD, is readily available in these cells (Kim et al., 1997). This implies either the presence of an unidentified vgQ repressor at the DV boundary or the absence of an additional essential activator. We have investigated the role of the *Drosophila* Drifter (DFR) POU domain protein (Anderson et al., 1995; Johnson and Hirsh, 1990) in regulation of the DPP target gene *vg* and shown that DFR functions as a direct activator of the vgQ enhancer in cells of the wing imaginal disc.

DFR, also referred to as Ventral Veinless (VVL) (de Celis et al., 1995), is a pleiotropic developmental regulator required for cell proliferation and wing vein patterning in the wing imaginal disc (de Celis et al., 1995). The DFR protein contains a conserved POU domain, a bipartite DNA-binding motif composed of a POU-homeodomain and an adjacent POU-specific domain connected by a flexible linker (Herr and Cleary, 1995). Members of the evolutionarily conserved POU domain family have been shown to function in a variety of essential roles during development (Herr and Cleary, 1995; Ryan and Rosenfeld, 1997). In keeping with the characteristic functional diversity of this family of transcriptional regulators, we have previously shown that DFR is required for directed migration of tracheal cells where it is essential for maintenance of Breathless receptor tyrosine kinase expression (Anderson et al., 1996). In the central nervous system (CNS), DFR is expressed in the midline glia of the ventral nerve chord and is required for correct commissure formation (Anderson et al., 1995; de Celis et al., 1995).

Results presented here show that DFR binds to a conserved sequence element immediately adjacent to a MAD binding site within the vgQ enhancer. Binding of both MAD and DFR is required for vgQ activation in the wing pouch and misexpression of DFR in cells at the DV boundary can activate ectopic *vgQ-lacZ* expression. These findings suggest that vgQ-dependent expression is excluded from the DV boundary due to the absence of the DFR activator.

MATERIALS AND METHODS

Drosophila stocks

Flies were raised on standard cornmeal-yeast-agar medium. All stocks and balancer chromosomes not specifically mentioned in the text are as previously described (Lindsley and Zimm, 1992). All genetic crosses were performed at 25°C unless otherwise specified. This study utilized the *TM3, Sb e* balancer chromosome as a representative of the *In(3LR)sep* allele, which is incorporated into all TM3 balancers (Lindsley and Zimm, 1992).

Transgenic fly strains used for this work were generated as previously described (Certel et al., 1996). Transformant flies were identified by screening for the appearance of w^+ eye color in the progeny of injectees crossed to w^{1118} adults. 3-5 independent transformant strains were established for each fusion construct. Strains were confirmed to contain single copy inserts of the appropriate P-element vector by Southern blot analysis of genomic DNA.

Molecular biology

The $Gal4^{Ser1}$ transgenic stock containing a *Serrate-Gal4* transposon was kindly provided by Robert Fleming (Hukriede et al., 1997). *UAS-DFR* transgenes were constructed by cloning the entire coding region of each *dfr* allele, utilizing synthetic *Eco*RI endonuclease sites flanking the coding sequence. Resulting fragments were cloned into the pUAST P-element transformation vector, generously provided by Andrea Brand (Brand and Perrimon, 1993). *UAS-*DFR^{B157} transgenic strains carry a *dfr* gene with a single amino acid substitution within α helix 2 of the DFR POU-specific domain (M. Anderson, unpublished data). This modified DFR protein retains the ability to bind to recognition elements with near wild-type affinity but is unable to transactivate. It therefore causes dominant wing patterning defects in heterozygous dfr^{B157} mutants and functions as a dominant-negative when overexpressed. Details of the dfr^{B157} mutation and the flies are available upon request and will be published elsewhere.

vgQ-lacZ reporter constructs were made by cloning mutants in the *vg* quadrant enhancer into the *Hsp-lacZ* CaSpeR plasmid (Nelson and

Laughon, 1993). Mutations and deletions were introduced by PCR. In deletion 2, the orange-shaded region in Fig. 3A was replaced by the sequence 5′CTA3′, to form an *Xba*I restriction site at the junction. In deletion 3, the green-shaded region in Fig. 3A was replaced by 5′-TCGAA-3′, to form a *Bst*BI restriction site. In the mutation of mad2,MS6 (see Fig. 3J) the sequence 5′-GCCGGC-3′ was replaced with 5′-ACTAGT-3′ (a *SpeI* site). To make Q12S6 (see Fig. 3J), the MS6 mutation was introduced into a reporter already mutant at mad1, with 5′-GCTGCCGTCGCG-3′ replaced with two tandem *Bgl*II restriction sites (Kim et al., 1997). M280 (Fig. 4B) has the sequence 5′-TGCATGCTG-3′ (bases 274-282) replaced with 5′-AGATCTAGA-3′. X-gal stainings of imaginal disks were performed as described (Halder et al., 1998) and incubated at 37°C for either 24 hours (deletion 2, Q12S6, and M280) or 2 hours (all other disks); all lines were homozygous.

Immunohistochemistry

Third instar larval wing imaginal discs were fixed and labeled using modifications of a previously published protocol (Sturtevant et al., 1993). Imaginal discs were dissected from crawling third instar larvae in 1× PBS and fixed with 0.4% formaldehyde in 0.1 M Pipes, pH 6.9, 2 mM $MgSO_4$, 1 mM EGTA, 1% Triton X-100 at room temperature for 30 minutes. Following five washes in incubation buffer (1× PBS, 0.5% NP-40), discs were blocked in incubation buffer containing 5 mg/ml BSA at 4°C for 2 hours. Block solution was replaced with incubation buffer with 1 mg/ml BSA containing the primary antibody and the discs were gently agitated overnight at 4°C. Discs were then washed five times for 10 minutes each with the incubation buffer containing 1 mg/ml BSA. Fluorescent conjugated secondary antibodies were incubated with the discs for 2 hours at room temperature. After five 10 minute washes at room temperature, discs were mounted with Vectashield (Vector Laboratories). DFR expression was detected using a preabsorbed anti-DFR rat serum at 1:3000 dilution (Anderson et al., 1995). β-gal protein was detected either by a rabbit polyclonal anti β-gal antibody (Cappel) at 1:500 or by a mouse monoclonal supernatant (Developmental Studies Hybridoma Bank, University of Iowa, Iowa City, USA) at a dilution of 1:2. Cy5-conjugated anti-rat (Jackson Laboratories), rhodamine-conjugated anti-rabbit (Biosource) and FITC-conjugated anti-mouse (Jackson Laboratories) secondary antibodies were all used at 1:200 dilution. Images of labeled discs were captured using a BioRad 1024 laser scanning confocal microscope at the University of Iowa Central Microscopy Facility.

