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TONDU (TDU), a novel human protein related to the product of *vestigial* (*vg*) gene of *Drosophila melanogaster* interacts with vertebrate TEF factors and substitutes for Vg function in wing formation

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SUMMARY

The mammalian TEF and the *Drosophila scalloped* genes belong to a conserved family of transcriptional factors that possesses a TEA/ATTS DNA-binding domain. Transcriptional activation by these proteins likely requires interactions with specific coactivators. In *Drosophila*, Scalloped (Sd) interacts with Vestigial (Vg) to form a complex, which binds DNA through the Sd TEA/ATTS domain. The Sd-Vg heterodimer is a key regulator of wing development, which directly controls several target genes and is able to induce wing outgrowth when ectopically expressed. Here we show that Vg contains two distinct transcriptional activation domains, suggesting that the function of Vg is to mediate transcriptional activation by Sd. By expressing a chimeric GAL4-Sd protein in *Drosophila*, we found that the transcriptional activity of the Vg-Sd heterodimer is negatively regulated at the AP and DV boundary of the wing disc. We also identify a novel human protein, TONDU, which contains a short domain homologous to the domain of Vg required for interaction with Sd. We show that TONDU specifically interacts with a domain conserved in all the mammalian TEF factors. Expression of TDU in *Drosophila* by means of the UAS-GAL4 system shows that this human protein can substitute for Vg in wing formation. We propose that TDU is a specific coactivator for the mammalian TEFs.

Key words: Vestigial, Scalloped, TEF, Wing patterning, Transcription factor

INTRODUCTION

The growth and patterning of the *Drosophila* wing is dependent on the formation of compartments in the wing imaginal disc which are defined by the restricted expression of the selector genes *engrailed* and *invected* in the posterior compartment and *apterous* in the dorsal compartment (reviewed in Blair, 1997). Interaction between cells at the boundary of these compartments leads to the production of the signaling molecules Wingless (Wg) at the dorsoventral (DV) boundary and Decapentaplegic (Dpp) at the anteroposterior (AP) boundary and to the activation of the Notch (N) pathway at the DV boundary (Lecuit et al., 1996; Neumann and Cohen, 1996, 1997; Serrano and O'Farrell, 1997).

These proteins will regulate the expression of several genes amongst which *vestigial* (*vg*) and *scalloped* (*sd*) play a crucial role in wing morphogenesis. These two genes are expressed in the wing primordia with identical patterns and mutations of both genes are associated with a loss of wing tissue. *vg* expression is regulated by two separate enhancers: the boundary (BE) and the quadrant enhancer (QE) (Kim et al., 1996; Williams et al., 1994). The BE directs *vg* expression at the DV boundary whilst the QE drives *vg* expression in the rest of the wing pouch. These two enhancers are sequentially activated by the DV signaling pathway (*N*, *wg*) and the AP signaling pathway (*dpp*) and allow *vg* expression in the wing pouch with highest expression at the DV boundary (Kim et al., 1996, 1997).

It has been shown that ectopic *vg* but not *sd* expression, in the eye-antenna or leg imaginal discs induces wing tissue outgrowth, misexpression of wing-specific genes such as *sd*, *drosophila serum responsive factor* (*dsrf*), *spalt* (*sal*) and *spalt related gene* (*salr*), and its own expression (Kim et al., 1996; Halder et al., 1998; Paumard-Rigal et al., 1998; Simmonds et al., 1998). In addition, several observations demonstrate that Vg must interact directly with Sd to activate target gene expression and promote wing tissue proliferation: (i) in vitro protein interaction tests and two-hybrid experiments have shown that Vg and Sd form a dimer (Paumard-Rigal et al., 1998; Simmonds et al., 1998), (ii) *trans*-determination as well as gene induction associated with ectopic *vg* expression are suppressed in *sd* mutants (Paumard-Rigal et al., 1998; Simmonds et al., 1998), (iii) binding sites for Sd have been identified in the enhancers of several genes regulated by Vg and in the two *vg* enhancers. In S2 cells, induction of these enhancers by Vg requires the function of the Sd protein (Halder

et al., 1998). It can be inferred from these studies that *vg* integrates positional information conferred by the AP and DV signaling pathways and activates gene expression by forming a complex with Sd. However, the role of Vg in this complex has not been formerly determined. Here we show that Vg is a transcriptional activator in yeast suggesting that the function of Vg, in the Sd/Vg complex, is to activate transcription.

The *sd* gene encodes a protein belonging to the TEA transcription factors family (Campbell et al., 1992; Jacquemin and Davidson, 1997). Strong or null alleles are lethal (Campbell et al., 1991). During embryonic development *sd* is expressed in the precursors of the larval nervous system (Campbell et al., 1992). During larval development, a specific pattern of *sd* expression is observed in the leg, eye-antenna and wing imaginal discs (Campbell et al., 1992) suggesting a role for *sd* in the development of the corresponding adult structures. In the wing imaginal disc, *sd* is mainly expressed in the region that will give rise to the wing blade and in the presumptive scutellum region (Campbell et al., 1992; Williams et al., 1993). This expression pattern correlates with the wing defects in *sd* mutants (Campbell et al., 1991).

Expression in the leg imaginal disc is not associated with any defects in these appendages at least in hypomorph *sd* alleles while in the antenna-eye imaginal disc, only a slightly roughening of eyes has been reported in *sd* mutants (Lindsley and Zimm, 1992).

In the wing imaginal disc, it has been shown that Sd interacts with Vg to activate the transcription of target genes (Halder et al., 1998). However, in other imaginal discs such as the leg or antenna-eye discs, where *sd* but not *vg* is expressed, there may be other Sd-interacting coactivators, assuming that the function of Sd in these structures is to activate the transcription of specific target genes.

To determine in which structures Sd is transcriptionally active without using ex vivo system or ectopic gene expression, which could modify cell fate, we produced transgenic flies harboring a GAL4-responsive *lacZ* reporter gene and expressing a modified Sd protein in which the TEA/ATTS domain has been replaced by the DNA-binding domain of the GAL4 protein. We show that, in third instar larvae, ubiquitous expression of this fusion protein activates the cognate reporter gene only in the wing pouch of the wing disc where *vg* is specifically expressed, but does not activate transcription in the other discs where *sd* is expressed. This result indicates that, at this time, *sd* is transcriptionally active only in Vg-expressing cells. Consequently, no functional homologues of Vg are present in other discs and the molecular function of *sd* in these discs, if any, is likely different from its role in wing morphogenesis. Surprisingly, we found that, in the wing disc, even if Vg and Sd are present, the activity of the Vg/Sd complex is reduced at the DV and AP boundaries in late third instar larvae. We propose that the ratio between Vg and Sd regulates the activity of Sd/Vg dimer during wing development.

