Molecular interactions between Vestigial and Scalloped promote wing formation in *Drosophila*

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Scalloped (Sd) and Vestigial (Vg) are each needed for *Drosophila* wing development. We show that Sd is required for Vg function and that altering their relative cellular levels inhibits wing formation. In vitro, Vg binds directly to both Sd and its human homolog, Transcription Enhancer Factor-1. The interaction domains map to a small region of Vg that is essential for Vg-mediated gene activation and to the carboxy-terminal half of Sd. Our observations indicate that Vg and Sd function coordinately to control the expression of genes required for wing development, which implies that Vg is a tissue-specific transcriptional intermediary factor of Sd.

The *Drosophila vestigial* (*vg*) and *scalloped* (*sd*) genes are expressed in similar patterns during wing development, and mutations in either gene lead to loss of wing tissue (Campbell et al. 1991, 1992; Williams et al. 1991, 1993). Vg is a developmentally regulated nuclear protein of previously unknown function and is required principally for the development of the wing and haltere (Williams et al. 1991). Sd is part of a highly conserved family of transcription factors, the TEA/ATTS domain proteins and is an essential protein with a wider developmental role (Campbell et al. 1991, 1992).

Expression of *vg* in cells of the developing wing primordia is established by a number of conserved signaling pathways and is required for subsequent cell proliferation and patterning. Expression of *wingless* (*wg*), as well as interactions between dorsal and ventral cells that activate the Notch receptor, initially directs limited *vg* expression along the dorsal–ventral (D/V) wing boundary (Williams et al. 1994; Kim et al. 1995, 1996; for review,

see Irvine and Vogt 1997). Subsequent *vg* expression in the wing primordia occurs in response to both the D/V Wg signal and Decapentaplegic (Dpp), a member of the Transforming Growth Factor-β (TGF-β) protein family, secreted by cells along the anterior–posterior (A/P) border. (Blair 1994; Kim et al. 1996, 1997; Zecca et al. 1996; Neumann and Cohen 1997). By the late third larval instar, maximal amounts of Vg are seen in cells at the D/V wing disc boundary, whereas cells located farther from this border produce progressively less Vg (Williams et al. 1991). *vg* is also required to maintain *sd* expression in the wing progenitor cells, and *sd* is similarly required for the maintenance of elevated *vg* expression (Williams et al. 1993).

A cellular role for Sd can be inferred from studies of its human homolog Transcription Enhancer Factor-1 (TEF-1). TEF-1 binds to SV40 enhancer sequences via a TEA/ATTS class DNA-binding domain, and has been shown to require transcriptional intermediary factors (TIFs) for proper function (Xiao et al. 1991; Ishiji et al. 1992; Hwang et al. 1993; Gupta et al. 1997). Interestingly, it has been reported that the Sd TEA/ATTS domain does not bind the same enhancer DNA sequences in vitro as TEF-1, although TEF-1 can substitute for Sd during *Drosphila* wing development (Hwang et al. 1993; Deshpande et al. 1997). This suggests that other factors within *Drosophila* wing cells interact with and modify the specificity of both Sd and TEF-1 in a similar fashion.

Results and Discussion

Previous analysis has shown that ectopic expression of Vg, under control of a *dpp* enhancer, can induce transformation of some cells in the eye, antenna, leg, and genital imaginal discs into wing-specific fates, as well as causing tissue overgrowth (Fig. 1b; Kim et al. 1996). Importantly, whereas Vg expression is normally restricted to the wing and haltere imaginal discs, a subset of cells within almost all imaginal discs normally express *sd* (Campbell et al. 1992). Thus, when Vg is ectopically produced a supply of Sd is already present in those tissues. As Sd is required for formation of the normal wing, we tested whether there is a similar requirement for Sd in the formation of Vg-induced ectopic wings. The induction of wing tissue overgrowths by ectopic Vg was partially suppressed in animals heterozygous for a strong viable allele of *sd* (Fig. 1d), *sd^58^*, and was completely suppressed in *sd^58^* hemizygotes (Fig. 1e). These observations demonstrate that Vg requires Sd to transform cells to wing fates. This requirement does not appear to reflect a role for Sd as a downstream effector of Vg function, as expression of Sd alone, whether under the control of *dpp* or other promoters, does not induce the formation of ectopic wing tissue (Fig. 1c; data not shown). Instead, these observations suggest that Sd and Vg could act in parallel to induce wing cell fates.

