Molecular and Functional Analysis of *scalloped* Recessive Lethal Alleles in *Drosophila melanogaster*

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ABSTRACT

The *Drosophila melanogaster scalloped* (*sd*) gene is a homolog of the human TEF-1 gene and is a member of the TEA/ATTS domain-containing family of transcription factors. In Drosophila, *sd* is involved in wing development as well as neural development. Herein, data are presented from a molecular analysis of five recessive lethal *sd* alleles. Only one of these alleles complements a viable allele associated with an *sd* mutant wing phenotype, suggesting that functions important for wing development are compromised by the noncomplementing alleles. Two of the wing noncomplementing alleles have mutations that help to define a VG-binding domain for the SD protein *in vivo*, and another noncomplementing allele has a lesion within the TEA DNA-binding domain. The VG-binding domain overlaps with a domain important for viability of the fly, since two of the *sd* lethal lesions are located there. The fifth lethal affects a yet undefined motif lying just outside the VG-binding domain in the C-terminal direction that affects both wing phenotype and viability. This is the first example linking mutations affecting specific amino acids in the SD protein with phenotypic consequences for the organism.

THE *scalloped* (*sd*) gene was first identified because of mutations that resulted in a loss of wing margin structures, giving the wing a scalloped appearance (Grunberg 1929). With respect to the wing phenotype, both strong and weak *sd* alleles that are viable as homozygotes exist. Strong alleles cause the wings to be drastically reduced in size, as in sd^{58d}, whereas weak alleles result in only partial loss of the wing margin structures, as in sd^{ETX4} (Campbell *et al.* 1991). All extant viable mutations of *sd* that have been characterized appear to affect regulatory elements upstream of the transcriptional start site (Campbell *et al.* 1991). Thus, they provide little information about the molecular nature of the protein and domains that are important for its function.

The *sd* locus produces multiple transcripts as a consequence of alternative splicing (Campbell *et al.* 1991). The E21 isoform (Campbell *et al.* 1991, 1992) is the best studied so far and encodes a protein product of 440 amino acids. This protein belongs to a family of highly conserved transcription factors containing the TEA/ATTS DNA-binding domain (Burglin 1991). *In silico* studies and *in vitro* binding experiments have provided some information pertaining to the location of functional motifs within the E21 isoform. The N-terminal region contains the TEA/ATTS DNA-binding domain (Campbell *et al.* 1992; Simmonds *et al.* 1998). The evolutionarily conserved C-terminal half is known to contain a domain that is necessary for binding VESTIGIAL (VG) *in vitro* (Simmonds *et al.* 1998). The *sd* gene is expressed in the embryonic nervous system and in the larval wing, eye-antennal, and leg imaginal discs, thus implicating it in some important role during development of these structures (Campbell *et al.* 1991, 1992). Furthermore, the existence of lethal alleles (Campbell *et al.* 1991) suggests that *sd* has a vital function in tissues other than the wing, which is not, in itself, essential. In the wing disc, *sd* is expressed in a pattern that encompasses the entire wing pouch (Campbell *et al.* 1992), overlapping with the expression pattern of the *vestigial* (*vg*) gene product (Williams *et al.* 1991).

The SD and VG proteins bind to each other (Paumard-Rigal *et al.* 1998; Simmonds *et al.* 1998) to form a functional transcription complex. In this complex, SD provides the DNA-binding activity as well as the nuclear localization signal whereas VG provides the activation function (Halder *et al.* 1998; Simmonds *et al.* 1998; Srivastava *et al.* 2002). The binding of SD and VG involves a 56-amino-acid SD interaction domain (SID) in VG with a region of SD within the C-terminal half (Simmonds *et al.* 1998). Apart from the SID, VG also harbors two domains that provide the activation function of the SD/VG transcription complex (Vaudin *et al.* 1999; MacKay *et al.* 2003). In this respect VG appears to act as a transcription intermediary factor (TIF; Hwang *et al.* 1993). TIFs are proposed to be tissue-specific molecules that are required to activate transcrip-

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tion by SD in Drosophila (Simmonds *et al.* 1998) and by its homologs in other organisms in tissues in which they are expressed (Hwang *et al.* 1993). Thus, in addition to VG, other proteins are likely involved in the activation of target genes by the SD protein in nonwing tissues. In addition to its function in wing development, VG is also involved in muscle development (Bate and Rushton 1993; Sudarsan *et al.* 2001) and possibly this function also is mediated by SD.