Wg misexpression clones

Clones expressing WG protein were generated using a flip-out Gal4 transposon (Pignoni and Zipursky, 1997) and UAS-WG (Hays et al., 1997). Females with the genotype hsp70-flp; UAS-WG were mated to Actin>CD2>Gal4; +; + males (Pignoni and Zipursky, 1997). Progeny were grown at 25°C, heat-shocked for 30 minutes at 35°C 30 to 54 hours after egg laying, then allowed to grow at 25°C until the late third instar stage. Wing discs were dissected, fixed and labeled as described above using: mouse anti-CD2 (Vector laboratories) at1:5000, preabsorbed at 1:100 against embryos; rat anti-Drifter (Anderson et al., 1995) at 1:3000, preabsorbed at 1:50 against 0- to 2-hour-old embryos. Secondary antibodies were donkey anti-rat LRSC and donkey anti-mouse FITC (Vector laboratories) used at 1:200 each.

DNAse I footprinting assays

Full-length DFR protein fused to the glutathione-s-transferase (GST) epitope was produced in protease deficient *Epicurian coli* BL21 cells (Stratagene) using protocols recommended by Pharmacia for use of the pGEX-6P-1 vector. Bacterial strains carrying the GST-DFR plasmid were induced with 0.4 mM isopropyl-β-D-thiogalactopyranoside (IPTG), lysed by sonication and GST-DFR protein purified using glutathione-sepharose beads (Pharmacia). Purified GST-DFR fusion protein yielded a single band on SDS-polyacrylamide gels with the expected M_r of 65×10^3. Concentrations of purified proteins were quantitated using the Bio-Rad protein assay system and used in DNAse I protection assays as previously described (Johnson and Hirsh, 1990; Johnson et al., 1989). Single-end-labeled footprinting probes were generated from subclones of vgQ DNA using polynucleotide kinase (NEB) and [γ-^{32}P]dATP (end labeling grade, ICN) following previously described protocols (Certel et al., 1996; Johnson and Hirsh, 1990). DNAse I digestion fragments were separated on 6% sequencing gels, dried and exposed to Kodak X-Omat X-ray film.

Gel mobility-shift assays

Binding of DFR-GST fusion protein to vgQ conserved sequence elements was examined using a gel mobility-shift assay. Single stranded oligonucleotidess were annealed and end-labeled with [γ-^{32}P]dATP and polynucleotide kinase. The sequence of oligonucleotides used was: vgQ^{WT}-5′ GAGTGTGCCATGCATGC-TGATGACGATG 3′; vgQ(M280)-5′ GAGTGTGCCAAGATCTAG-AATGACGATG 3′.

Binding reactions were carried out in 14 μl reactions of binding buffer (0.1% dried milk, 0.7% PVA, 300 ng poly(dI-dC), 25 mM Hepes, pH 7.6, 12.5 mM MgCl, 0.1 mM EDTA, 10% glycerol, 1 mM DTT, 0.1 M KCl). After 15 minutes incubation at room temperature, binding reactions were resolved on a 5% polyacrylamide gel run in 1× TBE at room temperature. Gels were dried and exposed to Kodax X-Omat X-ray film overnight.

RESULTS

Drifter is required for cell proliferation and patterning in the wing imaginal disc

The DFR protein with its highly conserved POU domain DNA-binding motif is an essential embryonic regulator, as indicated by the embryonic lethality associated with severe loss-of-function mutations (Anderson et al., 1995). However, DFR protein is expressed at all stages of development and has previously been shown to be required for vein formation in the adult wing using *dfr* mutant mitotic clones (de Celis et al., 1995). Postembryonic DFR functions can also be examined using certain adult viable heteroallelic combinations. The *Df(3L)XBB70* chromosome carries a small deficiency at region 65D uncovering the *dfr* locus (Anderson et al., 1995). The *TM3* balancer chromosome carries *In(3L)sep*, a molecularly uncharacterized chromosomal aberration disrupting the *dfr* locus and functioning as a hypomorphic *dfr* allele (Diaz-Benjumea and Garcia-Bellido, 1990). Flies carrying the *dfr* heteroallelic combination *Df(3L)XBB70*/*TM3, Sb e* will eclose as adults but cannot be maintained as a viable stock.

The wild-type adult wing displays a characteristic pattern of five longitudinal veins (L1-L5) as well as anterior (acv) and posterior (pcv) crossveins (Fig. 1A). Decreased levels of DFR activity in *Df(3L)XBB70*/*TM3, Sb e* adults resulted in a reduction in wing size to 75-80% of normal with disruptions in wing vein patterning. Mutant wings consistently displayed incomplete posterior cross vein (pcv) formation as well as thinning or breaks in veins L2 and L4 (Fig. 1B). This hypomorphic phenotype manifested by decreased wing size and vein defects, along with published reports that *dfr* mutant mitotic clones in the wing show defects in cell proliferation and wing vein patterning (de Celis et al., 1995), suggested that the DFR protein is required for cell growth and differentiation in the developing wing.

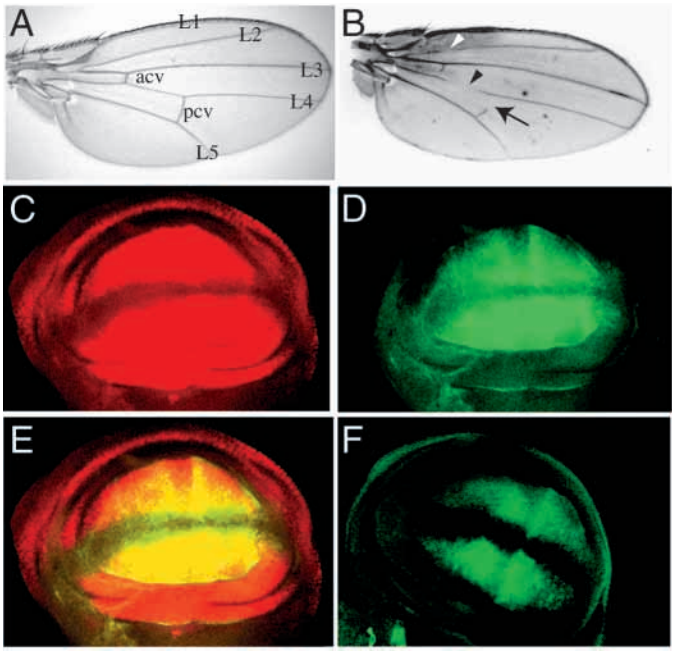

Fig. 1. Colocalization of DFR and vgQ expression in the third instar wing imaginal disc. (A) Wild-type adult wing showing the uniform pattern of cell proliferation and venation. The five longitudinal veins are indicated as L1-L5. Pcv, posterior cross vein; acv, anterior cross vein. (B) Viable hypomorphic *dfr* heteroallelic combination *Df(3L)XBB70/TM3, Sb* adult wing. Mutant wings show a slight reduction in size corresponding to 75-80% of wild type. In addition, venation defects include an incomplete pcv (arrow) as well as breaks in the ventral veins L2 (white arrowhead) and L4 (black arrowhead). (C-E) Third instar wing imaginal disc carrying a *vgQ-lacZ* transgene double-labeled for DFR (red) and β-galactosidase (green) expression. All discs are oriented with ventral up and anterior to the left. (C) DFR protein expression in the third instar wing imaginal disc. (D) *vgQ-lacZ* expression in the same wing disc. (E) merged images of DFR expression (red) and *vgQ-lacZ* expression (green) showing the distinctive colocalization within the dorsal and ventral wing pouch (yellow). Note that additional DFR expression not colocalizing with *vgQ-lacZ* expression is seen outside of the wing pouch in regions destined to become the dorsal and ventral hinge and notum. (F) *vgQ-lacZ* expression in a *Df(3L)XBB70/TM3, Sb* wing imaginal disc. Note the decreased levels of *vgQ-lacZ* expression most clearly manifested by widening of the gap of nonexpressing cells at the DV boundary and narrowing of the expression domain along the AP axis.