The possibility that coordinate action of Sd and Vg is effected via a direct protein–protein interaction was ex-
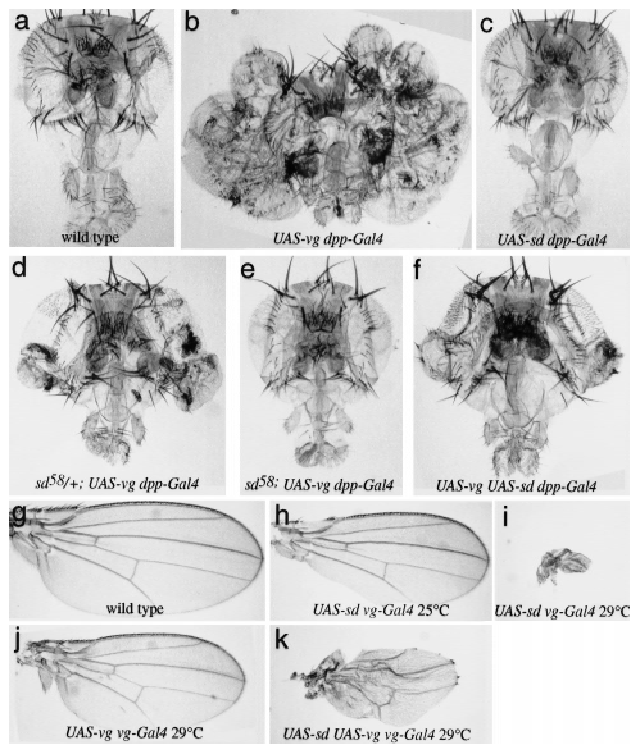
Figure 1. Vg requires Sd and is sensitive to Sd levels in vivo. (*a–f*) *Drosphila* heads; (*g–k*) *Drosphila* wings, in which the Vg and/or Sd proteins have been ectopically expressed using the UAS–Gal4 system (Brand and Perrimon 1993). (*a*) Wild type. (*b*) *UAS–vg dpp–GAL4*. A massive outgrowth of wing tissue from the eye occurs. (*c*) *UAS–sd dpp–GAL4*. No wing tissue is induced, and higher levels of expression (e.g., using *ptc–GAL4*) result in loss of head tissue. (*d*) *sd⁵⁸/+; UAS–vg dpp–GAL4*. The *sd⁵⁸* mutation reduces the amount of Sd produced, and formation of ectopic wing tissue is partially suppressed. (*e*) *sd⁵⁸; UAS–vg dpp–GAL4*. The formation of wing tissue is completely suppressed when no wild-type copy of *sd* is present. (*f*) *UAS–vg UAS–sd dpp–GAL4*. The formation of wing tissue is also partially suppressed when levels of Sd are increased. Raising or lowering the levels of Sd relative to that of Vg within the developing wing disc produces corresponding phenotypes. (*g*) Wild type. (*h*) *UAS–sd vg–GAL4* at 25°C. Elevating Sd levels in wing progenitor cells leads to incomplete formation of the wing margin. (*i*) *UAS–sd vg–GAL4* at 29°C. This effect becomes more severe in flies raised at 29°C, where almost no wing tissue is formed. (*j*) *UAS–vg vg–GAL4* at 29°C. The elevated Vg levels in the wings of these animals leads to a slight reduction in wing size. (*k*) *UAS–sd UAS–vg vg–GAL4* at 29°C. The loss of wing tissue induced by excess Sd (*i*) is partially suppressed by the simultaneous increase in Vg expression.

amined by in vitro-binding experiments. Microaffinity columns bound with bacterially expressed Sd protein selectively retain in vitro-translated Vg (Fig. 2a). Similarly, Vg columns show specific retention of Sd (Fig. 2b). TEF-1, the vertebrate homolog of Sd, was retained on Vg columns with similar affinity (Fig. 2b). Control columns containing the homeodomain proteins Engrailed (En) and Fushi tarazu (Ftz; not shown) did not retain Vg or Sd. Additionally, Luciferase (Luc) did not bind to Vg or Sd columns, confirming that the Sd–Vg interaction is spe-

cific (Fig. 2a–c). Notably, neither Vg nor Sd bound to themselves either, suggesting that they do not form homomultimers (Fig. 2a,b).