It has been proposed that the mammalian TEF factors require transcriptional intermediary factors (TIFs) to activate transcription (Chaudary et al., 1994; Hwang et al., 1993; Xiao et al., 1991). The existence of such TIFs have been deduced from transfection analysis where activation of a cognate reporter is severely reduced upon TEF-1 overexpression. This dominant effect is also observed with a truncated form of TEF-1 lacking the TEA/ATTS DNA-binding domain (Hwang et al., 1993). It has been shown that TEF-1 interacts with Vg in vitro and can substitute for Sd during *Drosophila* development (Deshpande et al., 1997; Simmonds et al., 1998). This functional conservation between Sd and TEF-1, and the fact that Vg is a likely TIF for Sd, suggests that a protein with homology to Vg may exist in mammalian cells. We have cloned a novel gene called TONDU (*TDU*) which possesses a short domain homologous to the region of the Vg protein required for interaction with Sd and TEF-1. We show that this novel human protein interacts with all the mammalian TEFs and with Sd both in yeast and in vitro and is able to functionally substitute for Vg in wing blade formation and induction of *sd* and *vg* expression in vivo in *Drosophila*. We conclude from this functional analysis in *Drosophila* that TDU could be a co-activator for the mammalian TEFs.

MATERIALS AND METHODS

Drosophila strains

The vg^{83b27} strain was provided by J. Bell (Williams et al., 1991) and the *vg-GAL4* was provided by M. Hoffmann and S. Morimura. The $sd^{P(ry+,ETX4)}$ strain is described in Anand et al. (1990) and Campbell et al. (1992), and the sd^{58} strain provided by K. Irvine (Simmonds et al., 1998). The *ptc-GAL4* and the *UAS-lacZ* strains were provided by the Bloomington Stock Center. The *UAS-reaper* strain is described in Van de Bor et al. (1999). The *vg*BE (for *vg* Boundary Enhancer) and *vg*QE (for *vg* Quadrant Enhancer) were provided by S. Carroll and are described in Williams et al. (1994) and Kim et al. (1996).

Cloning of TONDU (*TDU*)

TONDU cDNA (GenBank Accession number: AF137387) corresponds to the I.M.A.G.E. consortium clone ID 347406 (Lennon et al., 1995) which contains the entire coding sequence of TDU.

One- and two-hybrid assay

The different plasmids and methodological procedure used in this study were provided by R. Brent (Gyuris et al., 1993; Russel et al., 1995).

For one-hybrid assays: the yeast EGY48 holding the *lacZ* reporter plasmid pSH18-34 was transformed with pEG202 derivatives containing the lexA fused to the *vg* sequence corresponding to the amino acids 7-453, 7-127, 127-273, 273-357, 357-453. The β-galactosidase activity was detected with ONPG assay after growth on SCglu-UH. The LEU2 assay was performed by testing the growth on ScGlu-UHL.

For two-hybrid assays: the same yeast strain was co-transformed with a pEG202 derivative and a derivative containing the B42 fusion. To test the interaction between TEF-1, TEF-3, TEF-4, TEF-5 and TDU, we have constructed four pEG202 derivatives containing hTEF-1, hTEF-3, mTEF-4 and hTEF-5 sequences corresponding to the amino acids 136-426, 137-427, 146-445, 141-435, respectively, and pJG4-5 derivative containing TDU sequence corresponding to the amino acids 24-50. The β-galactosidase activity was detected with ONPG assay after growth on SCGal-UHW. The LEU2 assay was performed by testing the growth on ScGal-UHWL.

In vitro interaction test

Bacteria harboring plasmid pGEX2T-GST-TONDU, pGEX2T-GST-TEF-1 or pGEX2T-SdΔTEA were induced with IPTG 1mM. The fusion protein was purified using glutathione-sepharose 4B beads (Pharmacia) as described by the manufacturer.

0.5 μg of the appropriate pXJ40-TEF vectors (described in Hwang et al., 1993) or of the appropriate pXJ40-TDU vectors were used in a

TnT-coupled rabbit reticulocyte lysate system (Promega) according to the supplier's recommendations in the presence of [^{35}S]methionine. pXJ40-TDU plasmid and the three pXJ40TDU derivatives (TDU-Δ24-50, TDU-Δ24-40, TDU-Δ41-50) were produced by PCR and verified by sequencing. All details of cloning are available upon request. Interaction test procedures were performed as described in Monnier et al. (1998).

Transient transfection of *Drosophila* S2 cells

The reporter construct pUAST-*lacZ* vector was obtained by inserting the *lacZ* gene of *E. coli* into the pUAST vector. The pCaSpeR-hsp-GAL4$_{db}$-TDU vector encodes for a chimeric protein formed with the amino acids 1-147 of GAL4 and the amino acids 1-256 of TDU, under the control of *hsp* promoter. *Drosophila* S2 cells were maintained in Schneider medium with 5% heat-inactivated fetal bovine serum. Transfections were performed by using DOTAP liposomal transfection reagent kit (Boehringer) according to the supplier's recommendations. Following incubation for 24 hours at 25°C, cells were heat shocked at 37°C for 1 hour and incubated for an additional 24 hours incubation at 25°C. The β-galactosidase activity was evaluated with ONPG assay. All transfections were performed in duplicate in at least three independent experiments. Transfection efficiency ranged from 5 to 10% as assayed with pCaSpeR-hsp-GFP vector.

Germline transformation

The pCaSpeR-hsp-GAL4$_{db}$-Sd vector encodes for a chimeric protein formed with the amino acids 1-147 of GAL4 and the amino acids 213-440 of Sd, under the control of *hsp* promoter. The pUAST-TDU vector was obtained by insertion of the entire TDU cDNA in the pUAST vector.

Northern blot analysis

Northern blot Human Fetal Multiple Tissue Northern (MTN^{TM}) BlotII was purchased from CLONTECH Laboratories inc. Hybridization was carried out according to the supplier's recommendations with the entire cDNA of TDU as a probe.

X-gal staining and immunochemistry

X-gal staining was performed as described in Paumard-Rigal et al. (1998) and viewed using Nomarski optics. Immunostaining, using rabbit polyclonal anti-Vg (Williams et al., 1991), was performed according standard protocols.

RESULTS

Vestigial is a transcriptional activator in yeast

Previous analysis have shown that Vg and Sd physically interact (Paumard-Rigal et al., 1998; Simmonds et al., 1998). Furthermore, expression of Sd and Vg in S2 cells synergistically activates the three enhancers of *dSRF* or *vg* genes (Halder et al., 1998). These results support a model in which the two proteins form a heterodimer that binds DNA through the TEA domain of Sd, but the role of Vg remains unclear. To assess the possibility that Vg is a transcriptional activator, we tested the ability of a fusion protein between Vg and the LexA DNA-binding domain (LexA-Vg) to activate the expression of the *lacZ* and *LEU2* reporter genes in yeast. As shown in Fig. 1, significant activation was observed. To determine which region of the Vg protein is involved in transcriptional activation, we used four LexA-Vg derivatives. Significant activation was only observed with the N-terminal and C-terminal domains of the Vg protein (Fig. 1). No activation was observed with the domain of Vg that interacts