Examination of DFR protein in the third instar wing imaginal disc showed broad expression in cells of the wing pouch but complete exclusion from cells at the DV boundary making up the future wing margin (Fig. 1C). This selective pattern of expression with two symmetrical expression domains flanking the DV boundary was similar to the previously reported *vestigial* quadrant enhancer (vgQ) expression pattern (Fig. 1D) (Kim et al., 1996). A comparison of DFR and *vgQ-lacZ* expression patterns showed a complete overlap within the wing pouch itself, with all cells expressing *vgQ-lacZ* also expressing DFR protein (Fig. 1E). Additional DFR expression not colocalizing with *vgQ-lacZ* expression is seen outside of the wing pouch in regions destined to become the dorsal and ventral hinge and notum.

To determine whether DFR protein is necessary for vgQ-dependent expression within the wing pouch, we examined the levels of *vgQ-lacZ* expression in a *dfr* loss-of-function background. A marked reduction in β-galactosidase (β-gal) expression was observed in wing discs isolated from *Df(3L)XBB70/TM3, Sb e* viable hypomorphs (Fig. 1F). Even though the *Df(3L)XBB70/TM3, Sb e* heteroallelic combination retains significant levels of DFR activity, we observed a distinctive widening of the gap in *vgQ-lacZ* expression at the DV boundary and narrowing of the expression domain along the AP axis corresponding to an overall decrease in expression within cells of the pouch. This suggested that the DFR protein lies genetically 'upstream' of the *vg* gene and is necessary for vgQ-dependent expression, however, considering the complex combinatorial nature of gene regulation during pattern formation in the wing imaginal disc, DFR could be acting either as a direct activator of vgQ or could lie one or more steps upstream from the vgQ enhancer itself.

Drifter regulation of vgQ-dependent expression

The ability of the DFR protein to function as an activator of vgQ-dependent expression was tested using the Gal4-UAS system (Brand and Perrimon, 1993) and the *vgQ-lacZ* reporter. Full-length cDNAs encoding either wild-type DFR or a dominant-negative DFR isoform, DFR^{B157}, were placed under the control of Gal4-UAS sequences and used to generate transgenic flies. The DFR^{B157} protein is capable of binding to DFR recognition elements but is defective in transactivation and therefore can function to disrupt the function of endogenous wild-type protein (M. Anderson, personal communication; see Materials and Methods). As mentioned previously, both DFR and *vgQ-lacZ* expression are excluded from a strip of cells at the DV boundary (Fig. 1C,D). If the absence of *vgQ-lacZ* expression at the DV boundary is due to the absence of DFR protein then ectopic expression of DFR at the margin should aberrantly activate *vgQ-lacZ* expression. A *Serrate-Gal4* (*Ser-Gal4*) transposon was used to drive DFR expression within the prospective wing margin and the dorsal half of the wing pouch (Fig. 2B) (Hukriede et al., 1997).

We first examined the effects of ectopic DFR and DFR^{B157} expression in the adult wing. Adults carrying both the *Ser-Gal4* and *UAS-DFR* transposons grown at 25°C produced wings with occasional blistering within the region of *Ser-Gal4* expression at the distal end of the wing. Ectopic growth of intervein tissue was consistently observed resulting in a small outgrowth at the most distal wing tip (Fig. 2C). These outgrowths did not appear to be organized with the characteristics of a partial wing duplication but seemed to result from aberrant cell proliferation at the most distal tip of the wing.

When the dominant-negative DFR^{B157} protein was expressed using the same *Ser-Gal4* transposon, we observed striking defects in cell proliferation manifested as a severely notched wing in which cells from within the *Ser-Gal4* expression domain are absent (Fig. 2D). The *Ser-Gal4/UAS-*DFR^{B157} adult wing phenotype is essentially identical to wings produced by *Ser* mutations in which all cells within the SER expression domain fail to proliferate (Lindsley and Zimm, 1992). Based upon the observed colocalization of DFR and *vgQ-lacZ* expression in the wing pouch (Fig. 1) as well as the essential function of the VG protein for cell proliferation and

patterning in the wing disc (Kim et al., 1996), misexpression phenotypes observed for both wild-type DFR and DFR^{B157} were consistent with direct DFR-dependent regulation of *vg*.

We were able to correlate activation of the vgQ enhancer with the observed adult wing phenotypes by examining expression of the *vgQ-lacZ* reporter gene in third instar wing imaginal discs. Ectopic expression of wild-type DFR protein in *Ser-Gal4/UAS-DFR* larvae showed expansion of *vgQ-lacZ* expression into the region of the DV boundary where *vgQ-lacZ* and DFR expression are both normally absent (Fig. 2E,F). Although wing discs from *Ser-Gal4/UAS-DFR* larvae express ectopic DFR across the entire width of the DV boundary (Fig. 2B), aberrant *vgQ-lacZ* expression was observed only in the medial region where DPP levels would be at a maximum. The vgQ enhancer has previously been shown to bind activated MAD protein and is dependent upon the DPP signaling pathway (Kim et al., 1997). Our results suggest that ectopic DFR-dependent activation of *vgQ-lacZ* expression within DV boundary cells relies upon a threshold level of activated MAD protein near the AP boundary.

In contrast, expression of the dominant-negative DFR^{B157} protein under the control of *Ser-Gal4* caused a nearly complete suppression of *vgQ-lacZ* activation in the dorsal half of the wing disc, while β-gal expression in the ventral wing pouch was relatively unaffected (Fig. 2G,H). The region of *vgQ-lacZ* suppression consistently corresponded exactly to cells expressing DFR^{B157} protein and was emphasized by small patches of cells in the dorsal pouch that fail to express DFR^{B157} and consequently retain *vgQ-lacZ* expression (Fig. 2G,H, white arrows). A small number of DFR^{B157} expressing cells at the border of the *Ser-Gal4* expression domain occasionally retain some *vgQ-lacZ* expression. This may be due to lower levels of DFR^{B157} expression in these cells that were insufficient to compete with endogenous DFR expressed in the ventral wing pouch.