To map Vg–Sd and Vg–TEF-1 interaction domains, a Far Western blotting assay was used to screen 15 deleted proteins that remove terminal or internal regions of Vg (Fig. 3a–c). Only Vg proteins that contain amino acids 279–335 have any significant affinity for Sd (Fig. 3b,c). The Vg–Sd interaction appears to be limited to this 56-amino-acid domain, as Sd does not bind to a deleted Vg protein missing only these amino acids, and a construct encoding only this portion of the protein will still bind to Sd. Significantly, a duplicate panel of Vg deletion proteins probed with TEF-1 (Fig. 3b,c) shows that TEF-1 interacts with Vg via the same protein domain. Affinity columns containing this protein fragment of Vg bind Sd and TEF-1 protein as well as full-length Vg does (Fig. 2c). This Sd/TEF-1-binding domain of Vg is serine rich and includes putative phosphorylation sites (Williams et al. 1991). Phosphorylation of Vg at these sites may potentially modify the Vg–Sd interaction. This region is highly conserved in Vg proteins from *Drosphila virilis* (Fig. 3d) and *Aedes aegypti* (S. Carroll, pers. comm.). Similar sequences also occur in mammalian genomic and expressed sequence tag databases (Fig. 3d). The amino- and carboxy-terminal portions of Sd were also tested to map which region of Sd interacts with Vg. Previous studies with TEF-1 demonstrated that regions mediating interaction with cell-specific TIFs were separable from the DNA-binding TEA/ATTS domain (Xiao et al. 1991). The Vg-binding region of Sd maps to the carboxy-terminal half of the protein, separable from the TEA/ATTS domain in the amino-terminal half (Fig. 3e). The carboxy-terminal portion of Sd is also highly similar to TEF-1 (Campbell et al. 1992; Hwang et al 1993; Despande et al.
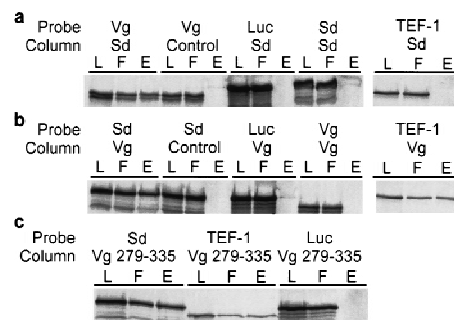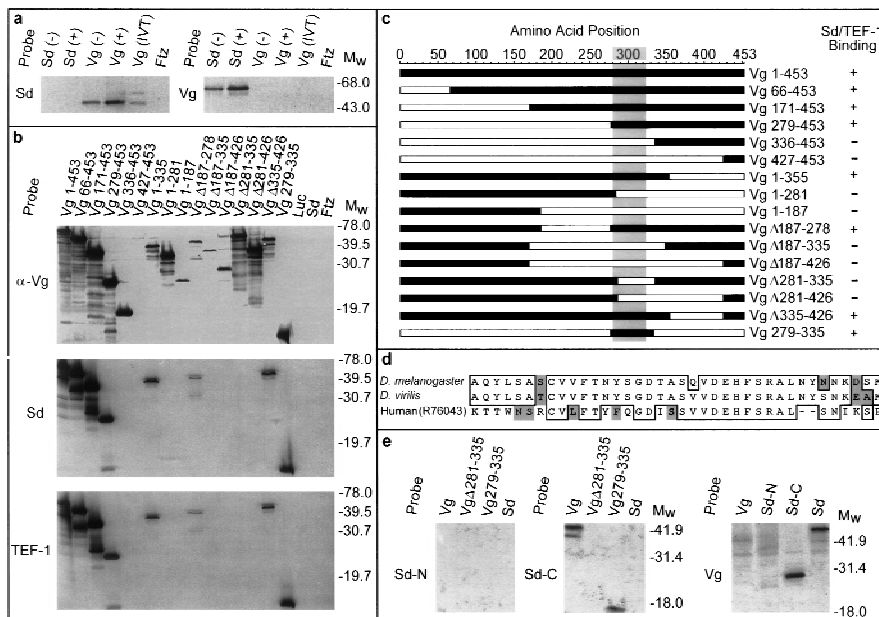

