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Binding of the Vestigial co-factor switches the DNA-target selectivity of the Scalloped selector protein

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SUMMARY

The formation and identity of organs and appendages are regulated by specific selector genes that encode transcription factors that regulate potentially large sets of target genes. The DNA-binding domains of selector proteins often exhibit relatively low DNA-binding specificity in vitro. It is not understood how the target selectivity of most selector proteins is determined in vivo. The Scalloped selector protein controls wing development in *Drosophila* by regulating the expression of numerous target genes and forming a complex with the Vestigial protein. We show that binding of Vestigial to Scalloped switches the DNA-binding selectivity of Scalloped. Two conserved domains of the Vestigial protein that are not required for Scalloped binding in solution are required for the formation of the heterotetrameric Vestigial-Scalloped complex on DNA. We suggest that Vestigial affects the conformation of Scalloped to create a wing cell-specific DNA-binding selectivity. The modification of selector protein DNA-binding specificity by co-factors appears to be a general mechanism for regulating their target selectivity in vivo.

Key words: Selector gene, Co-factor, Transcription, Wing formation network, *Drosophila*

INTRODUCTION

Organs and other animal body parts often develop from sets of cells that are determined as a group to form a particular structure. A class of genes, termed field-specific selector genes, has been identified that determine the fates of entire fields of cells and direct the development of whole organs and body structures (reviewed by Carroll et al., 2001; Mann and Morata, 2000). In *Drosophila*, some field-specific selector genes and the structures whose formation they regulate include *Pax6*/*eyeless* (*ey*) in the eye (Halder et al., 1995; Quiring et al., 1994), *tinman* (*tin*) in the heart (Azpiazu and Frasch, 1993; Bodmer, 1993; Frasch, 1999), *Distal-less* (*Dll*) in the limbs (Cohen et al., 1989; Gorfinkiel et al., 1997), and *vestigial/scalloped* (*vg/sd*) in the wing (Halder et al., 1998; Kim et al., 1996; Simmonds et al., 1998; Williams et al., 1991). A second class of selector genes, the Hox genes, act in specific domains along the anteroposterior body axis and in developing appendages to specify their identity but not their formation (Carroll et al., 1995; Lawrence and Morata, 1994; Lewis, 1978; Manak and Scott, 1994; McGinnis and Krumlauf, 1992).

The known selector proteins are transcription factors that exert their prominent effects by regulating presumably large but specific sets of target genes. However, the DNA-binding domains of selector proteins often show promiscuous DNA-binding specificity in vitro. For example, recognition sequences for the homeodomains of Hox proteins are typically only 6 bp long (Biggin and McGinnis, 1997; Ekker et al., 1994; Gehring et al., 1994; Mann, 1995; Mann and Affolter, 1998). Similarly, the consensus sequence bound by the TEA domain of the Scalloped (Sd) protein is 8 bp long but degenerate (reviewed by Jacquemin and Davidson, 1997). Potential binding sites for these proteins are predicted to occur once every 2-4 kb in a random sequence, and therefore may be found in *cis*-regulatory regions of virtually every gene. However, selector proteins presumably do not regulate all genes in a genome. Furthermore, the activity of many selector proteins, particularly Hox proteins, is not restricted to a single field, but may be required during the development of several structures to regulate distinct sets of target genes (Azpiazu and Frasch, 1993; Bodmer, 1993; Halder et al., 1995; Morata and Sanchez-Herrero, 1999) reviewed in (Mann and Morata, 2000). Understanding how the target selectivity of selector proteins is determined in vivo is thus fundamental to understanding how they control gene expression and pattern formation.

Interactions with specific co-factors may be a major determinant of selector protein target selectivity. The DNA-binding specificity of transcription factors is often increased by cooperative interactions with specific co-factors that are also DNA-binding proteins. The Hox proteins and their PBC (Pbx, ceh-20, Extradenticle (Exd)) and MEIS (Homothorax (Hth), Meis, Prep) co-factors provide a prominent example (Mann,

1995; Mann and Affolter, 1998; Mann and Chan, 1996; Mann and Morata, 2000; Wilson and Desplan, 1999). Like the Hox genes, the PBC genes encode homeodomain proteins. They bind cooperatively with Hox proteins to a bipartite DNA sequence. Importantly, they selectively form heterodimers with different Hox proteins, depending on differences within the sequence of the DNA-binding site (Knoepfler et al., 1996; Ryoo and Mann, 1999). The Hth/Meis and Prep1 homeodomain proteins appear to form ternary complexes with Hox and PBC proteins (Berthelsen et al., 1993; Ferretti et al., 2000; Ryoo et al., 1999). Unlike Hox proteins themselves, these complexes bind DNA with higher specificity, which thereby increases the discrimination between target and non-target *cis*-regulatory elements (Mann, 1995; Mann and Affolter, 1998; Mann and Morata, 2000). Post-translational modifications can also modify DNA-binding and the interactions of Hox proteins with co-factors (Berry and Gehring, 2000; Jaffe et al., 1997). These observations suggest that interactions with and activity regulation by co-factors may be a major determinant of Hox protein selectivity. Little is known, however, about the mechanisms that mediate the target specificity of field-specific selector proteins.

The Scalloped protein (Burglin, 1991; Campbell et al., 1992) controls wing development by directly regulating the expression of a network of genes in the imaginal wing disc (Guss et al., 2001; Halder et al., 1998). Sd binds to essential sites in numerous wing-specific *cis*-regulatory elements of its target genes (Campbell et al., 1992; Inamdar et al., 1993). Sd is the *Drosophila* homolog of the vertebrate transcription enhancer factor (TEF) family of transcription factors that contain a TEA DNA-binding domain (Burglin, 1991; Campbell et al., 1992; Jacquemin and Davidson, 1997) and the Sd and TEF-1 proteins possess similar DNA-binding specificities in vitro (Halder et al., 1998). In developing wing cells, Sd forms a complex with Vestigial (Vg) (Paumard-Rigal et al., 1998; Simmonds et al., 1998), a protein with no informative homologies (Williams et al., 1991). This complex is wing specific, because Vg and Sd are not co-expressed in other tissues. The Vg-Sd complex acts as a selector for wing development (Halder et al., 1998; Paumard-Rigal et al., 1998; Simmonds et al., 1998; Bray, 1999; de Celis, 1999). The wing field fails to develop in *vg* or *sd* loss-of-function mutants (Campbell et al., 1992; Williams et al., 1991; Williams et al., 1993) and targeted expression of Vg to regions where Sd is also active induces wing-like outgrowths on other structures (Halder et al., 1998; Kim et al., 1996). Sd and Vg physically interact in solution (Paumard-Rigal et al., 1998; Simmonds et al., 1998), but it is not known if they form a complex on DNA. Vg activates transcription in yeast one-hybrid experiments and it has been postulated that Vg acts as a transcriptional activator that is recruited by Sd (Vaudin et al., 1999).

We have examined whether interaction with Vg affects Sd DNA-binding and target gene specificity. We found that Vg and Sd formed a complex on DNA that had a different DNA-binding specificity than Sd alone. We also show that Vg-Sd complex formation on DNA requires protein domains of Vg that are not required for Sd binding in solution. The Vg-Sd complex on DNA appears to be a heterotetramer, and Vg exerts its effect without contacting bases outside the Sd-binding sites. Vg interaction thus switches the DNA target selectivity of Sd, so that Sd and the Vg-Sd complex bind to different sets of binding sites. The presence or absence of Vg in a particular cell is therefore a key determinant of the set of *cis*-regulatory elements, which are bound and regulated by Sd. The tissue-specific modification of selector protein DNA-binding specificity by co-factors may be a general mechanism for increasing their target selectivity in vivo.