These results would be consistent with the DFR protein functioning as a transcriptional activator at the vgQ enhancer. To demonstrate that the loss of *vgQ-lacZ* expression did not result from non-specific cell death induced by toxic overexpression of DFR^{B157}, we examined the expression of an *omb-lacZ* transposon expressing in a broad domain centered on the AP boundary (Fig. 2I). The pattern of *omb-lacZ* expression does not extend to the most lateral cells of the wing pouch but is restricted to a narrower band of cells closer to the center of the pouch. This is thought to be due to significant differences in how *omb* regulatory sequences respond to the DPP signal (Grimm and Pflugfelder, 1996; Nellen et al., 1996). In addition, *omb-lacZ* expression extends across the DV boundary where both DFR and *vgQ-lacZ* expression is absent (Fig. 2I).

When examined in *Ser-Gal4/UAS-DFR^{B157}* wing discs expressing DFR^{B157} at the DV boundary and in the dorsal wing

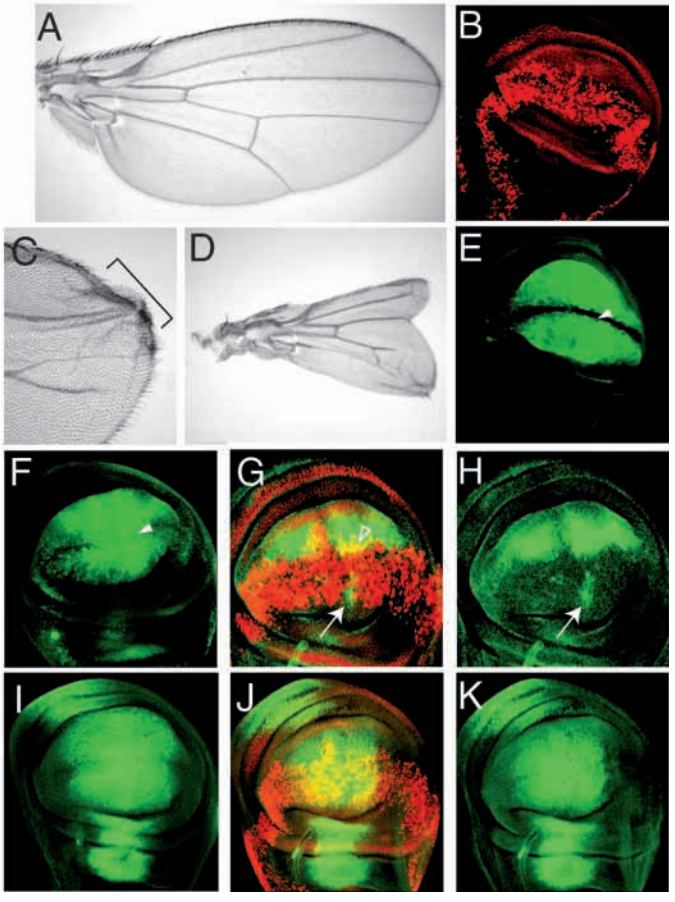

Fig. 2. DFR misexpression at the DV boundary. (A) Wild-type adult wing. (B) *Ser-Gal4/UAS-DFR^{WT}* wing disc labeled with anti-DFR polyclonal antiserum to visualize endogenous DFR protein as well as ectopic overexpression of DFR protein across the DV boundary and in the dorsal wing pouch. Compare to Fig. 1C for wild-type endogenous DFR expression pattern. (C) *Ser-Gal4/UAS-DFR^{WT}* adult wing showing ectopic outgrowth of intervein tissue at the distal wing tip (bracket). (D) *Ser-Gal4/UAS-DFR^{B157}* adult wing displaying severe truncation of the distal wing. Same magnification as A. (E-K) Third instar wing imaginals discs labeled with either anti-DFR polyclonal antiserum (red) and/or anti-β-galactosidase (green). All discs are mounted with anterior to the left and ventral up. (E) Wild-type *vgQ-lacZ* expression in dorsal and ventral domains within the wing pouch separated by cells at the DV boundary, which do not express β-galactosidase(white arrowhead). (F) *vgQ-lacZ* expression in a *Ser-Gal4/UAS-DFR^{WT}* wing disc ectopically expressing DFR protein across the DV boundary. Dorsal and ventral expression domains are connected by aberrant β-galactosidase expression extending across the DV boundary (white arrowhead). (G) *vgQ-lacZ* expression (green) in a *Ser-Gal4/UAS-DFR^{B157}* wing disc expressing the dominant-negative DFR^{B157} protein (red). (H) Same disc displaying only the green channel to clearly show that cells expressing DFR^{B157} do not label for *vgQ-lacZ* expression. β-gal expression is absent in the dorsal wing pouch but remains relatively normal in cells of the ventral pouch where DFR^{B157} is not expressed. Note the small patch of *vgQ-lacZ* expressing cells remaining in the dorsal pouch (white arrow), which do not express DFR^{B157}. A small number of cells at the ventral boundary of the SerGal4 expression domain often still retain *vgQ-lacZ* expression (yellow; open white arrowhead). It is assumed that cells in these patches may be expressing DFR^{B157} at lower levels, which are insufficient to compete with endogenous DFR protein in the ventral pouch cells. (I) Wild-type expression of *omb-lacZ* (green). (J) *omb-lacZ*; *Ser-Gal4/UAS-DFR^{B157}* wing disc double-labeled for DFR protein (red) and β-galactosidase (green). This disc is expressing the dominant-negative DFR^{B157} protein across the DV boundary and in much of the dorsal wing pouch. Note that *omb-lacZ* expression (green) is retained in cells expressing DFR^{B157} (red) with the overlap shown as the broad domain of yellow cells. (K) Isolation of *omb-lacZ* (green) channel from same disc shown in J. Expression of *omb-lacZ* is unaffected by DFR^{B157} overexpression.