Figure 2. Vg and Sd exhibit a strong protein–protein interaction in vitro. (*a*) Affinity chromatography was performed using bound bacterially expressed Sd protein [(L) column load; (F) column flowthrough; (E) column eluate]. Sd columns selectively retain Vg and not Sd or TEF-1. As a control for nonspecific binding, Luc was expressed in the same in vitro system and shown to not bind to the Sd column. Similarly, the column matrix alone does not retain significant amounts of labeled Vg. (*b*) Vg columns specifically bind Sd protein but not Vg or Luc. The human TEF-1 protein, a homolog of *Drosophila* Sd, also shows a similar specific affinity for Vg. (*c*) Affinity columns bound with a protein consisting of Vg amino acids 279–335 (see Fig. 3) can selectively retain Sd or TEF-1 but not Luc.

Figure 3. Mapping the Vg–Sd interaction domain. (*a*) Far Western blots of bacterial or in vitro-translated (IVT) Vg or Sd were probed with labeled Vg or Sd probes. Ftz protein expressed under similar conditions was also included on each blot to detect nonspecific probe binding. Sd selectively binds bacterially produced and IVT Vg, whereas a corresponding blot probed with Vg shows binding only to Sd. As the detected band is more intense in cell extracts after induction (+) of the expression plasmid than before (–), this confirms that each is plasmid and not endogenously encoded. (*b*) All but one of the specific deletions of Vg (*c*) were recognized by anti-Vg antibody (α-Vg), confirming that Vg is present in each lane. Expression of the Vg 427–453 protein was verified by Coomassie staining. When these deleted proteins were probed with Sd or TEF-1 under conditions similar to those above, only proteins retaining amino acids 279–335 of Vg were detected. Luc, Sd, or Ftz control lanes did not show any signals. (*c*) A map summarizing the position and relative size of the deletions (open bars) within Vg assayed for binding (+ or –) to Sd or TEF-1. The shaded area denotes a region that is highly similar to Vg from *D. virilis* and *A. aegypti* (S. Carroll, pers. comm.). (*d*) This region is also highly similar to sequences identified in mammalian genomic and expressed sequence tag databases (GenBank/EST accession nos. Z798880, Z97632, AA474871, AA571483, W81241, R65857, R73306, R76043). In addition to the 38 amino acids indicated, the smallest tested portion of Vg that has Sd-binding ability includes an amino-terminal Q and amino acids (ESSSPMSSRN-FPPSFWN) carboxy-terminal to this homologous region. (*e*) Probes corresponding to the amino-terminal half of Sd (amino acids 1–242, Sd-N) show no interaction with Vg. The carboxy-terminal half of Sd (amino acids 240–440, Sd-C) binds to Vg protein containing only amino acids 279–335 (Sd-binding domain). A reciprocal blot using full-length Vg (Vg) as a probe shows interaction only with the carboxy-terminal half of Sd (Sd-C).

1997), which is consistent with the observation (Fig. 3c) that TEF-1 binds to Vg with the same affinity as Sd does. To confirm the direct protein–protein interaction between Vg and Sd in a cellular environment, a yeast two-hybrid assay was used. In yeast, Vg and Sd proteins show a specific and reciprocal interaction when fused to either Gal4-binding domain (pGBDU) or Gal4 activation domain (pACT) fusion constructs (not shown; James et al. 1996). Each construct alone or paired with nonspecific (pSNF1BD) bait sequences does not activate a *lacZ* reporter.