MATERIALS AND METHODS

Protein production

Full-length ^{35}S-labeled Vg and Sd proteins were produced using the T7 in vitro transcription and translation system (Promega). The T7plink expression vector contains the T7 promoter, the 5′ untranslated leader from the β-globin gene fused to a Kozak consensus ATG followed by multiple cloning sites (Dalton and Treisman, 1992). Vg- and Sd-coding regions were PCR amplified and subcloned into T7plink. The Sd protein contained an extra six amino acids at the N terminus (MAGSEF) encoded by the T7plink vector. Sd^{myc} contained an N-terminal Myc tag (MEQKLISEEDLNM-AGSEF) fused to the Sd ORF. All Vg proteins containing the Vg N-terminus started with the Methionine of Vg itself and did not contain any extra residues. Vg proteins that had N-terminal deletions started with the vector encoded peptide MAGSEF fused to Vg. The breakpoints, indicated by an asterisk, of the Vg deletions are: ΔSID, 5′ breakpoint is DTASQ* and 3′ breakpoint is *NYVHP; position 66, *SVSAN; 5′SID, *QAQYL; 3′SID, PIPAP*; position 73, NAAAA*; position 176, *THQTK; and position 274, *GSGQGQ. The HA tag (SMAYPYDVPDYASLG) was inserted at position 153 between S and H. The Sd TEA domain was purified as a $(His)_6$-tagged protein on nickel chelate columns (Novagen), as described earlier (Halder et al., 1998; Jacquemin et al., 1996).

DNA probes

DNA probes for EMSAs were labeled with ^{32}P-αATP by fill in reaction of double T overhangs at both ends of double-stranded oligonucleotides using the Klenow fragment of DNA polymerase I. Single strand oligonucleotides were annealed at concentrations of 10 μM in 10 mM Tris pH 7.5 and 50 mM NaCl. Labeled probes were purified over Sephadex G50 columns (Princeton Separations). Different probes were diluted to the same specific activities with cold labeled oligonucleotides. Sequences of the upper strand oligonucleotides were 5′ to 3′: 2×GT (TTCGATACACTTGT-GGAATGTGTGGAATGTGTTAGCCCCG), 1×GT (TTCGATACA-CTTGTGGAATGTGTTTGATTTGTTAGCCCCG), GTspaceGT (TTCGATACACTTGTGGAATGTTATGATCGAAGTGGAATGTG-TTAGCCCC), cut-564 (TTGTCAATGTAATTCGAAAAATGTCGT-CAG), cut-341 (TTGGCGGCAGATAAAATTATTGAAATTACATT-GGCAAGAC); sal-750 (TTTGCTTTCTCTAATCAGACTAATG-AGGATT); sal-862 (TTGTTCGCATAACTTATTAAAAA); kni-268 (K. Guss and E. Bier, personal communication; TTCCCCT-CTTACATTTGTCGCATAGTTCCCATCTTGGCCA); DSRF (CGA-TACACTTAAACTATGCCAGGAATTTCTTAGCCCCG); cTNT (T-TCCCAGAGAGGAATGCAACACTTGT); and αMHC (TTGCAGG-CACGTGGAATGAGCTAT).

Electrophoretic mobility shift assays (EMSAs)

EMSA reactions with TNT produced proteins were carried out in 20 μl binding buffer (8% glycerol, 15 mM Hepes pH 7.9, 150 mM KCl, 1 mM EDTA, 100 μg/ml bovine serum albumin (BSA)) containing 0.7 μl TNT reaction, 0.3 μg dIdC and 3 fmol DNA probe. Equimolar amounts of ^{35}S-labeled proteins were added by diluting the TNT reactions accordingly with unprogrammed TNT extract. Binding reactions were incubated for 15 minutes at room temperature and complexes were separated on 5% polyacrylamide gels and standard

0.5× TBE buffer. Gels were run at 15 V/cm at room temperature. Run gels were dried and exposed with intensifying screens at −70°C overnight. EMSAs with purified TEA domain protein were carried out in essentially the same way, except that 1 fmol probe was used and the buffer contained 100 mM KCl, 2 mM $MgCl_2$ and no dIdC. TEA domain shifts were run on 6% polyacrylamide gels. For supershifts, 100 ng antibody was added to the binding reaction.

Co-immunoprecipitations

To preclear, 10 μl of TNT product were incubated with 400 μl IP buffer (15 mM Hepes pH 7.9, 150 mM KCl, 1 mM EDTA, 1% Triton) and 20 μl of protein A-Sepharose suspension (Amersham Pharmacia Biotech) at 4°C for 20 minutes shaking. Reactions were centrifuged for 2 minutes and 1 μg of antibody (mAB α-Myc, mAB α-HA, both from Babco) was added to the supernatant, which was then incubated on a shaker at 4°C for 60 minutes. 20 μl of protein A-Sepharose were added and the reaction was incubated on a shaker at 4°C for 60 minutes. Agarose beads were pelleted by centrifugation at 1500 ***g*** for 2 minutes. Supernatant was removed and beads were washed four times with 700 μl IP buffer. Bound proteins were eluted and denatured in 40 μl SDS sample buffer (with 200 mM DTT) by incubation at 68°C for 15 minutes. Proteins were separated by standard 12% and 18% SDS-PAGE. Gels were dried and exposed to BiomaxMR film (Kodak). The IP-buffer differs from the binding buffer used for EMSA only in that it contained 1%Triton X-100 and no BSA. The presence of 1% Triton X-100 had no effect on the EMSA results.

RESULTS

Vg binding switches the DNA-target specificity of Sd

We have identified essential native Sd-binding sites in several *cis*-regulatory elements that control the wing field-specific expression of Sd-regulated target genes (Guss et al., 2001; Halder et al., 1998). These sites were identified by DNaseI footprinting using the TEA domain of Sd. In these analyses, we were struck by the finding that essential sites occurred most often as tandem double sites, for example, in the *cut*, *spalt* and *DSRF* (*bs* – FlyBase) genes (Guss et al., 2001; Halder et al., 1998). Despite substantial differences in sequence, the TEA domain of Sd bound cooperatively to all of these doublet sites with high affinity, and with similar affinity to single, nonessential sites and to native single vertebrate TEF-1-binding sites in muscle-specific *cis*-regulatory elements and the SV40 enhancer (Guss et al., 2001; Halder et al., 1998; Table 1). From these studies, we have inferred a consensus binding site sequence of $^{T}/_{A}$ $^{A}/_{G}$ $^{A}/_{G}$ $^{T}/_{A}$ AT $^{G}/_{T}$ T for the TEA domain of Sd, which is very similar to that of the TEA domain of TEF-1 (Guss et al., 2001; Jacquemin and Davidson, 1997; Jiang et al., 2000).

In contrast to the isolated TEA domain, however, the full-length Sd protein (produced by in vitro translation, see Materials and Methods) did not bind equivalently to all of these sites but rather showed a restricted DNA-binding specificity (Fig. 1; summarized in Table 1). Full-length Sd bound specifically to the doublet site in the *DSRF* enhancer and to most of the single binding sites (Fig. 1, lanes 14, 18; Table 1), but binding to the *cut*, *sal*, *kni* and other native templates with doublet sites was weak or nearly undetectable (Fig. 1, lanes 2, 6, 10; Table 1). The difference in DNA-binding activity between the TEA domain and Sd protein indicates that there are motifs within the native Sd protein that affect the activity of the TEA domain and restrict its binding to certain sites. We refer to sites that are bound by Sd as A-sites.