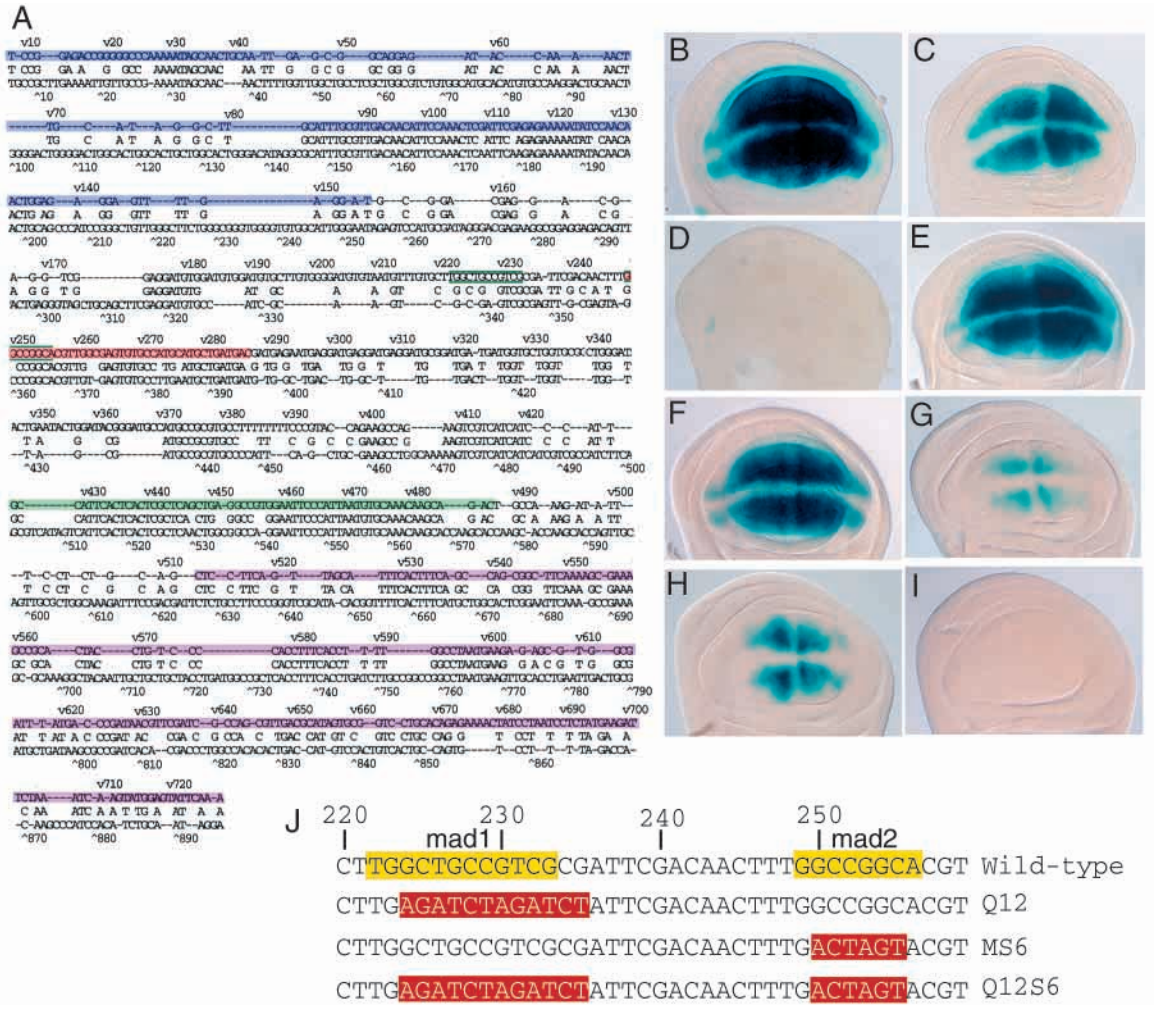

Fig. 3. Functional analysis of evolutionarily conserved vgQ sequence elements. (A) Alignment of vgQ enhancer sequence isolated from *D. melanogaster* (top) and *D. virilis* (bottom) with conserved nucleotides indicated (middle). Deleted sequences are indicated by colored shading: blue, deletion 1(Δ1-154); orange, deletion 2(Δ249-287); green, deletion 3(Δ427-488); purple, deletion 4 (Δ512-727). MAD binding sites are indicated by green lines at nucleotides 222-233(mad1) and nucleotides 249-257(mad2). (B-I) Third instar wing imaginal discs carrying wild-type or mutant *vgQ-lacZ* transgenes labeled with X-Gal. All discs are shown with anterior left and ventral up. (B) Wild-type *vgQ-lacZ* expression. (C) Deletion 1 (Δ1-154). (D) Deletion 2 (Δ249-287). (E) Deletion 3 (Δ427-488). (F) Deletion 4 (Δ512 through the end of the enhancer). (G) Mutation Q12 disrupting mad1 binding site. (H) Mutation MS6 disrupting mad2 binding site. (I) Mutation Q12S6 disrupting both mad1 and mad2. (J) DNA sequence comparison of mutations (red) disrupting the mad1 and mad2 binding sites (yellow).

pouch, *omb-lacZ* expression is unaffected (Fig. 2J,K). This result demonstrates two important points concerning the specificity of DFR and DFR^{B157} overexpression. The loss of *vgQ-lacZ* expression in wing discs overexpressing DFR^{B157} does not result from nonspecific cell death at this stage, since *omb-lacZ* expression is still present at wild-type levels within the domain of DFR^{B157} overexpression. In addition, this result would be consistent with a direct regulatory function for DFR at the vgQ enhancer, implying that the DFR protein is not functioning as an inherent component of the DPP signaling pathway. OMB expression in the wing pouch is also dependent upon DPP signaling from the AP boundary, therefore if the DFR protein were functioning well upstream of the vgQ enhancer to somehow accentuate or block DPP function it might be expected that some effect upon other DPP targets such as *omb* might be observed. This result suggests that the DFR protein may be acting selectively and directly upon the vgQ enhancer.

The direct nature of DFR function in vgQ-dependent expression is also supported by additional control experiments addressing the possibility that the observed adult wing phenotypes and effects upon *vgQ-lacZ* expression by DFR could be due to increased expression of either DPP or the *Drosophila* type I receptor Thick Veins (TKV). In control experiments using a *dpp-lacZ* transposon and a *tkv* enhancer trap line, we failed to detect any alterations in DPP or TKV expression in response to DFR misexpression (data not shown). This result demonstrates that the observed misexpression phenotypes are not mediated by DFR activation of DPP or TKV expression but may be due to direct activation of vgQ by DFR protein. To test for a direct relationship between DFR and the vgQ enhancer, we carried out a further characterization of the vgQ enhancer to identify essential sequences in addition to the previously identified MAD binding site.

Evolutionary conservation of the *vestigial* quadrant enhancer

The vgQ enhancer is thought to respond to multiple signaling inputs including the DPP (Kim et al., 1997) and WG (Kim et al., 1996; Zecca et al., 1996) morphogen gradients as well as the epidermal growth factor receptor (EGFR) pathway (Nagaraj et al., 1999). A binding site for the *Drosophila* MAD protein at nucleotide 222 was previously identified and shown to be responsible for vgQ regulation by DPP signaling (Kim et al., 1997). Comparison of vgQ sequence isolated from *D. melanogaster* and the evolutionarily diverged *D. virilis* genomes revealed several regions displaying a high degree of sequence conservation (Fig. 3A). Such strict evolutionary conservation of limited stretches of enhancer sequence would be expected for essential sequence elements required for vgQ function.