We employed the UAS–Gal4 system (Brand and Perrimon 1993) to further examine the in vivo significance of the Sd–Vg interaction. As *vg* is normally required for *sd* expression in wing imaginal discs (Williams et al. 1993),

we examined the influence of ectopic Vg expression on *sd*. Normally, *sd* expression appears confined to the wing pouch region of the wing imaginal disc, although very low levels can sometimes be detected elsewhere. Strong *sd* expression is induced outside of the wing pouch in wing disc cells where *vg* is expressed ectopically under the control of the *patched* (*ptc*) promoter (*UAS–vg ptc–GAL4*) (Fig. 4 a–d). Activation of a downstream target gene by ectopic expression of Vg and Sd was observed using expression of the *cut* (*ct*) gene as an assay. *ct* is likely a direct Vg–Sd target in *Drosphila*, as *sd*, *vg*, and *ct* interact genetically, and the Sd TEA/ATTS domain has been shown to bind to *ct* wing margin enhancer DNA sequences (Morcillo et al. 1996). Consistent with our observation of ectopic *sd* expression and the hypothesis that Sd and Vg function coordinately to regulate gene expression, ectopic expression of Vg in the wing under *ptc–GAL4* control also produces activation of *ct* (Fig. 4e). However, when a *UAS–vg* construct that is missing the 56-amino-acid Sd binding domain is expressed, *sd* and *ct* are not induced (Fig. 4g,h). Thus, activation of both target genes, *sd* and *ct*, by Vg requires the Sd–Vg interaction domain identified in vitro, implying that this is Sd dependent. Moreover, the *UAS–vg* construct with the Sd-binding domain deleted is also unable to induce the formation of ectopic wing tissue (results not shown), consistent with the observation that this induction is *sd* dependent (Fig. 1e). Interestingly, Vg protein deleted for the Sd-binding domain is found in the cytoplasm rather than in the nucleus (Fig. 4f), which implies that association with Sd is required for nuclear localization of Vg.

Further support of a direct Sd–Vg interaction in vivo comes from the observation that elevated amounts of Sd protein can also result in programmed cell death and wing tissue loss (Fig. 1g–i; data not shown). These phenotypes are very similar to those of *sd* or *vg* loss-of-function mutants (Williams et al. 1991, 1993; Campbell et al. 1992). The apparent dominant-negative effect of Sd overexpression suggests that a Vg–Sd heterodimer is functional in vivo and that Sd alone can compete with the functional heterodimer for binding to DNA or other essential cofactors. This suggestion is consistent with studies of the transcriptional activity of TEF-1 and Sd in
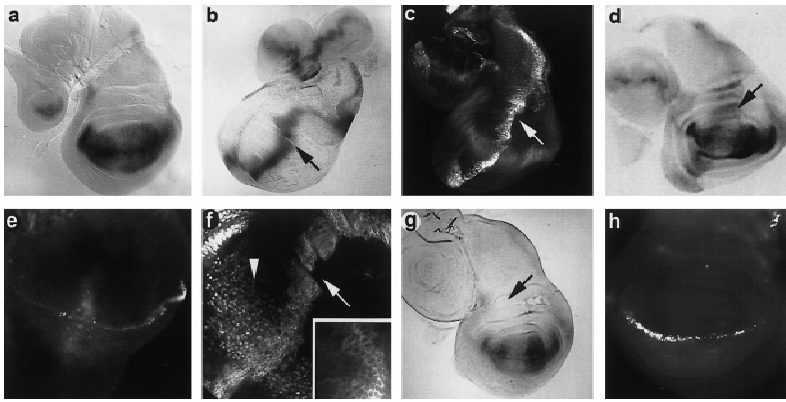
Figure 4. Influence of ectopic Vg on gene expression. (*a*) Third instar larval wing discs of genotype *sd–lacZ*; *ptc–GAL4* showing a normal pattern of high levels of *sd–lacZ* expression in all cells of the presumptive wing pouch (Campbell et al. 1992). Using in situ hybridization to *sd* mRNA, low levels of *sd* expression are also detected outside of the wing pouch (not shown). A localized depression of *sd* activity is usually seen where the D/V intersects the A/P boundary. (*b*) *ptc–GAL4* activates a *UAS–lacZ* reporter along the A/P axis of the wing disc. Note that the highest levels of activation occur in cells immediately adjacent to the border (arrow); lower levels of *lacZ* expression are seen in more anterior cells. (*c*) A *ptc–GAL4*; *UAS–vg* wing imaginal disc shows a similar graded pattern of ectopic *vg* expression along the A/P margin (arrow). (*d*) Third instar wing discs of genotype *sd–lacZ*; *ptc–GAL4*; *UAS–vg*. The ectopic *vg* expression activates *sd* along the A/P margin, causing a nongraded level of activation of the *sd–lacZ* reporter gene in *vg*-expressing cells (arrow). (*e*) A wing imaginal disc of the same genotype as *c* stained with anti-*ct* antibody. Ectopic activation of *ct* occurs in some cells that express ectopic Vg and Sd along the A/P margin. (*f*) VgΔ281–335 protein (driven by *ptc–GAL4*) is found primarily in the cytoplasm [arrow and *inset* (high magnification)] compared to the nuclear localization of endogenous wild-type Vg (arrowhead). (*g*) In discs containing *sd–lacZ*, *ptc–GAL4* mediated expression of a *UAS–vgΔ281–335* construct (missing the Sd-binding domain) does not induce *sd* expression along the A/P margin (arrow). (*h*) Where high levels of ectopic VgΔ281–335 and Sd overlap, *ct* expression remains in the wild-type pattern along the D/V border.