The finding that most of the doublet-binding sites were not bound by the full-length Sd protein was surprising, considering that these templates were bound with high affinity by the TEA domain and that these sites are essential for enhancer activity in vivo (Guss et al., 2001). The observations that the activity of these *cis*-regulatory elements in vivo and in cell culture depends on co-expression of Vg with Sd (Halder et al., 1998), and the finding that Vg and Sd interact physically (Paumard-Rigal et al., 1998; Simmonds et al., 1998), raised the possibility that interaction of Vg with Sd changed its DNA-binding properties and enabled binding to these sites. However, previous Vg-Sd protein interaction studies have been performed in the absence of DNA and the possible effect of the interaction between Vg and Sd on DNA-binding has thus not been addressed. We have tested whether Sd and Vg form a complex on DNA in vitro and whether this complex has different DNA-binding properties from the Sd protein alone.

Co-translation of Sd with Vg produced a Vg-Sd complex

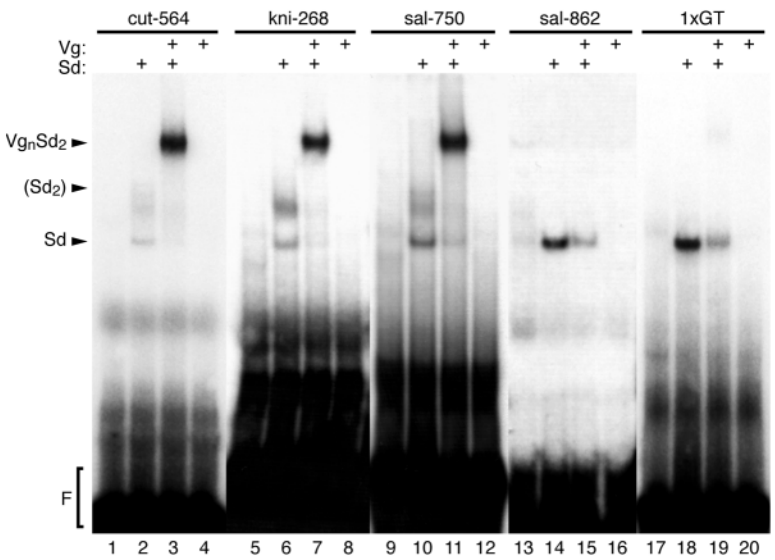

Fig. 1. Vg binding switches the DNA-target specificity of Sd. EMSAs using in vitro transcribed and translated (TNT) Sd and Vg proteins binding to templates shown in Table 1. Four lanes of EMSA are shown for each DNA template. From left to right, DNA templates are incubated with unprogrammed TNT extract (lanes 1,5,9,13,17); Sd alone (lanes 2,6,10,14,18); Sd co-translated with Vg (lanes 3,7,11,15,19); and Vg alone (lanes 4,8,12,16,20). Unprogrammed TNT extract does not shift any of these probes. Sd alone binds poorly to doublet sites in *cut-564* (lane 2), *kni-268* (lane 6) and *sal-762* (lane 10), but Vg-Sd complexes binds well to all three probes (lanes 3,7, 11); Sd alone binds to single sites in *sal-862* (lane 14) and 1×GT, but co-expression of Sd with Vg did not result in higher order complexes (lanes 15,19). On the contrary, Vg inhibited Sd from binding and reduced the amount of Sd-DNA complexes observed. The residual binding activity migrates at the position of the Sd-DNA complexes and is thus due to uncomplexed Sd. None of the probes are bound by Vg in the absence of Sd. F, free probe. Proteins expressed and probes used are indicated above gels.

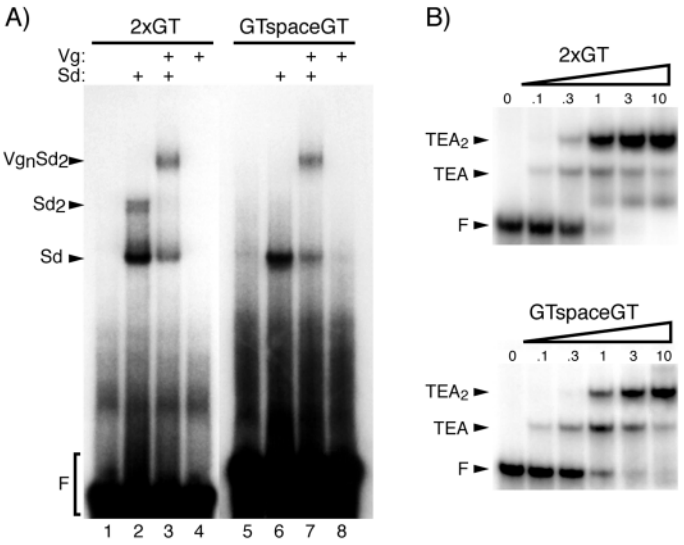

Fig. 2. Cooperativity of Sd binding is not required for Vg-Sd complex formation on DNA. (A) EMSAs of Sd, Sd and Vg, and Vg binding to the 2×GT and GTspaceGT probes. Sd bound to the 2×GT template as a monomer and as a dimer (Sd and Sd_2; lane 2). Incubation of a co-translated mixture of Sd and Vg produced an additional complex that migrated more slowly (Vg_nSd_2; lane 3), while expression of Vg alone did not result in any detectable DNA-binding activity (lane 4). The Vg-Sd complex bound to GTspaceGT with similar affinity as to 2×GT (lanes 3,7), although cooperativity of Sd binding is reduced in the GTspaceGT probe, as it does not bind two molecules of Sd, in contrast to 2×GT (lanes 2,6). Labeling and arrangement of lanes is as in Fig. 1. (B) EMSAs showing titrations of purified TEA domain binding to 2×GT and GTspaceGT. Both probes are shifted by 1 ng TEA domain added and thus have similar affinities. However, two TEA molecules bind cooperatively to 2×GT but non-cooperatively to GTspaceGT. TEA, one molecule TEA domain bound; TEA_2, two TEA molecules bound. Protein concentrations are indicated in ng/20 μl. F, free probe.

that bound to these other sites (referred to as B-sites). In contrast to Sd alone, complexes containing Sd and Vg bound strongly to the *cut*, *sal* and *kni* elements (Fig. 1, lanes 3, 7, 11). Quantification of the bound complexes showed that Vg increased Sd binding to these doublet sites by about 10-fold. In addition to enabling binding to B-sites, interaction with Vg reduced Sd binding to the single site templates by at least fivefold (Fig. 1). Importantly, we have not observed binding of Vg alone to any of the binding sites described in this report or to any other DNA templates tested (Fig. 1, data not shown). Therefore, Vg binding to Sd switches the DNA target preference of Sd from the single A-sites to the doublet B-sites.

Only two of the eleven templates that we tested were bound by both Sd and Vg-Sd and thus possessed A- and B-site properties. These were the synthetic 2×GT (see below) and the *DSRF* probes (data not shown). Thus, while most native templates have either A- or B-site character, sites with both A- and B-site properties also occur.