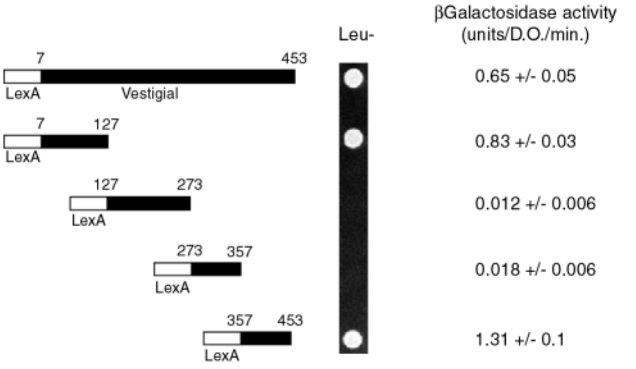

Fig. 1. One-hybrid assay shows that Vg activates transcription in yeast. Yeast one-hybrid assay: the EGY48-pSH18-34 strain was transformed with several pEG202 derivatives containing LexA-Vg fusions and analyse for β-galactosidase activity and leucine auxotrophy. Growth in the absence of leucine and β-galactosidase activity indicate a transcriptional activity of the Vg protein. The activation of the two reporter genes is observed with the LexA-Vg protein, which contains the entire sequence of Vg. To determine which domain of Vg is transcriptionally active, four pEG202-Vg derivatives (see Materials and Methods) were transformed in yeast. Two domains of Vg, corresponding respectively to the amino acids 7-127 and 357-453, are able to induce transcription of the two reporter genes.

with Sd. This result indicates that Vg contains two separate autonomous domains that are able to activate transcription in yeast.

Sd activates transcription only in Vg-expressing cells of the *Drosophila* wing imaginal disc

In the wing imaginal disc, it has been shown that Sd interacts with Vg to activate the transcription of target genes (Halder et al., 1998). However, in other imaginal discs such as the leg or antenna-eye discs, where *sd* but not *vg* is expressed, there must be other Sd-interacting coactivators if Sd function is to activate the transcription of specific target genes. To assess this possibility, we developed a genetic approach to allow us to determine in which structures Sd is transcriptionally active. We produced transgenic flies expressing a modified Sd protein in which the TEA DNA-binding domain has been replaced by that of the GAL4 yeast protein, under the control of the *hsp70* promoter, We used two GAL4-responsive reporter strains; *UAS-lacZ* and *UAS-reaper*. In this latter line, the expression of the cell death gene *reaper* is under the control of *UAS* sequences and will produce cell ablation in the cells where Sd is transcriptionally active.

In a first step towards determining where Sd is active, heat shock was applied at the mid third larval instar and larvae collected 24 hours later, which corresponds to late third instar. We found that, in *hsp-GAL4$_{db}$-sd/UAS-lacZ* larvae, *lacZ* expression was restricted to the wing pouch in the wing and halter discs (Fig. 2C). When the experiment was done in a *vg* mutant context (*hsp-GAL4$_{db}$-sd; vg^{BG}/vg^{BG}; UAS-lacZ*), X-gal staining in the wing and halter discs was strongly reduced or absent (Fig. 2I). No expression was detected in other imaginal discs (data not shown). With *hsp-GAL4$_{db}$-sd/UAS-reaper* flies, we observed, in pharate adults, a loss of wing tissue (see below for a more precise description), but no visible effect on the