The functional significance of conserved vgQ sequences was evaluated in vivo using a series of mutant *vgQ-lacZ* transposons containing sequence deletions or clustered point mutations. Both deletion 1 (Δ1-154) at the 5′ end and deletion 4 (Δ512-727) removing sequences at the 3′ end had little effect upon *vgQ-lacZ* expression (Fig. 3C,F). The internal deletion 3 (Δ427-488) also showed a wild-type expression pattern (Fig. 3E). This would imply that major portions of vgQ sequence, including significant stretches of highly conserved sequence, are either nonessential or redundant for vgQ function. In contrast, the relatively small internal deletion 2 (Δ249-287) showed no β-gal expression (Fig. 3D), suggesting that essential sequences had been removed.

Sequences removed by deletion 2 (Δ249-287) did not include the previously identified MAD binding site, mad1, located at nucleotides 222-233 (Kim et al., 1997). Previous results have shown that disruption of the mad1 binding site by a *Bgl*II restriction site (mutation Q12) resulted in a severe reduction in *vgQ-lacZ* expression (Fig. 3G,J) (Kim et al., 1997). An additional MAD binding site of lower affinity can be detected in DNAse I protection assays (J. Kim, unpublished observation) and is removed by deletion 2 (Figs 3A, 4B). We mutated the second MAD binding site, mad2, spanning nucleotides 249-256 (GGCCGGCA), by replacing the central six base pairs with a *Spe*I restriction site (mutation MS6; Fig. 3J). A *vgQ-lacZ* reporter containing this mutation in transgenic flies showed reduced expression compared to wild type, but a much higher residual expression level than when mad1 had been mutated (Fig. 3H,J). When both mad1 and mad2 were simultaneously disrupted (mutation Q12S6; Fig. 3J), however, no expression was observed (Fig. 3I), suggesting that both sites are capable of binding MAD in vivo and responding to DPP signaling.

Since specific disruption of the mad2 binding site results in only mildly reduced expression of vgQ (Fig. 3H), removal of the mad2 binding site alone cannot account for the complete lack of expression of the deletion 2 reporter. Therefore, there must be another essential element within this region. Because the deletions were made without replacement, it is possible that the lack of expression of the deletion 2 reporter is due to a change in alignment between upstream and downstream factors and not due to the sequence itself. To control for this, we made another reporter construct, in which the original spacing was restored with heterologous sequence (*d2srvgQ-lacZ*). This construct also showed no β-gal expression in transgenic flies (data not shown), indicating removal of an essential sequence element by deletion 2.

Regulation of the *vestigial* quadrant enhancer by direct binding of DFR protein

Results from analysis of vgQ expression in a *dfr* loss-

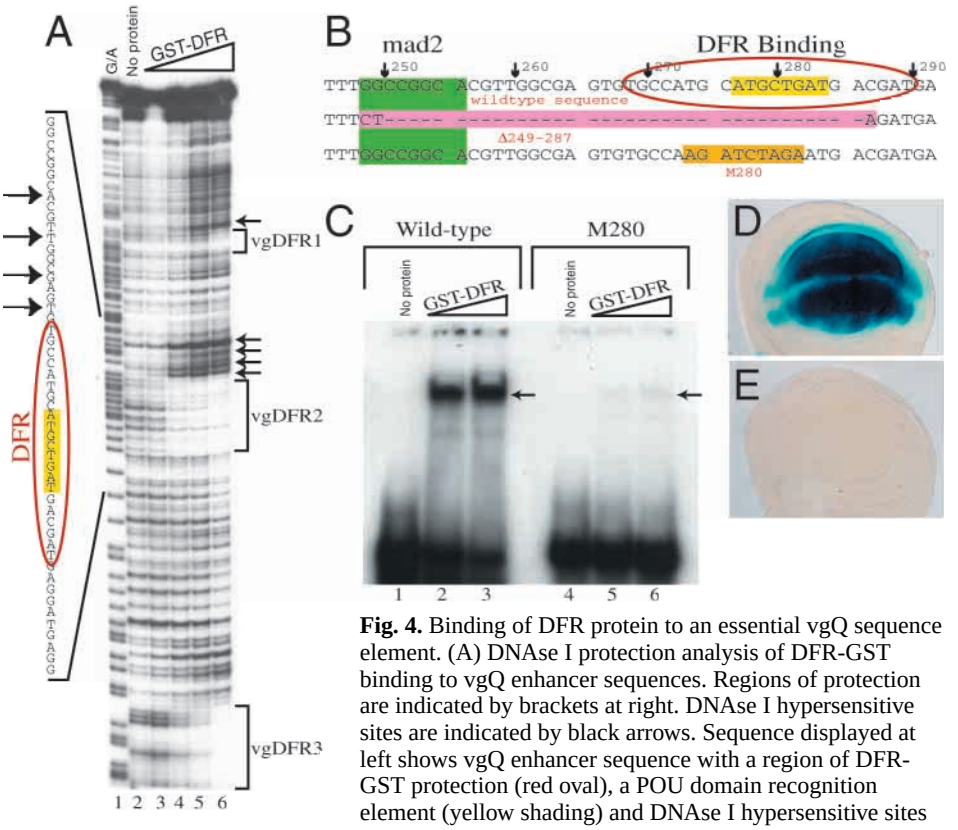

Fig. 4. Binding of DFR protein to an essential vgQ sequence element. (A) DNAse I protection analysis of DFR-GST binding to vgQ enhancer sequences. Regions of protection are indicated by brackets at right. DNAse I hypersensitive sites are indicated by black arrows. Sequence displayed at left shows vgQ enhancer sequence with a region of DFR-GST protection (red oval), a POU domain recognition element (yellow shading) and DNAse I hypersensitive sites (black arrows) indicated. (B) Schematic representation of vgQ sequence removed by deletion 2 (Δ249-287). mad2 binding site (green); region of DFR-GST DNAse I protection (red oval); POU domain recognition element (yellow); deletion 2 (Δ249-287; pink); M280 clustered point mutation disrupting the DFR recognition element (orange). (C) Gel mobility-shift assay for DFR-GST binding to wild-type and M280 mutant sequences. A ^{32}P-labeled double-stranded oligonucleotide corresponding to nucleotides 265-290 of the vgQ enhancer was used as a probe. The position of a single slower migrating complex representing the DFR-GST-bound oligonucleotide is indicated by the black arrow. Lanes 1-3: wild-type oligonucleotide; Lanes 4-6: M280 oligonucleotide. (D,E) Third instar larval wing imaginal discs labeled with X-Gal. Discs are shown with anterior left and ventral up. (D) Wild-type *vgQ-lacZ* expression. (E) *vgQ(M280)-lacZ* expression.