Two binding sites but not cooperativity of Sd binding are required for Vg-Sd complex formation on DNA

The observation that the DNA templates that were bound by the Vg-Sd complexes (B-sites) contained two binding sites arranged in tandem to which the TEA domain bound cooperatively, raised the possibility that cooperative binding and the presence of two binding sites are required for the Vg-Sd complexes to form on DNA. To test this, we analyzed binding to a series of probes derived from the GT-IIC high-affinity TEF-1 site identified in the SV40 enhancer (Davidson et al., 1988). We selected this probe because templates composed of two GT-IIC-binding sites arranged in tandem (referred to as 2×GT) are bound cooperatively and with high affinity by the TEA domain, full-length Sd and TEF-1, as well as by the Vg-Sd complex (Fig. 2A) (Davidson et al., 1988; Halder et al., 1998; Jacquemin et al., 1996; Xiao et al., 1991). We also designed derivatives of the 2×GT probe that either had

Table 1. Sequences bound by the TEA domain, Sd and the Vg-Sd complex

Site		TEA	Sd	Sd-Vg
cut-564	TCAATGTAATTCGAAAAATGTCGTC	+++	+/–	++++
cut-341	CAGATAAAATTATTGAAATTACATT	++++	+	+++
sal-750	TTTCTGGAATCCCACGAATGTCCAT	++++	+	++++
kni-268	CCTCTTACATTTGTCGCATAGTTCC	++++	+	+++
sal-862	CATAACTTATTAAAAA	++	+++	–
cTNT	AGAGAGGAATGCAACA	++	++	–
αMHC	CACGTGGAATGAGCTA	++	++	–
1×GT	CTTGTGGAATGTGTTT	++++	++++	+/–
2×GT	CTTGTGGAATGTGTGGAATGTGTTA	++++	++++	+++
GTspaceGT	CTTGTGGAATGTTATGATCGAAGTGGAATGTGTTA	++++	++++	++
DSRF	CTTAAACTATGCCAGGAATTTCTTA	++++	+++	++++

The shaded boxes and arrows indicate the Sd binding sites, as inferred from the nucleotides required for TEF-1 binding to cTNT - defined by scanning mutagenesis (Butler and Ordahl, 1999). The black bar above the sequences identifies the region in the DSRF *cis*-regulatory element protected by the Sd TEA domain from DNAseI digestion (Halder et al., 1998). Circles indicate G-residues in cTNT that, when methylated, interfere with TEF-1 binding (Larkin et al., 1996). Relative binding affinities, as determined by EMSA (Fig. 1) and densitometry, are shown on the right. Sequences are from Guss et al. (2001) (cut, sal, kni), Cooper and Ordahl (1985) (cTNT), Molkentin and Markham (1994) (αMHC), Davidson et al. (1988) (GTIIC, from which 1×GT, 2×GT and GTspaceGT are derived) and Halder et al. (1998) (DSRF).

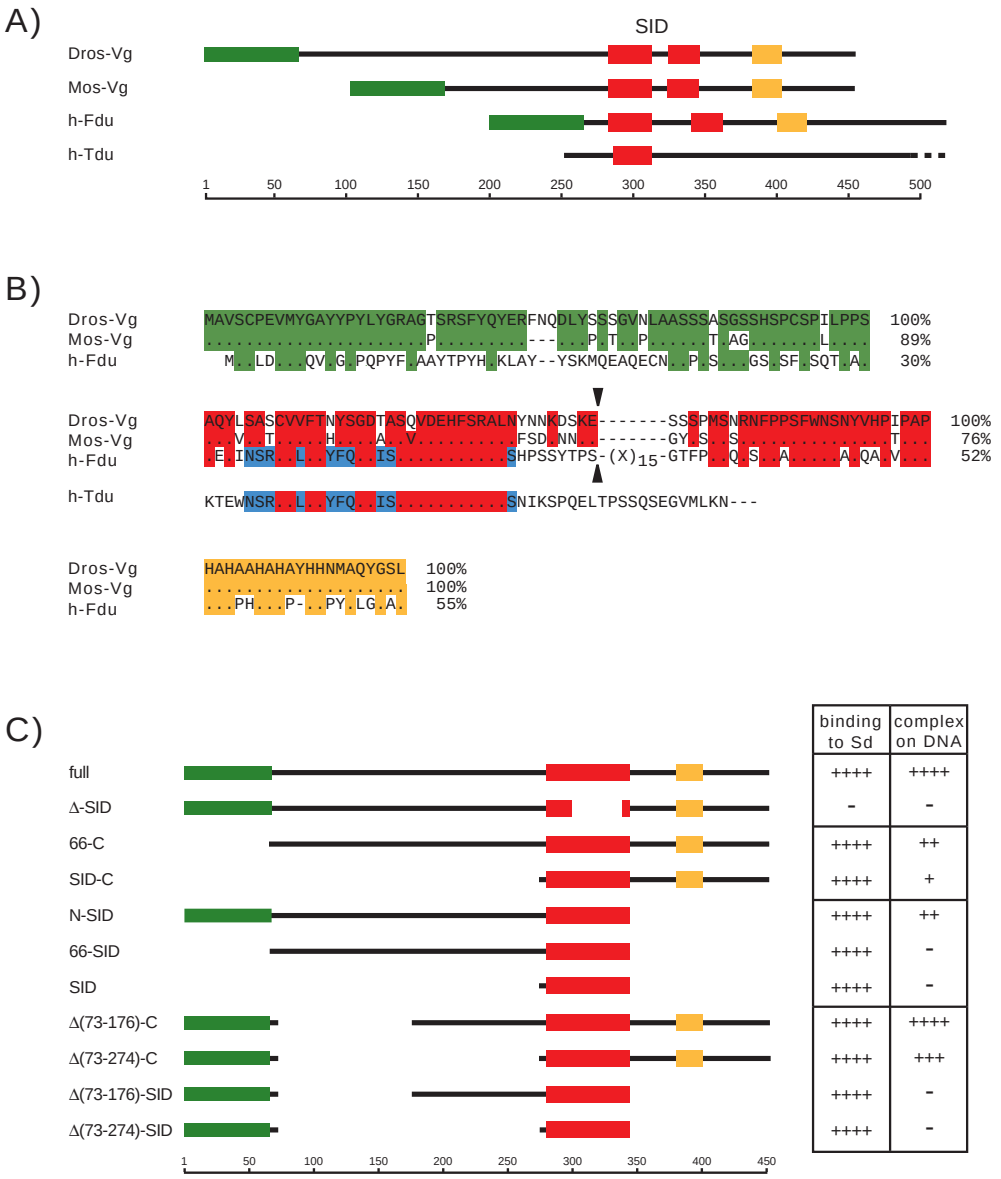

Fig. 3. Domains conserved between fly and vertebrate Vg homologs have different functions in Sd binding and Vg-Sd complex formation on target DNA. (A) Schematic structure of *Drosophila* Vg and its homologs from Mosquito (Mos-Vg) and human: Fondue (h-Fdu) and Tondu (h-Tdu), respectively. Conserved domains are boxed in color: the Sd interaction domain (SID) in red: and the N- and C-terminal domains in green and orange, respectively. Protein lengths are to scale and an amino acid ruler is shown at the bottom. (B) Sequence comparison of the conserved domains between Dros-Vg, Mos-Vg, h-Fdu and h-Tdu. Identical residues are boxed in color and indicated by a dot. Percent identity over entire domains are indicated to the right. Dros-Vg and h-Fdu have an intron at similar positions in the SID (arrowheads). Residues shared between h-Fdu and h-Tdu are boxed in blue. (C) Schematic of the series of Vg deletion mutants tested for interaction with Sd in solution and for Vg-Sd complex formation on DNA. A summary of their activities is indicated on the right.

only a single Sd binding site (1×GT, resembling the native GTII-C-binding site, Table 1) or that had a 10 bp spacer between the two Sd-binding sites (GTspaceGT) that abolishes cooperative binding (Fig. 2; Davidson et al., 1988; Jiang et al., 2000).