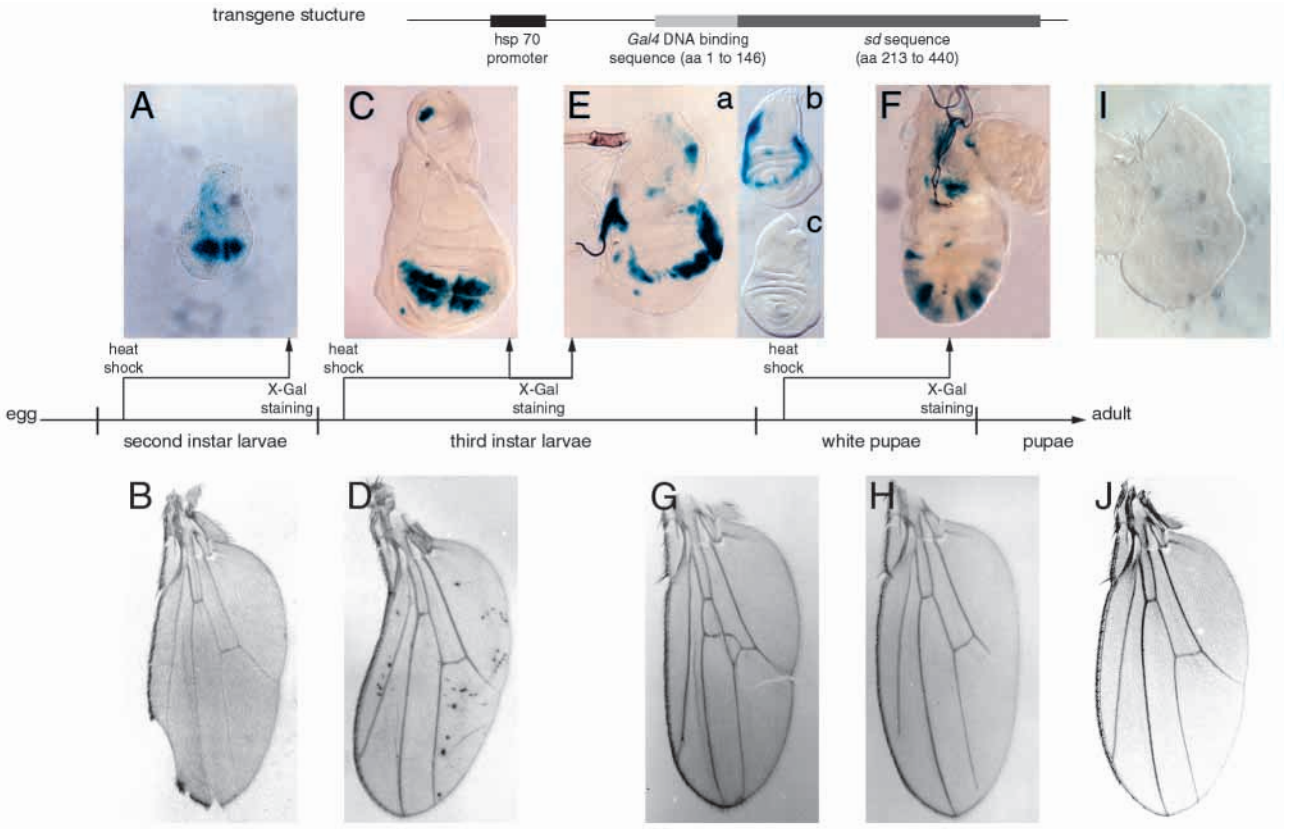

Fig. 2. Functional analysis of the Sd/Vg dimer activity. Transgene structure is described at the top of the figure. This transgene encodes, under the control of the *hsp* promoter, a modified Sd protein in which the TEA/ATTS DNA-binding domain was substitute by the GAL4 DNA-binding domain. To investigate where and when the protein is transcriptionally active, *GAL4$_{db}$-Sd* flies were crossed either with *UAS-lacZ* flies (A,C,F) or with *UAS-reaper* flies (B,D,G,H) and larvae were heat-pulsed for 2 hours at 37°C at mid second instar (A,B), mid third instar (C,D) or prepupal stages (F-H) as shown in the time scale. In the case of *UAS-lacZ* reporter gene, β-galactosidase staining was performed 24 hours after heat shock, whilst in the case of *UAS-reaper*, wing phenotypes were observed at adult stage and compared with wild-type wing in J. (A) When GAL4$_{db}$-Sd is induced at mid second instar, X-gal staining is observed at the DV boundary and in a part of the wing pouch. (B) Wings appear with gaps in the wing margin. Wing blade and veins are not affected. This result, with the one obtained in A, support the fact that the Sd/Vg dimer is active only at the DV boundary of the wing disc. (C) X-gal staining is displaced in the entire wing pouch except at the DV and AP boundaries, when GAL4$_{db}$-Sd is induced at mid third instar stage. Staining is also observed in the haltere disc. (D) Wings show necrosis in the wing blade; wing margin and veins are not affected. Together with the result obtained in C, this shows that the Sd/Vg dimer is preferentially active in the wing disc region, which gives rise to the wing blade. (E-a) β-galactosidase staining was observed at the DV boundary and in the presumptive hinge region of the wing disc when larvae of the genotype *sd^{58}/Y; GAL4$_{db}$-sd/+; UAS-lacZ/+* were heat pulsed at mid third instar stage. (E-b) activity of the *vg*BE was detected in *sd^{58}/Y* mutant wing discs. (E-c) *vg*QE is not activated in a *sd^{58}/Y* mutant wing disc. (F) β-galactosidase is produced in the wing pouch except at the DV boundary when GAL4$_{db}$-Sd is produced at the prepupal stage. Note a more intensive staining in provein regions. (G,H) Disruptions in veins L2, L4 and L5 or ectopic veins were observed and indicate that, in prepupal stage, the Sd/Vg dimer is involved in the vein and intervein cell fate determination. (I) No β-galactosidase staining was observed when *vg^{BG}/vg^{BG}; GAL4$_{db}$-Sd/+; UAS-lacZ/+* larvae were heat pulsed at mid third instar stage. This result indicates that Sd transcriptional activity in the wing disc is dependant of Vg.

thorax, legs or eyes. This result indicates that, in third instar larvae, Sd activates transcription of reporter genes only in the wing discs where *vg* is specifically expressed. This suggests that, in other imaginal discs where *sd* is endogenously expressed, it is not transcriptionally active and that its function is different from that in wing morphogenesis. For example, Sd may behave as a transcriptional repressor in these discs. Furthermore, as we analyzed the ability of Sd to activate transcription in third instar larvae, we cannot exclude the possibility that Sd interacts with factors other than Vg and to activate transcription at the embryonic, early larval or pupal stages. However, the absence of clear phenotype associated with *sd* hypomorph mutations in cuticular structures such as eyes or legs indicates that *sd* is likely not required for the development of these structures, but this has to be more precisely determined by clonal analysis.

To determine in which cells the Sd/Vg dimer is active during wing imaginal disc development, we applied heat shock at different stages. When heat shock was applied at the beginning of the second larval instar, we observed a disruption of the wing margin, but an intact wing blade in the *hsp-GAL4$_{db}$-sd/UAS-reaper* flies (Fig. 2B). This shows that, at this time, the Sd/Vg complex is preferentially active and required at the presumptive wing margin. X-gal staining performed on *hsp-GAL4$_{db}$-sd/UAS-lacZ* discs supports this conclusion (Fig. 2A).

In late third instar wing discs, X-gal staining was restricted

to the wing pouch, but interestingly, no or lower staining was observed at the DV and AP boundary (Fig. 2C). This indicates that although Vg and Sd are present**,** the complex formed is not active at the DV and AP boundaries. This was confirmed with the *hsp-GAL4$_{db}$-sd/UAS-reaper* flies, which show a necrosis of the wing blade, but an intact wing margin when heat shock was done at the beginning of the third instar (Fig. 2D).

With *hsp-GAL4$_{db}$-sd/UAS-reaper* flies heat pulsed at the end of third larval instar, we observed vein disorganization, with both ectopic or absent veins (Fig. 2G,H). In prepupal discs, X-gal staining was more intense in stripes, which likely correspond to the presumptive vein regions (Fig. 2F). This result suggests a role of the Sd/Vg complex in the determination of vein/intervein cell fate and is supported by experiment from Halder et al. (1998), which shows that the dSRF gene is a target gene of the Sd/Vg dimer. In addition, we observed that *vg* surexpression in wing disc induces disorganisation of vein patterning (data not shown).

Altogether, these results indicate that, at the beginning of the third larval instar, the Sd/Vg complex is active at the DV boundary, whilst its activity later decreases at the DV and AP boundary to be finally restricted to specific regions of the wing blade.

How can these results be explained in terms of a functional Sd/Vg complex? Several observations suggest that the Sd/Vg ratio is critical for the function of the Sd-Vg complex. First, it has been shown that overexpression of *sd* by means of the *UAS/GAL4* system suppresses the wing outgrowth induced by ectopic *vg* expression (Halder et al., 1998). Second, their stoichiometry is crucial in cell culture for the activation of target genes (Halder et al., 1998; Simmonds et al., 1998). In fact, in our system, Vg can bind either to endogenous Sd or to the GAL4$_{db}$-Sd chimeric protein. If the lack of X-gal staining that we observed at the DV boundary reflects an imbalance between Sd and Vg, we would predict that reducing *sd* expression will increase the quantity of GAL4db-Sd/Vg dimer and therefore lead to X-gal staining at the DV boundary. To test this possibility, we analyzed *lacZ* reporter expression driven by GAL4$_{db}$-Sd in the *sd^{58}* strain, which is a strong hypomorph *sd* mutant. In this context, we observed a staining at the DV boundary and in the presumptive hinge region (Fig. 2E-a) at the third instar. This experiment confirms that the stoichiometry between Sd and Vg is critical for the formation of an active complex. No staining was observed in the remaining of wing pouch suggesting that *vg* is not expressed in these cells in a *sd^{58}* mutant context. To confirm this, we analyzed *vg*BE and *vg*QE activity and found that *vg*BE but not *vg*QE was activated in a *sd^{58}* context (Fig. 2E-b,E-c). Although it has never been described before, we found that less Vg is produced at the DV and AP boundaries (inset in Fig. 5D) in late third instar larvae. As *vg* is itself regulated by the Vg/Sd heterodimer, this decrease in Vg expression could be due to the lack of activity of this complex at the DV boundary.