Homologs of *sd* from several organisms have been cloned and characterized (Mirabito *et al.* 1989; Xiao *et al.* 1991; Chaudhary *et al.* 1995; Azakie *et al.* 1996; Gavrias *et al.* 1996) and show a remarkable degree of conservation within the DNA-binding domain as well as in the C-terminal region. There is some evidence that *sd* in Drosophila may be involved in somewhat similar functions as its mammalian homolog. Both *sd* (Campbell *et al.* 1992) and its vertebrate homolog, transcription enhancer factor-1 (TEF-1), are expressed in neural tissues (Yasunami *et al.* 1995). TEF-1 has also been shown to be important for both cardiac (Gupta *et al.* 1997; Ueyama *et al.* 2000; Maeda *et al.* 2002b) and skeletal muscle development (Stewart *et al.* 1994; Azakie *et al.* 1996; Hsu *et al.* 1996). Furthermore, hTEF-1 can functionally substitute for Drosophila *sd* during development and is able to rescue *sd* wing mutations and the lethality associated with an *sd* lethal allele (Deshpande *et al.* 1997). A role in muscle development for a human VG homolog, vestigial-like 2, has also been documented (Maeda *et al.* 2002a).

The evidence points to a functional evolutionary conservation of *sd* and related genes in other organisms for roles in developmental switches. Thus, studies of *sd* in Drosophila should provide valuable insights into the nature and function of homologs in other organisms. Studies involving the mammalian homologs of *sd* have primarily involved *in vitro* methodologies and the TEA domain has been the focus of intense *in vitro* investigations (Hwang *et al.* 1993; Berger *et al.* 1996; Gupta *et al.* 2000; Jiang *et al.* 2001). While domains in the C-terminal region of TEF-1 have been predicted, their biological function has not been ascertained (Simmonds *et al.* 1998; Vaudin *et al.* 1999). To gain further insight into the function of the *sd* gene product, five recessive lethal mutations were characterized molecularly. These alleles were previously mapped within the *sd* gene and predicted to affect the C-terminal portion of the SD protein (Campbell *et al.* 1991). A more detailed characterization of the lethal *sd* mutations herein links specific residues in the SD protein with a phenotypic consequence in the organism. Also, the data show that the molecular lesions associated with two of these alleles help to define a domain in SD that is responsible for binding VG (VG-binding domain; VBD) *in vivo*. This domain was previously predicted (Vaudin *et al.* 1999), but the prediction was based on indirect evidence comparing the amino acid sequence of TEF1 that binds to TONDU (now called Vgl-1; Maeda *et al.* 2002a) with the SD amino acid sequence. More directly, the previous Drosophila *in vitro* data localized the VBD to only the C-terminal half of SD (Simmonds *et al.* 1998). This VBD partially overlaps with a domain that is important for satisfying some vital function important for early development of the fly, since the lesions for two of the *sd* lethal alleles map there and a third lies just outside this domain. Yet, the absence of wings *per se* is not lethal. Another of the lethal mutations affects the TEA DNA-binding domain, the first such example from any experimental organism.

MATERIALS AND METHODS

***Drosophila melanogaster* stocks and crosses:** The genotypes of the *sd* stocks used in this study are described in detail in Campbell *et al.* (1991, 1992). The chromosomes carrying each of the five recessive lethal *sd* alleles were tagged with *yellow* (*y*) using standard genetic techniques and the *y*-tagged alleles were maintained as balanced stocks over an *FM7* or an *FM6* chromosome that also carries a *y* marker. The *y*-tagged females were crossed to y^+*BinSn* males and y^+ heterozygous *Bar* females were selected and then recrossed to y^+*BinSn* males. We used a dissecting microscope to visually examine mouth hooks to separate the hemizygous *y* male larvae carrying the respective *sd* lethal lesion from their y^+ sibs. The sd^{ETX4} enhancer trap has also been described (Campbell *et al.* 1992). The y^+*BinSn* stock was obtained from the Drosophila Stock Center at Bloomington, Indiana.

Genomic DNA isolation: We used a glass milk-based DNA extraction protocol to isolate genomic DNA from ~10–20 first instar *y* larvae from each *sd* larval lethal stock and 10 third instar *y* larvae from the pupal lethal stocks. In brief, genomic DNA was extracted with phenol and chloroform and incubated with glass milk (BIO 101, Vista, CA). The glass milk-treated DNA was eluted in 30 μl of distilled water. For PCR, 2 μl of the DNA solution obtained was used in each reaction.