Full-length Sd bound to the 2×GT site as a monomer and as a dimer (Sd and Sd_2; Fig. 2A, lane 2). Incubation of a co-translated mixture of Sd and Vg produced an additional complex that migrated more slowly (Vg_nSd_2; Fig. 2A, lane 3), while expression of Vg alone again did not result in any detectable DNA-binding activity (Fig. 2A, lanes 4, 8). Two molecules of the TEA domain bound to the 2×GT probe cooperatively (Fig. 2B, top). Upon titration of the TEA domain, only a small fraction of complexes containing a single TEA molecule was observed, while the shift to two TEA molecules bound occurred abruptly between 0.3 ng and 1 ng of TEA domain added, indicating cooperative binding. However, the GTspaceGT probe was bound by two TEA domains, but in a non-cooperative fashion (Fig. 2B, bottom). At lower TEA domain concentrations, only one TEA molecule bound and with increasing concentration the second site was gradually occupied. However, 2×GT and GTspaceGT bound with similar affinity, as the TEA concentrations required to shift them were approximately the same. Cooperative binding of full-length Sd on GTspaceGT was also reduced, as no complexes containing two Sd molecules were observed, in contrast to Sd binding to the 2×GT template (Fig. 2A, lanes 2, 6). Despite this reduction in Sd cooperativity, a Vg-Sd complex formed on the GTspaceGT probe as efficiently as on 2×GT (Fig. 2A, lanes 3, 7). Therefore, cooperative binding of Sd to DNA is not required for Vg-Sd complex formation on DNA. Importantly, however, two Sd-binding sites are required, as Vg-Sd complexes form on the GTspaceGT template but barely on 1×GT.

The Sd-interaction domain of Vg is sufficient for binding to Sd but not for complex formation on DNA

To identify domains within Vg that may be important for Sd interaction and complex formation on DNA, we first searched

for conserved domains in Vg homologs and other proteins. A Vg homolog from the mosquito (Jim Williams and S. B. C., unpublished) shows strong conservation of the first 79 amino acids and of the region beginning with the previously identified Sd-interaction domain (SID) to the very C terminus (Fig. 3A,B). The region from position 80 to 280 in the *Drosophila* protein shows no or only moderate similarity to mosquito Vg. A Vg homolog from vertebrates, Tondu (Tdu; Vaudin et al., 1999), shares the first part of the SID but no other domains (Fig. 3A,B). We have identified a novel vertebrate Vg homolog,

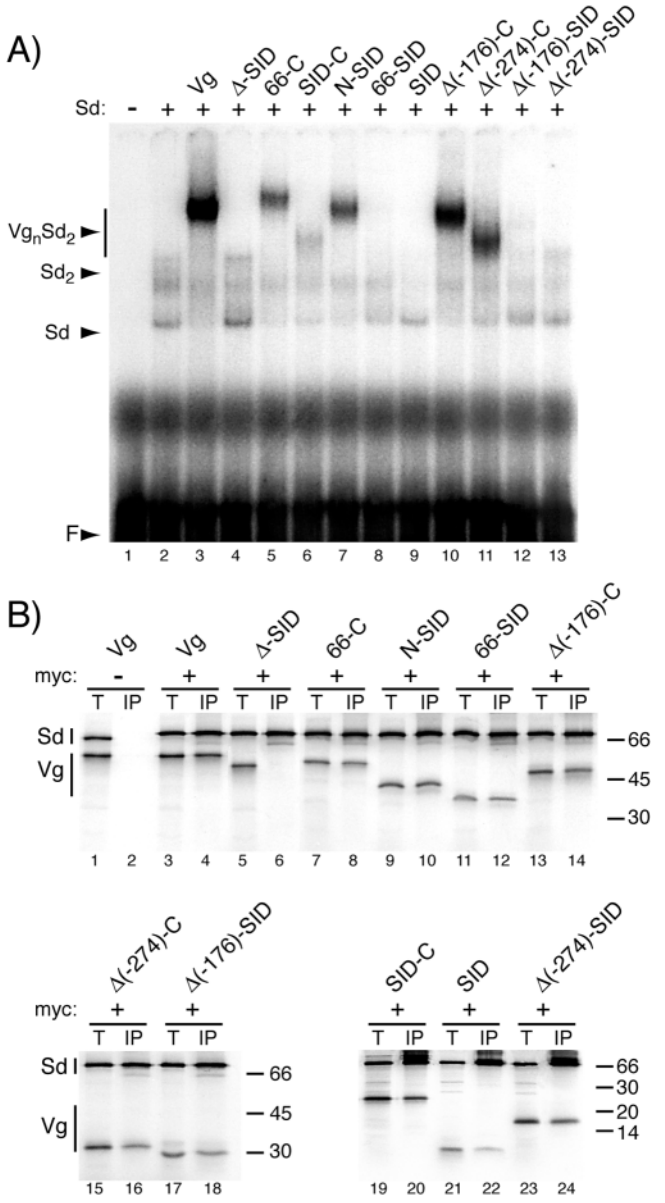

Fig. 4. Vg protein domains outside the Sd interaction domain are required for Vg-Sd complex formation on DNA but not for Vg-Sd interaction in solution. (A) EMSA of the binding to the *cut* template by the Vg deletion mutant proteins co-expressed with Sd. Only the two internal deletions Δ(73-176) and Δ(73-274) are as efficient in DNA complex formation as full-length Vg (lanes 3,10,11). The deletions 66-C, SID-C and N-SID showed partial activity, while all other deletions were not active. Lane 1, TNT extract; lane 2, Sd only; lanes 3-13 Sd co-expressed with the indicated Vg mutants. Labeling as in Fig. 1. (B) SDS-PAGE of Co-IPs of Sd and Vg mutants. The ^{35}S-labeled proteins produced by TNT (T) and the precipitated proteins (IP) are shown next to each other for each Vg mutant. Lanes 1 and 2: anti-Myc antibody does not precipitate untagged Vg or Sd. Lanes 3-24: precipitation of Myc-tagged Sd with anti-Myc antibody co-precipitates all Vg mutants, except the mutant deleted for SID (lanes 5,6). Top band is Sd, lower bands are Vg mutant proteins.

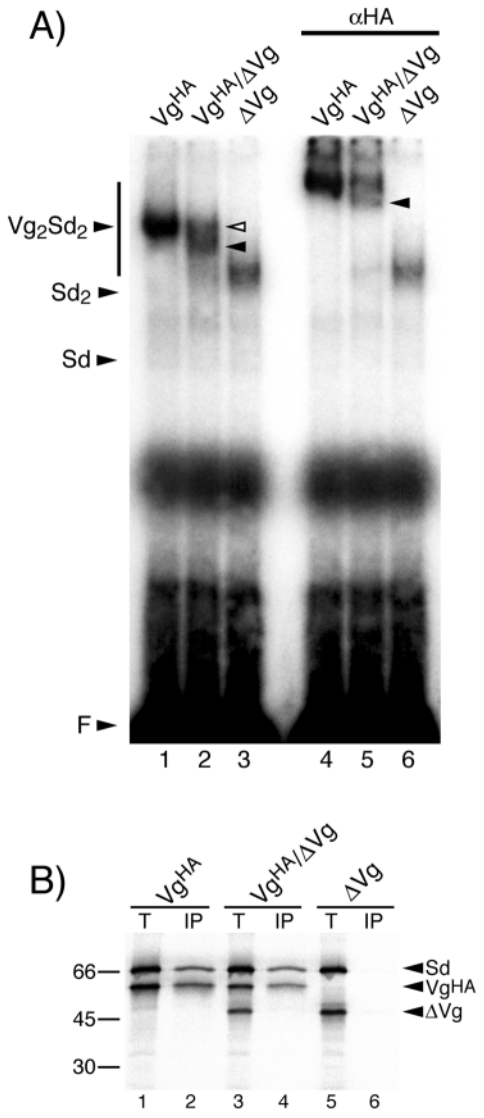

Fig. 5. Vg and Sd form multimeric complexes on DNA but heterodimers in solution. (A) Lanes 1-3: EMSA with Vg^{HA} and/or VgΔ(73-274) co-expressed with Sd binding to the *cut* template. Black arrowhead indicates complexes with intermediate mobility, presumably comprising the two Vg forms, Sd and DNA. Open arrowhead indicates complexes with the same mobility as those observed by co-expressing full-length Vg and Sd only. Lanes 4-6: same reactions incubated with anti-HA antibody. Complexes containing full-length Vg (lanes 4,5) as well as the intermediate complexes (lane 5, arrowhead), but not the untagged VgΔ(73-274) (lane 6), are supershifted. VgΔ(73-176) gave the same effect (not shown). (B) SDS-PAGE of Co-IPs of Sd co-expressed with Vg^{HA} and/or VgΔ(73-176). Precipitation of Vg^{HA} co-precipitated Sd but not the other Vg form demonstrating that Vg and Sd form complexes in solution that do not contain more than one Vg molecule. The anti-HA antibody did not precipitate the untagged Vg deletion (lanes 5,6). The VgΔ(73-274) protein gave the same result (not shown).