A human protein with homology to Vg interacts with Sd and mammalian TEF factors

The above results show that Vg is a coactivator for Sd in *Drosophila* (this work and Halder et al., 1998; Simmonds et al., 1998). The observation that the mammalian TEF factors are able to physically and functionally interact with Vg led us search for mammalian proteins with homology with Vg (Deshpande et al., 1997; Simmonds et al., 1998). Sequence comparison analysis with dbEST cluster database identified a putative human protein Q99990 that shows sequence homology with Vg. This protein was deduced from EST sequences and corresponds to a unique gene included in PAC 196E23 that was found to localize on chromosome Xq26.1-27.2. Interestingly the similarity between the two proteins is restricted to the Vg domain that interacts with Sd or TEF-1 suggesting that this human protein could interact with different TEF proteins. By using I.M.A.G.E. consortium cloneID 347406 (Lennon et al., 1995), we cloned a 1147 bp cDNA comprising the complete open reading of this gene, designated TONDU (*TDU*) encoding a 258 amino acid protein (Fig. 3A). In northern blot experiments, this cDNA reveals a 1.35 kb transcript which is expressed in fetal lung and kidney, but not in brain and liver (Fig. 3B), indicating that the isolated cDNA is almost complete and that TDU is not ubiquitously expressed.

To investigate possible interactions between TDU and the

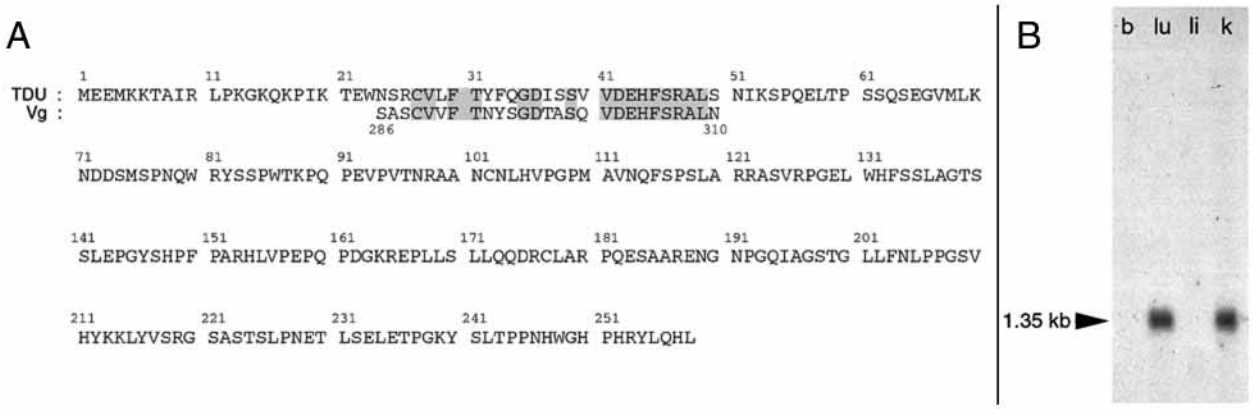

Fig. 3. Comparison of TDU sequence and Vg sequence and Northern blot analysis. (A) Protein sequence of TDU (GenBank Acc: AF137387) was deduced from the I.M.A.G.E. consortium clone ID 347406 (Lennon et al., 1995). TDU encodes a 258 amino-acid protein, which contains a homologous region with the region 286 to 310 of Vg (in grey). TDU shares no more homology with other known protein sequences.
(B) Northern blot analysis. A northern containing poly(A)+ RNA from human fetal brain (b), lung (lu), liver (li) and kidney (k) was hybridized with a TDU cDNA probe. This reveals a 1.35 kb RNA in lung and in kidney.