cultured cell lines, in which dominant-negative effects (squelching) appear to occur when they are overexpressed (Xiao et al. 1991; Ishiji et al. 1992; Hwang et al. 1993; Halder et al. 1998). Direct in vivo support for this hypothesis comes from the observation that overexpression of Sd is able to suppress the consequences of ectopic Vg expression almost as efficiently as loss of *sd* function (Fig. 1f). Moreover, overexpression of Vg in the wing is able to partially suppress the tissue loss otherwise associated with Sd overexpression (Fig. 1, cf. k and i). Together, these observations argue that balanced levels of Sd and Vg are essential for normal wing development and further support the conclusion that the specific interaction between Vg and Sd identified in vitro is essential for function in vivo. The exquisite sensitivity of wing tissue growth to the Sd/Vg ratio also raises the possibility that variation of this balance could be a mechanism of growth control during normal development.

A wide variety of studies have suggested that TEA/ATTS domain proteins require tissue-specific TIFs, although relatively little progress has been made toward identifying and characterizing these TIFs (Xiao et al. 1987, 1991; Ishiji et al. 1992; Hwang et al. 1993; Chen et

al. 1994; Farrance and Ordahl 1996; Gavrias et al. 1996; Jacquemin et al. 1996; Stewart et al. 1996; Gupta et al. 1997). According to the definitions established by the analysis of TEF-1 (Xiao et al. 1987, 1991; Ishiji et al. 1992), a TIF for Sd would be expected to bind directly to Sd, to show a restricted pattern of expression, and to be required for Sd function in vivo. Previous studies have shown that *sd* and *vg* have similar mutant phenotypes and patterns of expression in the developing wing (Williams et al. 1991; Kim et al. 1996, 1997). The results presented here demonstrate that Sd and Vg proteins bind to each other and that the cooperative action of Sd and Vg is required in vivo for *Drosphila* wing development. These observations, together with the analysis of the coordinate regulation of downstream target genes by Sd and Vg (Halder et al. 1998), argue that Vg functions as a tissue-specific TIF for Sd. Although it is possible that Vg interacts with proteins other than Sd, genetic studies argue against this, because all *vg* mutant phenotypes are shared by *sd*. In contrast, *sd* is required for the development of other tissues in which *vg* is not required (Campbell et al. 1991; Williams et al. 1991). Thus, it is likely that there are other *trans*-acting factors in *Drosophila* that interact with Sd.

Although the DNA target sequence of Sd is as yet uncharacterized, one target of a yeast TEA/ATTS domain protein (TEC-1) is an element in the TEC-1 promoter (Madhani and Fink 1997). Likewise, in flies, one target of Sd–Vg is likely to be the *sd* promoter itself, as activation of *sd* during early development is dependent on Vg (Williams et al. 1993), and ectopic Vg induces elevated expression of *sd* (Fig. 4). This suggests a model whereby low levels of Sd expression within wing imaginal discs are elevated in the presence of Vg by positive autoregulation. The dependence of elevated levels of Vg in the wing disc on *sd* suggests that *vg* is also a target of positive autoregulation, and direct evidence for this now been obtained (Halder et al. 1998).