Fondue (Fdu) (G. H. and S. B. C., unpublished). The similarity of the SID in Fdu to the SID in Vg is more extensive than in Tdu and spans two exons. In fact the splice site in Vg and Fdu occur at nearly identical positions (Fig. 3B, arrowheads). We now define the extent of the SID by the region that is conserved between insect Vg and Fdu (Fig. 3B). In addition to the SID, Fdu and Vg share two other domains: the N-terminal 66 amino acids (green domain in Fig. 3A,B) and a domain rich in histidine and alanine residues C-terminal to the SID (orange domain in Fig. 3A,B). Therefore, the newly identified Fdu protein is more similar to Vg than is the Tdu protein.

Using these domain boundaries as guidelines, we constructed a series of Vg protein deletion mutants and tested them for interaction with Sd in solution (Fig. 4B) and for complex formation on DNA (Fig. 4A). Fig. 3C shows a schematic of the mutant proteins and a summary of their activities that are presented in Fig. 4. We assayed for Sd-Vg interaction in solution by co-immunoprecipitation (Co-IP). We co-expressed ^{35}S labeled Myc-tagged Sd with the Vg deletion mutants and immunoprecipitated Sd using an anti-Myc antibody (Fig. 4B). The same buffer conditions and protein concentrations were used for the EMSA and Co-IP assays. We found that only the SID is required for interaction with Sd in solution because all Vg deletion mutants, except for the deletion in SID itself, are still co-immunoprecipitated with Sd (Fig. 4B). In fact, the SID by itself was able to bind to Sd (Fig. 4B, lane 22). This demonstrates that the SID is necessary and sufficient to mediate Vg-Sd interaction in solution.

In contrast to Sd binding in solution, the SID by itself, however, was not able to substitute for full-length Vg and to mediate the formation of a complex with Sd on the *cut* DNA template (Fig. 4A, lanes 1-3,9). In fact, SID interaction inhibited Sd from binding to A-sites (not shown). Therefore, domains in addition to the SID are required for complex formation on DNA. Indeed, deletion of the green domain or of the N-terminus up to the SID reduced DNA complex formation, indicating that the green domain is important for this activity (Fig. 4A, lanes 5, 6). Similarly, deletion of the region C-terminal to the SID reduced complex formation on DNA (Fig. 4A, lane 7). Deletion of the green domain or of the N terminus up to the SID in the context of the C-terminal deletion completely abolished complex formation (Fig. 4A, lanes 8, 9). These results indicate that the green domain and C-terminal residues act redundantly and/or cooperate in complex-forming activity.

We also created four deletion mutants to test whether the non-conserved residues between the green domain and the SID have essential functions. Internal deletions of residues 73-176 and 73-274 had essentially no effect on Vg activity in the DNA-binding assay. However, in combination with the C-terminal deletion, they abolished complex formation (Fig. 4A, lanes 10-14). Thus, the internal region may be required to correctly position the green domain, so that it can interact with C-terminal residues to form the Vg-Sd-DNA complex.

Taken together, these data identify three regions in Vg that are required for complex formation with Sd on DNA: the Sd interaction domain (SID), which is sufficient to mediate binding to Sd in solution, the N-terminal 66 residues and the region C-terminal to the SID, both of which are required specifically for forming a Vg-Sd complex on DNA. All three domains show conservation between insect Vg and vertebrate Fdu.

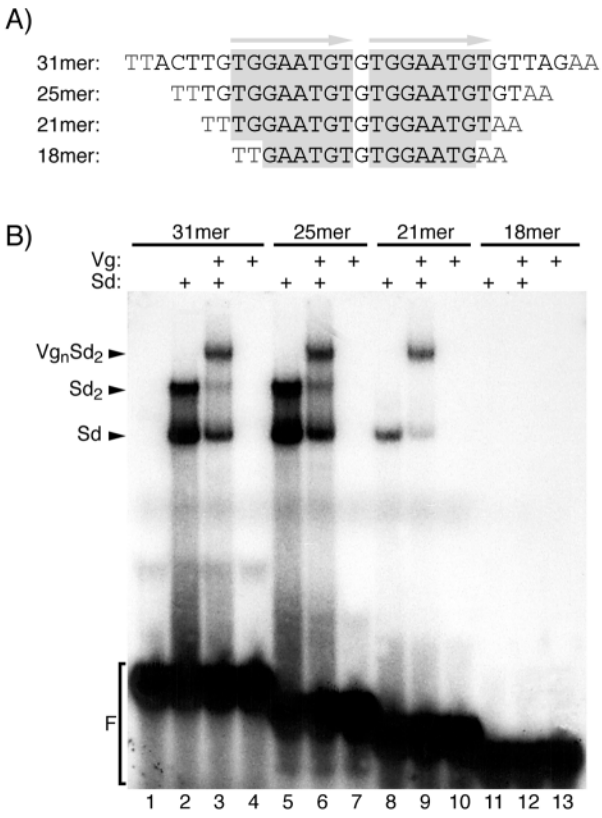

Fig. 6. Vg-Sd complex formation on DNA does not require bases outside of the Sd-binding sites. (A) Top strands of the series of truncated templates. TT and AA bases at the ends of the probes were added in order to label the probes with ^{32}P α-dATP. The extent of the Sd binding sites (shaded regions) was defined by systematic mutational analysis (Butler and Ordahl, 1999). (B) EMSA using TNT produced Vg and Sd and the probes shown in A. The 31 and 25 mers bind strongly to Sd and the Vg-Sd complex (lanes 1-7). The 21 mer is weakly bound by Sd (lane 8), while the Vg-Sd complex forms nearly as efficiently as on the longer templates. The 18 mer is not bound by Sd or Vg-Sd. Labeling is as in Fig. 1.

Vg and Sd form a heterodimer in solution but a heterotetramer on target DNA

The observation that the Vg-Sd complex did not bind to the 1×GT probe was surprising given that it bound to the 2×GT probe, which contains two tandemly arranged 1×GT binding sites. This raises the question of why the Vg-Sd complex requires doublet sites but does not recognize single binding sites. One possibility is that DNA binding by the Vg-Sd complex requires more than one Sd and Vg molecule in the complex. For example, a single molecule of Vg could bridge two Sd molecules to enable DNA binding or it may be that Vg-Sd dimers interact to increase the affinity or stability of the complex on DNA.