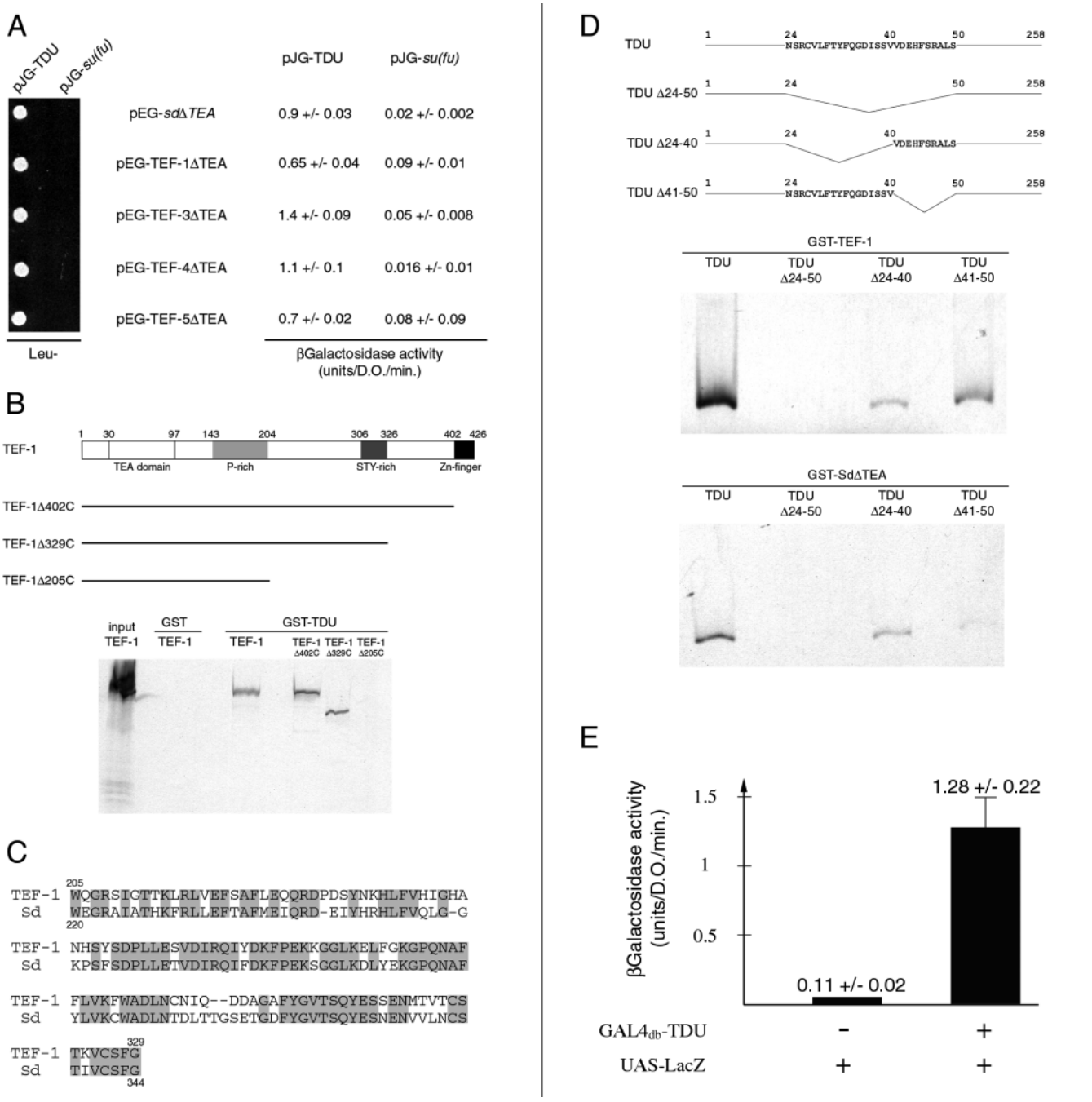

Fig. 4. Evidence for in vivo and in vitro interaction between TDU and TEF factors. (A) Yeast two-hybrid assay: the EGY48-pSH18-34 strain was co-transformed with several pEG202 derivatives containing LexA-SdΔTEA, LexA-TEF-1ΔTEA, LexA-TEF-3ΔTEA, LexA-TEF-4ΔTEA and LexA-TEF-5ΔTEA fusions and one pJG4-5 derivative containing B42-TDU fusion (see Materials and Methods for constructions). Interaction between TDU and the TEF family and Sd is revealed by the two assays seen in Fig. 1. Results indicate that TDU interacts specifically with all TEF and Sd. Indeed no interaction is observed between Su(fu) (Monnier et al., 1998) and the TEF family or with Sd. (B) In vitro interaction test: a schematic diagram indicating the structural features of the TEF-1 protein and of the three truncated ΔTEF peptides (TEF-1Δ402C, TEF-1Δ329C, TEF-1Δ205C) is indicated at the top of the figure. Full-length TEF-1 or truncated TEF-1 peptides was transcribed and translated in vitro with [^{35}S]methionine. The labelled peptides were incubated with GST or GST-TDU beads as indicated on figure. The materials retained by GST or GST-TEF-1 beads were analysed by autoradiography after SDS-PAGE. (C) The sequence of the region TEF-1 which interacts with TDU is aligned with the corresponding sequence of Sd (amino acids 220-344). Identical amino acids are indicated in grey. (D) The Vg homology region of TDU is required for interaction with TEF-1. Full-length TDU or the three deleted TDU proteins described at the top of the figure were transcribed and translated in vitro with [^{35}S] methionine and then incubated with GST-TEF-1 beads or GST-SdΔTEA beads. The materials retained by GST-TEF-1 beads or GST-SdΔTEA beads were analysed by autoradiography after SDS-PAGE. (E) TDU activates transcription in *Drosophila* S2 cells. A strong activation of the expression of the *UAS-lacZ* reporter was observed when *Drosophila* S2 cells were cotransfected with a vector expressing a chimeric GAL4$_{db}$-TDU protein (pCaSpeR-hsp-GAL4$_{db}$-TDU). The values represent the average ± s.d. of β-galactosidase activity, of three independent transfections.

different TEFs, we used the yeast two-hybrid system. As shown in Fig. 4A, co-expression of TDU-B42 with the different TEF-LexA chimeras (TEF-1, 3, 4, and 5) in yeast leads to the activation of the two reporter genes *lacZ* and *LEU2* (Fig. 4A). Similar results were obtained when we used a fusion protein including the complete sequence of TDU (data not shown) or only the Vg homology region. These results indicate that the Vg domain involved in the interaction with Sd is conserved in TDU and mediates interaction with the mammalian TEFs.

To determine if TDU-TEF-1 complex formation that we observed in yeast results from a direct interaction, a GST-TDU fusion protein was expressed in *E. coli* and immobilised on glutathione sepharose beads. In vitro ^{35}S-labeled TEF-1 was produced in a coupled transcription/translation reaction system and incubated with the beads. As shown in Fig. 4B, ^{35}S-labeled TEF-1 bound specifically to the GST-TDU beads, while no binding to GST beads was observed. In addition, when TDU was produced in vitro and incubated with GST-Su(fu) beads, no [^{35}S]TDU was recovered (data not shown). These results demonstrate that TEF-1 directly and specifically interacts with TDU.

To determine which domain of TEF-1 is involved in the interaction with TDU, three TEF-1 C-terminal deletion mutants were used (Fig. 4B and Hwang et al., 1993). As shown in Fig. 4B, ^{35}S-labeled TEF-1 (1-329) and (1-402) bound to GST-TDU, whereas no binding of TEF-1 (1-205) was observed. This indicates that a domain of TEF-1 within or overlapping with amino acids 205-329 is required for interactions with TDU. Comparison between Sd and the mammalian TEF factors shows that the region required for interaction with TDU is well conserved (64.6% identity and 89% similarity at the amino acid level, Fig. 4C).

To confirm that Vg homology region is required for interaction with TEF-1 and Sd, we tested three different TDU mutants, one with an 27 amino-acid deletion that removes all the Vg homology region (TDU-Δ24-50), one with an 17 amino-acid deletion that removes the N-terminal homology domain (TDU-Δ24-40) and one with an 10 amino-acid deletion that remove the C-terminal homology domain (TDU-Δ41-50), for their ability to interact with TEF-1. The full-length [^{35}S]TDU protein and the three [^{35}S]TDU deleted proteins were produced in a coupled transcription/translation reaction system and incubated with GST-TEF1 beads. As shown in Fig. 4D, deletion of the Vg homology region (TDU-Δ24-50) abolishes the binding of TDU to TEF-1. However, a lower but significant binding was observed when Vg homology region was partially deleted (TDU-Δ24-40, TDU-Δ41-50). Similar results were obtained when we used GST-Sd beads (Fig. 4D). These findings confirm that the Vg homology domain of TDU is required for interaction with TEF-1 and that both the C-terminal and the N-terminal domains of this conserved region contribute to this interaction.

If TDU interacts with TEF to form an active transcriptional factor, we would predict that TDU behaves as a transcriptional activator. Transfection of *Drosophila* S2 cells with a vector expressing a fusion protein between TDU and the DNA-binding domain of the GAL4 yeast protein (pCaSpeR-hsp-GAL4$_{db}$-TDU) resulted in a strong activation of expression from a GAL4-responsive pUAST-*lacZ* reporter (Fig. 4E). These results confirm that TDU interacts with TEF to form a transcriptional factor and that the Vg homology region of TDU is required for this interaction.

Altogether, these results indicate that this region of TEF/Sd and the conserved sequence of TDU/Vg constitute two evolutionary conserved protein-protein interaction domains. In addition, dimerization observed between Vg and TEF-1 (Simmonds et al., 1998) as well as with the other TEF factors (data not shown) and between TDU and Sd/TEF factors (Fig. 4A-C) further supports the conservation of these interaction interfaces during evolution.

Expression of TDU in *Drosophila* rescues loss of Vg function

The evolutionary conservation of the Sd/TEF-Vg/TDU interaction suggests that TDU may functionally substitute for Vg. To test whether TDU can substitute for Vg in *Drosophila*, we produced transgenic flies expressing TDU by means of the *UAS/GAL4* system (Brand and Perrimon, 1993).