The TEA/ATTS domain protein family is involved in developmental processes as diverse as mammalian neuronal and cardiac muscle development (Chen et al. 1994; Jacquemin et al. 1996; Yockey et al. 1996) to conidial formation in *Aspergillus* (Gavrias et al. 1996) and pseudohyphal growth of *Saccharomyces cerevisiae* (Madhani and Fink 1997). Although Vg homologs have not yet been identified in these organisms, we have found that genes containing sequences related to the Sd/TEF-1 interaction domain of Vg are conserved in mammals; these genes are thus candidate TIFs for mammalian TEF-1-related proteins (Fig. 3). One of these candidate TIFs is expressed in fetal heart tissue, which is intriguing given that gene-targeted mutations in TEF-1 result in cardiac defects (Chen et al. 1994). Future challenges will be to

determine whether genes encoding these putative Sd-interacting domains actually function as TIFs for TEF-1 or related proteins, and whether distinct regulatory TIFs have evolved that adapt the transcriptional activities of conserved Sd/TEF-1 homologs to specific functions in different tissues in their respective organisms.

Materials and methods

For the in vitro protein interaction assays, PCR fragments containing either full-length *sd* or *vg* coding region were cloned into pET16b (Novagen) and expressed in *Escherichia coli* (BL21–DE3 pLysE) to produce 6× His-tagged Vg or Sd protein. Inverse PCR, using primers that amplified specific portions of the *vg* or *sd* coding region shown in Figure 3, was used to create deleted Vg and Sd expression constructs. Recombinant proteins containing the amino-terminal histidine tag were purified on Ni^{2+} resin following the manufacturer's directions (Invitrogen). Affinity chromatography, Western, and Far Western blotting were performed as described previously (Guichet et al. 1997), with the following modifications. For microcolumn affinity chromatography, 500 µl of a 200 µg/ml solution of each protein to be tested was coupled to 100 µl of Ni^{2+} resin in binding buffer (20 mM NaH$_2$PO$_4$ at pH 7.8, 0.5 M NaCl) and rewashed according to the manufacturer's directions (Invitrogen). Columns with Ni^{2+} resin alone served as controls for nonspecific binding of the labeled probe. Each column was then equilibrated in 100 mM NaCl, 0.1 M Tris (pH 7.6), and 10% glycerol. Columns were subsequently blocked using a solution of 0.5% skim milk powder in equilibrium solution. ^{35}S-Labeled probes were made by cloning the relevant coding region of each protein to be tested into pBluescript SK(–) and then expressed using the TnT in vitro-coupled rabbit reticulocyte system (Promega). The resulting probes were purified using Sepharose G25 spin columns, and a 2-µl aliquot of each was then quantified using a PhosphorImager. Approximately 40–45 µl of each labeling reaction was combined with 80 µl of blocking solution and passed over the respective affinity column. Any bound protein was eluted using 100 µl of the equilibration solution plus 2% SDS. For Far Western blotting, ~10 µl of total lysate from bacteria expressing Vg, Sd, or control protein per lane was separated on a 13% SDS–polyacrylamide gel and blotted to nitrocellulose. Proteins on the resulting blot were serially renatured in a series of 6, 3, 1, and 0.1 M guanidine–HCl solutions containing 20 mM Tris (pH 7.6), 100 mM NaCl, 0.1% Tween 20, 2% skim milk powder, 10% glycerol, and 1 mM EDTA. Probes prepared as described were incubated with each blot for 2 hr at 4°C in blocking solution plus 1 mM DTT. Antibody staining of imaginal discs was performed as described previously (Simmonds et al. 1995). Rabbit anti-Vg was used at a dilution of 1/50 on imaginal discs and at 1/200 for Western blotting (Williams et al. 1991). Mouse anti-β-galactosidase (Promega) and mouse anti-Ct 2B10 (Blochlinger et al. 1990) were used at 1/500. *sd–lacZ* refers to the *sd^{ETX4}* line described by Campbell et al. (1992). *UAS–sd* and *UAS–vgΔ281–335* transgenes were constructed by cloning a 2.05-kb *Xmn*I–*Cla*I fragment of a *sd* cDNA (Campbell et al. 1992) or a PCR fragment consisting of the coding region of the VgΔ281–335 expression construct into pUAST (Brand and Perrimon 1993).

Acknowledgments

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