To determine how many Vg molecules are present in the respective complexes, we examined the mobility of complexes formed when two Vg molecules of different sizes were present. The design of this experiment was to test for the formation of

heteromeric complexes of intermediate size that would indicate the presence of two (or more) Vg molecules in the complexes. We made use of two internal deletion mutants that showed nearly normal activity in the DNA-binding and Sd interaction assays (Fig. 4). The Vg deletion Δ(73-274) formed complexes with Sd on DNA that migrate faster on EMSA gels than complexes containing full-length Vg, owing to the smaller size of the mutant protein (Fig. 5A, lanes 1,3). Because we wanted to use the same proteins for the EMSA and the Co-IPs described below, we used an HA-tagged Vg protein (Vg^{HA}), which gave the same results as native Vg (not shown). When full length Vg^{HA} was co-expressed with VgΔ(73-274) and Sd, complexes of intermediate mobility formed (Fig. 5A, lane 2 arrowhead). Complexes migrating at the position of the complexes formed with Vg^{HA} were still present (open arrowhead). However, little (if any) complexes with the Vg deletion were observed, which may be due to competition by Vg^{HA}, which binds with higher affinity than Δ(73-274) (Fig. 5A, compare lane 1 with lane 3). We interpret the intermediate complexes as hetero-complexes formed between Sd, a mixture of Vg^{HA} and VgΔ(73-274), and DNA. Results using the VgΔ(73-176) deletion were identical (not shown). Thus, more than one Vg molecule is present in the shifted complexes. More precisely, as one extra complex of intermediate size appeared, it most probably contains one of each of the two Vg forms and is a heterotetramer.

We next examined whether or not this heterotetrameric complex formed in solution independently of DNA binding. We inserted an HA-tag into the middle of the non-conserved and dispensable region of Vg at a position predicted to be exposed to the surface. We first evaluated the activity of the Vg^{HA} mutant and a possible negative effect of anti-HA antibody binding on DNA complex formation in EMSA supershifts. Addition of anti-HA antibody supershifted complexes from TNT reactions producing Vg^{HA}. Importantly, the anti-HA antibody also supershifted the intermediate complex containing the full-length and shortened forms of Vg (Fig. 5A, lane 5 arrowhead), demonstrating that the anti-HA antibody does not disrupt the formation of heterotetrameric complexes on DNA. Immunoprecipitation of Vg^{HA} in the absence of DNA template co-precipitated Sd as efficiently as Vg was co-precipitated by Sd^{myc} (compare Fig. 5B, lanes 1 and 2 with Fig. 4, lanes 3 and 4). Significantly, immunoprecipitation of Vg^{HA} from a TNT reaction containing Sd, Vg^{HA} and one of the Vg deletions Δ(73-176) or Δ(73-274) co-precipitated Sd but not the other Vg protein species (Fig. 5B, lanes 3, 4). Thus, the Vg-Sd complex is a heterodimer in solution but a heterotetramer on DNA.

Vg-Sd complex formation on target DNA does not require bases outside the Sd-binding site

The observation that the Vg-Sd complex has increased affinity for B-sites compared with the Sd protein alone raises the possibility that Vg makes DNA contacts outside the region that is contacted by Sd, which could enlarge the DNA interaction surface and thereby increase the affinity of the complex. It has not been possible to produce sufficient quantities of active Vg-Sd complexes for chemical interference and DNAseI footprinting assays that could localize exactly the region contacted by the Vg-Sd complex. Bacterially produced Sd and Vg are insoluble and do not form active complexes upon renaturation, either refolded together or refolded separately and then mixed together. As an alternative to a chemical interference assay, we designed a series of 2×GT probes with increasingly truncated ends to test whether bases outside of the Sd binding sites are required for Vg-Sd complex formation (Fig. 6A). We chose the 2×GT probe because this template allows observation of Sd and Vg-Sd binding. Our rationale was that, if Vg contacts DNA outside the Sd-binding sites, then the Vg-Sd complex may not form on shorter probes that are nevertheless bound by Sd alone. However, if Vg does not contact DNA, the minimal template length requirement should be similar for Vg-Sd and for Sd binding.

We found that the Vg-Sd complex still formed on a probe that was shortened sufficiently to reduce Sd binding (Fig. 6B). The 31 and 25 mer probes bound to Sd and Vg-Sd with high affinity (Fig. 6, lanes 1-7). The shorter 21 mer had strongly reduced Sd binding (Fig. 6, lane 8), but Vg-Sd complex formation was nevertheless nearly as efficient on this template as on the longer probes (Fig. 6, lanes 3, 6, 9). Neither the Vg-Sd complex nor Sd alone bound to the shorter 18 mer. Thus, Vg interacts with Sd and increases its binding to a template that is too short for efficient Sd binding. We conclude that Vg-Sd complex formation does not require bases outside the region required by Sd, and thus that Vg does not contact DNA, at least not outside of the Sd-binding sites.

DISCUSSION

We have analyzed the DNA-binding properties of Vg-Sd complexes, the full-length Sd protein, and of the TEA domain of Sd with respect to a number of binding sites, particularly the functional sites identified in native *cis*-regulatory elements. We have made four key findings. First, we found that the Sd protein has a more restricted DNA-binding specificity than its isolated TEA domain. Second, we showed that the Vg-Sd complex binds well to sites in native *cis*-regulatory elements to which Sd alone does not bind well. Third, we found that two domains of the Vg protein are required for Vg-Sd complex formation on DNA that are not required for Vg binding to Sd in solution. And fourth, that this complex is a heterotetramer on DNA while apparently a heterodimer in solution. Below we present a mechanistic model for the control of Vg-Sd DNA target selectivity that considers these findings.

Vg binding switches the DNA target selectivity of Sd

We propose a model in which Vg binding to Sd switches the DNA target selectivity of Sd (Fig. 7). We found that the Sd protein alone binds to sites with a particular composition, termed A-sites, which exist singly or as doublets. In the latter case, Sd may bind cooperatively if the two sites are arranged in tandem. When Vg is also present, Vg and Sd interact and form a dimer in solution (Fig. 5B). This complex has two distinct properties. First, the Vg-Sd dimer has a greatly reduced affinity for A-sites (Fig. 1). Vg may either induce a conformational change in Sd that inhibits the TEA domain from interacting with DNA, or Vg could directly mask the TEA domain. Second, the dimer forms a higher order complex on a different set of binding sites, termed B-sites (Fig. 1). These two activities of Vg are distinguished by their structural requirements. While the SID domain of Vg is sufficient to

inhibit Sd DNA-binding to A-sites, additional domains N- and C-terminal to the SID are required for complex formation on B-sites (Fig. 4). Importantly, B-sites are poorly bound by Sd in the absence of Vg. Thus, Vg binding to Sd inhibits binding to A-sites while enabling binding to B-sites, that is, Vg switches the DNA-binding preference from A-sites to B-sites.

How does Vg binding affect the target selection of Sd? Two, not necessarily mutually exclusive models, may be postulated. First, Vg may influence Sd through global effects on Sd DNA binding. That is, Vg may act to reduce the DNA binding affinity of Sd to any target DNA, while also enhancing cooperativity of neighboring Vg-Sd complexes on DNA. We found that Vg and Sd form dimers in solution and that these dimers do not bind single A-sites. We have never observed any complexes of Sd and Vg on DNA migrating at a position, indicating Vg-Sd dimers bound to DNA on either A- or B-sites (the Vg-Sd complexes bound weakly to 1×GT have the same mobility as the ones bound to B-sites, and thus also consist of heterotetramers). However, in spite of the negative effect of Vg on DNA binding, two Vg-Sd dimers bound strongly to doublet B-sites. Apparently, strong cooperative interactions between two Vg-Sd dimers allow binding to B-sites. The N- and C-terminal protein domains of Vg that are required in addition to the SID for complex formation on DNA may be required for these interactions, which could involve Vg-Sd and/or Vg-Vg interactions between the two dimers on DNA.