We first examined whether TDU expression can substitute for Vg in wing development. To do so, we used the *vg^{83b27}* allele, which is associated with an almost complete deletion of intron 2 and which removes the boundary enhancer and behaves as a null allele during wing development (Williams et al., 1991, 1994). We chose to express TDU by using a driver strain in which *GAL4* expression is under the control of the boundary enhancer (*vg-GAL4* strain). Indeed, we observed that *vg* expression driven by this *GAL4* driver in a *vg^{83b27}* mutant context leads to a complete rescue of the wing phenotype (Van de Bor et al., 1999). When TDU was expressed with this driver, we observed a partial, but significant rescue of the *vg^{83b27}* wing phenotype (Fig. 5A compared to 5B) in emergent flies. Importantly, the wings formed presented veins and wing margins, indicating that TDU not only induces wing tissue growth, but also allows correct wing patterning (inset in Fig. 5A). This result demonstrates that TDU can functionally substitute for Vg in wing development.

It has been previously shown that *vg* induces its own expression, by acting on boundary and quadrant enhancers, as well as *sd* expression (Halder et al., 1998; Paumard-Rigal et al., 1998). If TDU is able to interact with Sd to form a transcriptionally active complex, as shown for Vg, we would predict that it will be able to activate *sd* and *vg* expression. We first analyzed *vg* expression in *vg^{83b27}*; *UAS-TDU*; *vg-GAL4* wing imaginal discs. Indeed, Vg is not detectable in *vg^{83b27}* wing imaginal discs, but the *vg*-QE could be activated by TDU expression. As shown in Fig. 5C, we observed that TDU expression at the DV induces *vg* expression in the wing pouch except at the DV boundary. In fact, it has been shown that in the *vg^{83b27}* mutant, Vg and QE reporter expression is lost, suggesting that *vg* expression at the DV boundary mediates *vg* expression in the rest of the wing pouch non-autonomously (Kim et al., 1996; Nagaraj et al., 1999). Our results show that TDU expression at the DV boundary is able to confer an identity on the cells of the DV boundary that allows them to initiate *vg* expression through the QE. The correct wing margin that we observed in *vg^{83b27}*; *UAS-TDU*; *vg-GAL4* flies and the fact that TDU expression restores *cut* expression at the presumptive wing margin in a *vg^{83b27}* mutant context (data not shown) are consistent the notion that TDU substitutes for Vg in the establishment of a DV boundary.

To test the effect of TDU on *sd* expression, we expressed

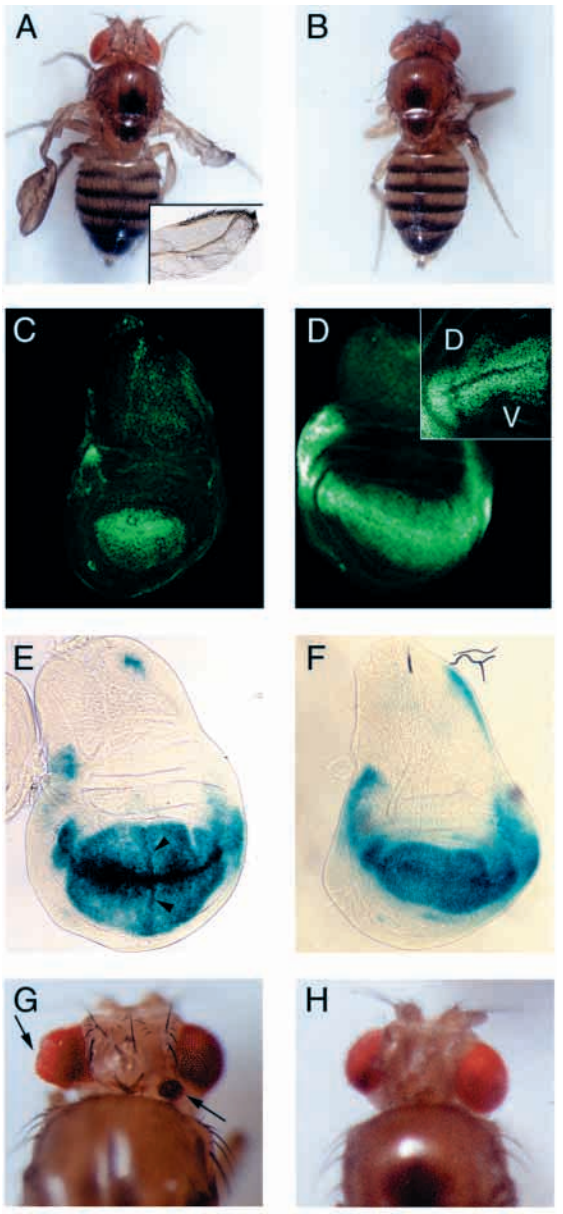

Fig. 5. TDU expression in *Drosophila* rescues a loss of Vg function. vg^{83b27}/vg^{83b27}; *vg-GAL4/+*; *UAS-TDU/+* flies (A) show a partial rescue of the no wing phenotype observed with vg^{83b27}/vg^{83b27} flies (B). The rescued wings contain veins and marginal bristles (inset in A). *TDU* activates *vg* transcription in the wing pouch. (C) vg^{83b27} *vg-GAL4/*vg^{83b27}; *UAS-TDU/+* wing discs stained with α-Vg antibodies shows presence of Vg protein in all the wing pouch even though no Vg protein is detected in vg^{83b27}/vg^{83b27} context. (data not shown). (D) A wild-type wing disc from late third instar larvae stained with α-Vg antibodies shows a reduced Vg expression in a strip of cells centered at the DV boundary (inset in D). (E,F) *TDU* activates *sd* transcription in the wing pouch. (E) To monitor *sd* expression, sd^{ETX4}*; ptc-GAL4/+*; *UAS-TDU/+* wing discs, at the AP boundary, were stained for β-galactosidase activity. An evident increase in staining at the AP boundary (black arrowheads), where *TDU* is expressed, is observed as compared to sd^{ETX4}*; ptc-GAL4/+* wild-type wing discs in F. (G,H) *TDU* induces an increase in eye size. (G) *ptc-GAL4/+*; *UAS-TDU/+* adult flies present enlarged eyes and ectopic eye tissue in the head (black arrows). (H) This effect of *TDU* expression depends on *sd*. sd^{ETX4}/Y*; ptc-GAL4/+*; *UAS-TDU/+* flies exhibit normal eyes.

TDU with the *ptc-GAL4* driver in a *sd-lacZ* strain (sd^{ETX4}). Expression of the *sd-lacZ* reporter gene normally occurs in the wing pouch with highest activity at the DV boundary (Fig. 5F). When the *lacZ* expression pattern was analyzed in *sd-lacZ*; *UAS-TDU; ptc-GAL4* discs, we observed an increase of X-gal staining at the AP boundary of the wing pouch in accordance with the *ptc-GAL4* expression pattern (Fig. 5E).

Ectopic expression of *vg* by different *GAL4* drivers leads to wing tissue outgrowth (Kim et al., 1996). In contrast, expression of TDU in other imaginal discs did not induce wing outgrowth. Nevertheless, using *ptc-GAL4* or *daughterless-GAL4*, we observed a large increase in eye size (Fig. 5G), which can lead to the formation of additional eye tissue in the head. This result indicates that, like Vg, TDU is able to induce cell proliferation although it is not capable of directing these structures to form wing tissue. This effect is partially suppressed in sd^{ETX4} males, showing that the eye enlargement induced by TDU is dependent on Sd (Fig. 5H).