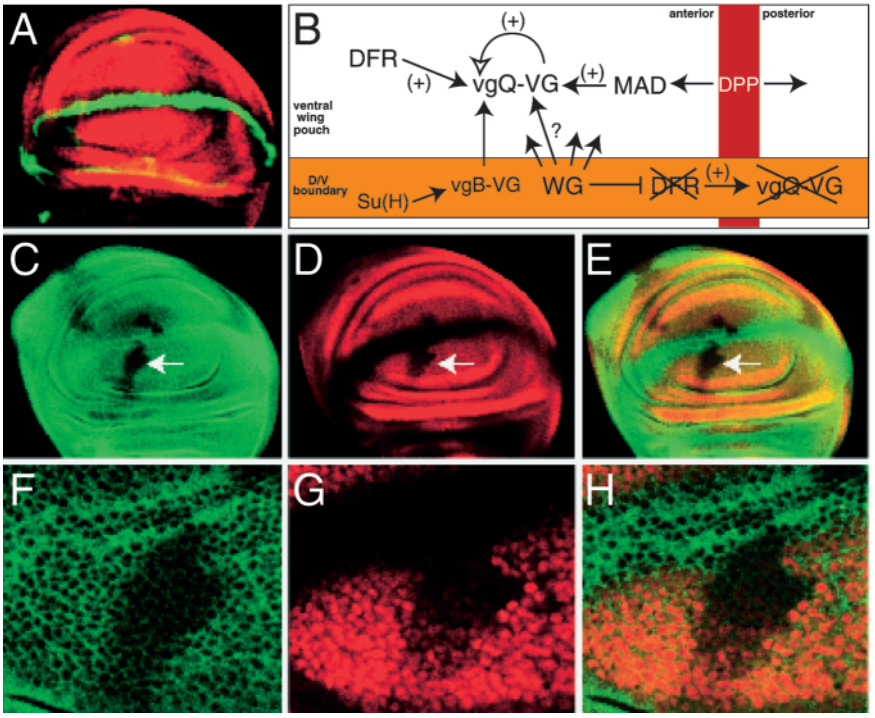

Fig. 5. Repression of DFR expression by Wingless. (A) Third-instar wing imaginal disc showing nonoverlapping expression of DFR (red) and *wingless-lacZ* (green). (B) Schematic representation of the ventral anterior quadrant of a developing wing imaginal disc. Arrows indicate known patterning signals activating vgQ-dependent expression in the wing imaginal disc. The repressive effect of WG upon DFR expression is indicated as a flat bar. The DV boundary is represented by the orange stripe and the AP boundary expressing DPP is shown as a red stripe. (C-H) Third-instar wing imaginal disc containing multiple flip-out Gal4 clones expressing WG protein double-labeled with anti-CD2 (green) and anti-DFR (red). (C) Loss of CD2 expression (green) indicates the clone boundaries corresponding to cells ectopically expressing WG. (D) DFR protein (red) is downregulated within the WG-expressing cells but is not completely eliminated. (E) Overlay of CD2 and DFR expression. (F-H) High magnification images of the dorsal clone from C-E, respectively.

of-function background and in DFR misexpression experiments suggested that the DFR protein may function as an essential regulator of vgQ. Close examination of vgQ sequence contained within the region removed by deletion 2 revealed a potential POU domain recognition sequence (ATGCTGAT) at the 3′ end of deletion 2 spanning nucleotides 277-284 (Fig. 4B). The ability of the DFR protein to bind to sequences within the vgQ was tested using a DNAse I protection assay and affinity-purified DFR-glutathione-s-transferase (GST) fusion protein. In initial experiments, three possible DFR binding sites were identified within the vgQ and designated as vgDFR1, vgDFR2 and vgDFR3 (Fig. 4A). We have focused upon vgDFR2, based upon its inclusion in deletion 2.

The vgDFR2 binding site corresponded to the potential POU domain recognition sequence removed by deletion 2 (Fig. 4B). DNAse I protection by DFR-GST bound at vgDFR2 extended from nucleotides 269-290 with four associated DNAse I hypersensitive sites (Fig. 4A). This region of protection is separated by only 12 nucleotides from the mad2 binding site, suggesting the possibility of a potential interaction between DNA-bound DFR and MAD. The functional significance of DFR binding to vgDFR2 was evaluated in vivo using a mutant *vgQ-lacZ* transgene containing a clustered point mutation, M280, in which 8 out of 9 basepairs were modified within the vgDFR2 binding site (Fig. 4B). The M280 mutation caused a near total loss of DFR binding affinity, as shown using a gel mobility-shift assay (Fig. 4C). The *vgQ(M280)-lacZ* transgene showed no detectable β-gal expression in vivo (Fig. 4D,E), demonstrating an essential function for DFR binding within the vgQ. These results demonstrate that both MAD binding and DFR binding to the adjacent vgDFR2 sequence element are necessary for vgQ-dependent expression in the wing pouch.

Repression of DFR expression at the DV boundary by Wingless

The results presented here indicate that exclusion of vgQ-dependent expression at the DV boundary is primarily due to the absence of DFR protein in boundary cells. A more thorough understanding of this regulatory circuit, therefore, raises the question of what signals regulate the expression of DFR, particularly in its exclusion from cells adjacent to the DV boundary. The DV boundary is initially defined by expression of the Apterous (AP) LIM-domain protein and subsequent Notch signaling to designate dorsal and ventral compartments within the developing wing pouch (Diaz-Benjumea and Cohen, 1993; Doherty et al., 1996; Kim et al., 1995). In response to Notch signaling at the DV boundary, a strip of cells expressing the secreted Wingless (WG) protein acts as a fundamental organizer of DV pattern (Neumann and Cohen, 1996, 1997). WG is thought to act as a morphogen, creating a gradient of activity very high near the DV boundary and decreasing with distance from the boundary (Zecca et al., 1996). Cells very close to the DV boundary are exposed to a higher concentration of WG and will take on the characteristics of the wing margin (Zecca et al., 1996).

Previous reports have suggested that the WG protein may act as a negative regulator of DFR/VVL expression (de Celis et al., 1995). Examination of WG and DFR expression in the third instar wing pouch using a *wg-lacZ* enhancer trap (Fig. 5A) shows that expression of these two proteins is nonoverlapping. DFR protein is completely excluded from WG-expressing cells, as well as a 2-3 cell wide band of cells flanking the stripe of *wg-lacZ* expression (Fig. 5A). This complete lack of coexpression would be consistent with a negative influence of high WG concentrations upon DFR expression.

To further examine the effect of WG signaling upon DFR

expression, cell clones ectopically expressing WG protein away from the DV boundary were generated using a flip-out GAL4 transposon driving expression of UAS-WG (Fig. 5C-H). Clones of WG-expressing cells within the dorsal and ventral wing pouch display a downregulation of DFR protein expression consistent with negative regulation of DFR by WG signaling (Fig. 5D,G). This result suggests that exclusion of DFR expression from cells near the DV boundary and, therefore, the resulting exclusion of vgQ-dependent expression, is mediated at least in part by high concentrations of WG protein (Fig. 5B).