Alternatively, Vg interaction may specifically enhance binding to doublet B-sites. We favor this model because we found that Vg-Sd had a similar affinity for several B-sites such as those in *cut* and 2×GT, even though 2×GT is a much better Sd binding site. The affinities of Sd for these sites therefore do not translate directly into the relative affinities observed for Sd-Vg binding, as would be expected if Vg only enhanced cooperativity. In addition, we found that the TEA domain binds several A- and B-sites with high affinity, but that full-length Sd has a strong preference for A-sites over B-sites. Thus, in the absence of any co-factor, Sd is in a conformation in which a domain of Sd separate from the TEA domain inhibits the TEA domain from binding to B-sites specifically (Fig. 1). In vitro, Vg interaction appears to be able to alleviate this inhibition because Vg-Sd complexes bind strongly to B-sites. This alleviation only occurs when complexes form on doublet sites, as Vg-Sd complexes do not bind to DNA as a dimers. We suggest that some sort of conformational change is associated with binding to doublet B-sites (Fig. 7). Our model is supported by the finding that the region of Sd that binds to the SID of Vg is homologous to a region of the vertebrate TEF-1 that negatively affects DNA binding (Hwang et al., 1993; Simmonds et al., 1998). This model is analogous in part to the role of Exd overcoming the inhibitory effect of the YKWM motif in the Labial Hox protein (Chan et al., 1996).

We have argued here that Sd and the Vg-Sd complex differentiate between A- and B-sites. What then are the distinguishing features of these sites? The sequences of the A- and B-sites are quite diverse and their alignment does not reveal different consensus sequence motifs. However, Sd clearly prefers binding to A-sites, and the inability of Sd to bind strongly to B-sites, such as that in the *cut* element, must therefore be due to the sequence of the template site. Vg-Sd complexes bind with high affinity to only two sites when arranged in tandem, and do not form on single A- or B-sites. Thus, Sd discriminates between A- and B-sites based on sequence, while the binding of Vg-Sd complex depends both on sequence and the arrangement of the sites. We have identified two sites (DSRF and 2×GT) that have A- as well as B-site properties, so these properties are not mutually exclusive. However, many sites exist that are bound well by Sd or Vg-Sd, but not by both. Most of the essential sites for Vg-Sd regulation in vivo have mainly B-site character and are bound poorly by Sd. The identification of the exact sequence requirements that distinguish native essential Sd sites from the known Vg-Sd target sites will require some knowledge of Sd-regulated target genes in other tissues (see below).

Vg as a determinant of the specificity of Sd action in vivo

Vg binding and its effect on the DNA target selectivity of Sd plays a major role in distinguishing the biological specificity of Sd action in the developing wing from Sd function in other tissues. Sd is required for the development of tissues other than the wing, for example, the eye and the PNS, where it is not co-expressed with Vg (Campbell et al., 1992; Inamdar et al., 1993). Based on our results, we postulate that Sd selects a different set of target genes there, at least in part because its DNA-binding specificity is different in the absence of Vg.

No direct target genes for Sd in these other tissues have been identified. However, many target genes for the vertebrate Sd homolog TEF-1 are known (reviewed by Jacquemin and

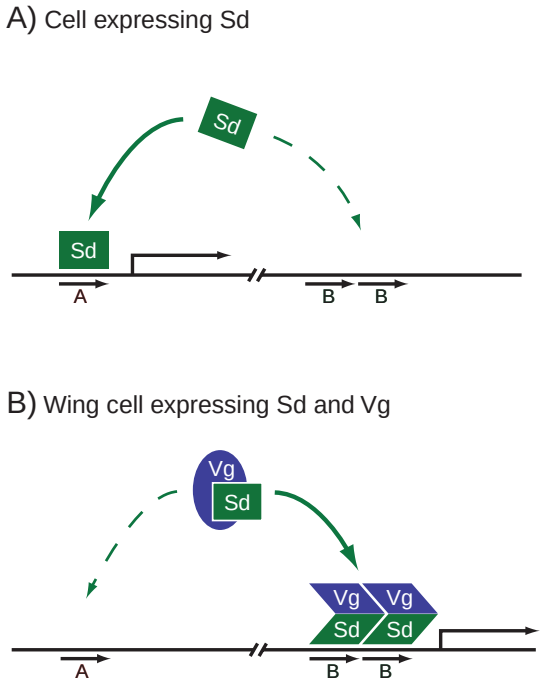

Fig. 7. Model for Vg-Sd interaction and DNA-binding selectivity. (A) Sd binds to A- but not B-sites in cells that express Sd. (B) Sd forms a 1:1 complex with Vg in developing wing cells that express both proteins. The interaction of Sd with Vg prevents Sd from binding to A-sites. However, the Vg-Sd complex is able to bind to B-sites. This activity requires two B-sites in close proximity. Binding to B-sites may be accompanied by conformational changes in Vg-Sd that are only induced when two B-sites are present, indicating that interactions take place between neighboring Vg-Sd complexes.

Davidson, 1997). Sd and TEF-1 may function very similarly, as their TEA domains are 99% identical (Campbell et al., 1992) and have indistinguishable DNA-binding properties in vitro (Halder et al., 1998), and TEF-1 can substitute for Sd in *Drosophila* (Deshpande et al., 1997). In mammals, TEF-1 directly regulates many genes expressed during muscle differentiation by binding to A-sites containing the so-called 'm-CAT' motif (CATTCCT) (Cooper and Ordahl, 1985; Farrance et al., 1992; Mar and Ordahl, 1990; Nikovits et al., 1986). Importantly, this motif is bound by a single TEF-1 molecule (Farrance et al., 1992). We tested two of these m-CAT sites for Sd binding and found that, as for other single A-sites, Sd alone bound well, but the presence of Vg inhibited Sd binding and did not result in complex formation on DNA. Because these sites are in vivo targets of TEF-1, this suggests that TEF-1 and Sd may directly regulate gene expression by binding to single A-sites alone or in complexes with other factors, but not in complexes containing the Vg/Fdu proteins. Interestingly, it has been found that vertebrate TEF-1 forms a complex with the bHLH protein Max in vivo, and that Max, or another bHLH protein, may be an obligatory co-factor for TEF-1 function during muscle differentiation (Gupta et al., 1997). Because Max contacts DNA sequence specifically, it increases the target selectivity of TEF-1 in muscle cells. The association of TEF-1 with Max may present another example of a tissue-specific co-factor that differentiates the DNA-target selectivity of a TEF transcription factor family member between different tissues.

Implications for the elucidation of regulatory networks on a genomic scale

One of the major aims of genome sequence analysis is to decipher genetic regulatory sequences involved in development and differentiation. One critical challenge in achieving this goal is the ability to correctly predict the in vivo target genes of transcription factors. Several types of data may be considered for such predictions, including the presence or absence of transcription factor binding sites in potential regulatory regions, gene expression profiles and detailed protein function studies. Searching genomic sequences for binding sites is obviously important; however, binding site consensus sequences are often short and degenerate, so that potential binding sites are predicted to occur in regulatory regions of virtually any gene. This also holds true for Sd. The consensus binding site of the TEA domain ($^{T}/_{A}$ $^{A}/_{G}$ $^{A}/_{G}$ $^{T}/_{A}$ AT $^{G}/_{T}$ T) is found once about every 2 kb, on average. However, we have argued that many, if not all, Vg-Sd-regulated target genes possess a doublet of Sd-binding sites. Requiring a second binding site in tandem decreases the frequency of potential biologically relevant Vg-Sd binding sites by a factor of ~2000. The fact that most of the Vg-Sd sites would not have been found using full-length Sd protein in footprint assays and that the Sd DNA-binding domain alone binds promiscuously is therefore a note of caution. Understanding the role of tissue-specific co-factors may be imperative to deciphering transcription factor-regulated networks on a genome-wide scale. Efforts are under way, using these new insights into the selectivity of the Vg-Sd complex, towards defining the network of Vg-Sd-regulated genes in the developing wing.

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