Together, these results indicate that TDU associates with Sd to form a bipartite transcription factor, which is able to partially substitute for Vg function in *Drosophila*.

DISCUSSION

The function of the Sd/Vg heterodimer is dynamically regulated during wing development

The development of appendages in *Drosophila* as well as limb development in vertebrates requires a complex and precise spatial and temporal regulation of gene expression. Studies of wing development in *Drosophila* constitute a powerful tool to better understand how these complex expression patterns are established and regulated. This latter aspect is well illustrated by studies showing that establishment of the apical ectodermal ridge (AER) of the vertebrate limb bud, like the DV boundary of the wing primordia, is dependent on Notch and Wg (reviewed in Shubin et al., 1997). It has been shown that expression of wing-specific genes such as *dSRF* or *vg* results from the activity of the Vg/Sd heterodimer, which restricts expression to the presumptive wing region, and from a combination of signaling outputs (Ci, Su(H) or Mad), which activates a specific pattern of gene expression within the wing field (Halder et al., 1998).

In this study, we have used a different approach, which can be considered as a functional test, to show that the activity of the Sd/Vg dimer is dynamically regulated during wing imaginal disc development. We show that the Sd/Vg dimer is active at the DV boundary at the end of the second larval instar, but is strongly downregulated at the DV and AP boundary in late imaginal disc development. Importantly, as these experiments were done without ectopic gene expression, they probably accurately reflect the wild-type situation for *vg* and *sd*. Our findings lead us to propose that the function of the Sd/Vg heterodimer is not solely to restrict the expression of target genes to the wing pouch, but that it also participates in setting up the pattern of gene expression within the wing pouch.

The modulation of Vg/Sd activity could also contribute to growth control during wing development. Indeed, in the wing disc, a zone of non-proliferating cells (ZNC), coinciding with the DV boundary of the wing pouch, is formed during the third

instar (O'Brochta and Bryant, 1985). This ZNC is established through the coordinate action of Notch and Wg and is required for a correct specification of the wing margin (Johnston and Edgar, 1998). Several lines of evidence point to a role for *vg* in the control of cell proliferation; clones with a loss of *vg* function fail to proliferate (Simpson et al., 1981), whilst ectopic *vg* expression induces cell proliferation. Recently, it has been shown that cell proliferation of wing pouch cells controlled by the EGF receptor pathway is mediated by regulation of *vg* expression (Nagaraj et al., 1999). As, *vg* induces cell proliferation, a negative modulation in Vg/Sd activity at the DV boundary could be a prerequisite to the formation of the ZNC.

Our results highlight the advantage of using a transcription factor complex combining a DNA-binding molecule (Sd) and an activator molecule (Vg) for dynamically controlling the activity of several cognate enhancers, which have to be precisely and dynamically expressed during wing growth and determination. The activity of such transcription factor complexes can be modulated by changing the expression levels of either component. In the case of Sd and Vg, an increase in Sd or a reduction in Vg may lead to diminished activation as Sd alone can bind to its cognate sites and act as dominant negative inhibitor of the Sd/Vg complex. Such a situation increases the combinatorial possibilities for the fine tuning of transcriptional regulation.

TDU, a putative cofactor for the mammalian TEFs

Sd belongs to an evolutionary conserved family of transcriptional factors. In mammals, the functions of these different TEFs are not completely understood, but they are probably involved in the transcriptional control of cardiac, skeletal muscle and placental genes. Indeed, it has been shown that the TEFs bind to the M-CAT element found in the regulatory region of several cardiac and skeletal muscle genes (Farrance and Ordhal, 1992) and the placenta-specific chorionic somatomamotrophin gene (hCS) (Jacquemin and Davidson, 1997; Jiang and Eberhardt, 1994). Furthermore, disruption of the *TEF-1* gene (*Tead1*) in mouse is associated with heart defects showing that TEF-1 is likely involved in the regulation of specific cardiac genes (Chen et al., 1994).

Like Sd, TEF function may depend on interaction with coactivators that are expressed in a specific pattern to activate transcription. Indeed transactivation by TEF-1 is cell-type dependent (Xiao et al., 1991) and could act as a transcriptional repressor in particular cell types (Jiang and Eberhardt, 1996). In addition, two factors, NEF-1 and NEF-2, negatively regulate transcriptional activation by TEF-1 in vitro, but none of these factors have been cloned (Chaudhary et al., 1994, 1995). Our results showing that TDU interacts with the TEF factors suggest that it is a good candidate coactivator. TDU is expressed in lung, kidney and placenta, and also probably in fetal heart since ESTs for TDU were originally identified in these tissues. Heart, skeletal muscle, lung, placenta and kidney are enriched in transcripts for various TEFs. It is therefore possible that TDU acts as a transcriptional coactivator for the TEFs in these tissues allowing that activation of specific target genes.

Two others factors have been identified which interact with the TEFs and positively regulate TEF-dependent transactivation of the rat cardiac α-myosin heavy chain (αMHC) and of the cardiac troponin T genes (Gupta et al., 1997; Butler and Ordahl, 1999); the bHLH protein Max and the chromatin-modifying protein PARP. For the troponin T promoter, gel-shift assays indicate that TEF-1 binds to M-CAT elements with different partners (Butler and Ordahl, 1999), one of these could be TDU.

Strong evidence that TDU is a transcriptional coactivator in vivo comes from our experiments which show that TDU is able to functionally substitute for Vg in *Drosophila* and is able to induce some of the effects observed with ectopic *vg* expression. Most importantly TDU expression in *Drosophila* is able to rescue a loss of Vg function in wing blade formation.

To some extent, the function of *vg* in wing morphogenesis is analogous to function of *eyeles*s (*ey*) in eye morphogenesis. These two genes are able to *trans*-determine a tissue and can be considered as master genes control (Kim et al., 1996; Halder et al., 1995). In the case of *eyeless*, strong homology in the coding and in the flanking sequences indicates that it is orthologous to the murine gene *Pax-6*/*Sey* and to the human gene ANIRIDIA, which are also involved in eye morphogenesis. Consistent with this notion, expression of *Pax-6* in *Drosophila* induces like *ey*, formation of ectopic eye structures (Halder et al., 1995). In contrast, the homology between Vg and TDU is limited to a short domain of 26 amino acids essential for the interaction with the TEFs. Primarily because of this low similarity between Vg and TDU, we do not consider that *TDU* is orthologous to *vg*. However, our results show rescue of *vg* function by TDU demonstrating that, in terms of molecular function, *TDU* is analogous to *vg*. This observation together with the fact that TDU interacts with all the TEFs supports the hypothesis that TDU is a TEF coactivator. The future challenge will be to determine the precise function of TDU in vertebrates. This will require biochemical analysis of the transcriptional properties of the TDU/TEF heterodimers and analysis of the role of TDU development and organogenesis by homologous recombination.

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