Positive regulation of DFR expression within the wing pouch is less well understood. Ectopic expression of either DPP or a constitutively activated form of the DPP receptor TKV using the GAL4/UAS system does not induce ectopic DFR expression (data not shown). Similarly, although the tight coexpression of DFR and vgQ-dependent VG expression (Fig. 1) suggests that DFR may be positively regulated by the VG protein, ectopic expression of VG protein does not cell-autonomously induce DFR expression (data not shown). Therefore, the *dfr* gene is not a direct regulatory target of VG. We have previously shown that *dfr* is activated by an autoregulatory enhancer in tracheal cells and midline glia of the ventral nerve cord (Certel et al., 1996); however, this enhancer does not activate expression in the wing disc (data not shown). Therefore, direct positive regulators of DFR expression in the wing pouch remain to be identified. Nonetheless, our results suggest that the DFR protein serves an essential function downstream of the organizing signals from the AP and DV boundaries to regulate the spatial patterning of VG expression within the developing wing pouch.

DISCUSSION

Regulation of cell proliferation and patterning: a balance between activation and repression

Previous work has demonstrated that a significant amount of patterning has already taken place by the time vgQ-dependent expression is initiated in the wing disc. At early stages of development, cells within the epithelial sheet making up the prospective wing have already been divided into AP and DV compartments, based upon expression of the Engrailed and Apterous proteins, respectively (Basler and Struhl, 1994; Capdevila and Guerrero, 1994; Diaz-Benjumea and Cohen, 1993; Tabata and Kornberg, 1994). Cells immediately adjacent to the AP boundary secrete DPP to establish a gradient of activity essential for both cell proliferation and organization of the anteroposterior pattern of the wing blade (Nellen et al., 1996). At the DV boundary, interactions between the Serrate, Fringe and Notch proteins designate a band of cells destined to become the wing margin (Doherty et al., 1996; Kim et al., 1995). Cells within the margin secrete the Wingless protein to establish an additional morphogen gradient perpendicular to the anteroposterior DPP gradient (Zecca et al., 1996).

Within this complex mix of regulatory signals, the *vg* gene serves as a useful target to examine the transcriptional response of individual cells. Based upon examination of its two regulatory enhancers, vgB and vgQ, the circular pattern of VG expression within the wing pouch has been shown to be dependent upon at least four different signals (Fig. 5B). The vgB enhancer directly binds the Su(H) protein and responds to Notch signaling to restrict early VG expression to the DV boundary (Williams et al., 1994). Subsequent activation of the vgQ enhancer depends upon the DPP morphogen gradient originating at the AP boundary (Kim et al., 1997) as well as a signal derived from the DV boundary likely to be the WG protein (Kim et al., 1996; Zecca et al., 1996). Recent results have also demonstrated a significant autoregulatory function for a VG/SD protein complex acting upon the vgQ enhancer (Halder et al., 1998; Klein and Arias, 1999; Paumard-Rigal et al., 1998; Simmonds et al., 1998).

In addition to this set of activating signals, recent reports have described an overlying gradient of repressor activity functioning in opposition to the DPP morphogen gradient. Expression of the Brinker (BRK) protein, proposed to function as a general repressor of DPP target genes, is itself repressed by DPP signaling, creating a complementary activity gradient (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a,b). Thus, one function of the DPP morphogen would be to relieve repression by BRK, allowing target genes to be activated either directly by DNA-bound MAD or by heterologous activators. Although direct binding of BRK to vgQ enhancer sequences has not yet been demonstrated, it has been proposed that *vg* is among the group of DPP target genes repressed by BRK (Campbell and Tomlinson, 1999; Jazwinska et al., 1999a,b).

To this diverse collection of activators and repressors controlling vgQ-dependent expression we now add the DFR POU domain transcription factor. Results presented here demonstrate that DFR binding to a sequence element adjacent to the mad2 binding site is essential for vgQ activation within the wing pouch. If the combined activation influences of MAD, DFR, WG and VG/SD acting upon the vgQ enhancer were simply additive then removal or modulation of the activity of any single component would be expected to cause a proportional decrease in transcriptional response. This might suggest a mechanism by which the graded modulation of target gene activation is determined, based upon the combined level of transcriptional activators bound to the enhancer. However, our results show that disruption of DFR binding caused a complete loss of *vgQ-lacZ* expression rather than a proportional decrease. The same requirement for MAD binding was observed in our experiments in which no *vgQ-lacZ* expression was seen when both mad1 and mad2 were simultaneously disrupted. These results imply that both MAD and DFR are incapable of activating vgQ-dependent expression in the absence of the other.

Our results indicate that DFR is clearly not an essential general component in the DPP signaling pathway and does not function as an activator of all DPP target genes. Despite the striking alterations in *vgQ-lacZ* expression observed in wing imaginal discs expressing ectopic DFR or DFR^{B157} proteins, we observed essentially no effect upon two other DPP target genes, *omb* (Fig. 2K) and *sal* (K. Certel, unpublished observation). The DFR protein is a pleiotropic regulator during development, however, and is expressed in a number of DPP-responsive tissues (Anderson et al., 1995). Functional colocalization of DFR activity and DPP signaling in multiple tissues raises the possibility of yet other enhancer-specific interactions involving MAD and DFR. Both DFR and DPP signaling are required for wing vein patterning at pupal stages

(de Celis, 1997; de Celis et al., 1995) and tracheal cell migration in embryos (Affolter et al., 1994; Anderson et al., 1996, 1995; Vincent et al., 1997). Since neither of these developmental events utilize the *vg* gene, this may be evidence of additional instances in which DNA-bound MAD and DFR cooperate to regulate distinct cell-specific enhancers analogous to the vgQ enhancer.

Results presented here describe an essential role for the DFR POU domain transcription factor in activation of the vgQ enhancer in the *Drosophila* wing imaginal disc. This function requires a synergistic interaction with DPP-dependent *vgQ* activation, contributing to our understanding of how patterns of gene expression are generated in response to multiple developmental signals. In this example, a distinctive pattern of DPP target gene expression, represented by the *vgQ* enhancer, is generated not only by a direct response to nuclear-localized MAD protein but also by a restricted response to the essential synergistic activator DFR (Fig. 5B). We have also presented evidence that the exclusion of DFR expression from the DV boundary may be due to repression by high concentrations of WG protein. The DFR protein therefore serves as an additional regulatory link required to orient the vgQ-dependent expression pattern relative to the DV boundary.

Thus, certain DPP target genes may not merely interpret spatial position by exposure to the graded DPP signal, but must also satisfy requirements for additional activators contributing to the potential for selective cell-specific patterns of gene expression. As a result, the absence of the DFR activator at the DV boundary results in exclusion of vgQ enhancer activation, despite the presence of adequate levels of DPP signal. By combining requirements for multiple activators, a single morphogen gradient can control a broad variety of expression patterns specific for individual targets.

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