PCR amplification of scalloped regions: For PCR amplification, Platinum PCR super mix (Invitrogen, San Diego) was used in a reaction containing 2 μl of genomic DNA (isolated above) from each *sd* allele and 5 ng of each primer. The sequences of PCR primers used for amplification of the different regions of *sd* are shown in Table 1 and their respective locations within *sd* are depicted in Figure 1C. These primers span most of the coding region including the TEA domain. The first three exons of the *sd* lethal alleles were not sequenced as the *sd* alleles studied herein were previously mapped to the remainder of the *sd* locus (Campbell *et al.* 1991). The PCR reactions were performed on a Perkin-Elmer (Norwalk, CT) thermal cycler using the following amplification program: 95° for 5 min followed by 40 cycles at 94° for 1 min, 60° for 1 min, and 72° for 3 min. This was followed by a hold of 1 cycle at 72° for 7 min and then storage at 4°.

Sequencing of the amplified products: The amplified product was either sequenced directly or cloned into pGEMT (Promega, Madison, WI) and then sequenced using an Amersham (Arlington Heights, IL) Dyenamic ET kit according to the manufacturer's instructions. For products that were cloned, multiple independent clones were sequenced to detect possible errors incorporated during cloning manipulations. Once a particular putative change was identified it was confirmed by sequencing DNA amplified from a heterozygote.

Generation of sd^{68L} clone by PCR replacement: Site-directed inverse PCR mutagenesis was performed on *sd* cDNA to create

TABLE 1

PCR primers used for amplification of *sd* regions

Primer designation	Sequence, 5′–3′	T_m (°)
1	CCAGGATCCCACCACCCATCACATATACC	73.4
2	GCGAATTCGCTCTGATTGTGTCGTTCCTTGC	77.2
3	CCAGGATCCCAATTCTGGCAACCTGGACTACAGC	78.8
4	GCGAATTCACGGTATATGAGATGGGTGGTGC	75.2
5	CCAGGATCCGGCATACGTCAGTTCTTAGATGTGTGC	78.3
6	GCGAATTCCATGCTCATCCAACCCAAGGATCTAGC	78.8

See Figure 1C for locations of these primers.

the sd^{68L} lesion from the wild-type version of the gene using the following primers: 5′-AGACGTAGCGATTGTTCTCC-3′ and 5′-GAAAGCGAG**A**ACTCTCGACT-3′. The location of the desired point mutation is indicated with the uppercase A in boldface type. Sequencing was done to ensure the accuracy of this change and that no other changes were produced.

Sequence analysis: All sequence analysis was done using DNAMAN sequence analysis software by Lynnon BioSoft (Montreal). The assembled sequence was aligned against wild-type sequence in the database using the blast server at http://www.ncbi.nlm.nih.gov/blast.

Immunohistochemistry: All immunohistochemical staining was done according to a published protocol (Williams *et al.* 1991). The anti-VG antibody was a gift from Sean Carroll (University of Wisconsin) and has been previously described (Williams *et al.* 1991). The secondary antibody was a Cy3-conjugated anti-rabbit antibody (Jackson ImmunoResearch, West Grove, PA).

GST-pull-down assays: GST-SD and GST-SD^{68L} fusions were created by cloning either *Bam*HI-digested SD or SD^{68L} coding regions into the *Bam*HI site of pGEX4T-1 (Amersham). *Escherichia coli* (BL21) cultures containing each of these plasmids were grown to an A_{600} optical density of 1.8 and induced with 1 mm isopropyl-L-thiogalactoside. The resulting glutathione S-transferase (GST)-protein fusions were isolated on glutathione-agarose (Pharmacia, Piscataway, NJ) following the manufacturer's instructions. The resulting yield of each protein was calculated by eluting the protein from 10 μl of a 50% GST-bead/PBS mixture using reduced glutathione (Sigma, St. Louis). The eluted protein was quantified using a micro-BCA assay (Pierce, Rockford, IL) and the resulting concentration of GST or GST-fusion per microliters of beads was calculated. ^{35}S-labeled probe proteins were created using SD and VG open reading frames cloned into Bluescript SK (Stratagene, La Jolla, CA) as in Simmonds *et al.* (1998) and the TNT T7 Quick Coupled in-vitro transcription and translation kit (Promega) and [^{35}S]methionine (Amersham). The resulting proteins were purified using a Sephadex G-25 spin column (Roche). The probe protein was incubated with 10 μl GST or GST-SD or GST-SD^{68L} beads in affinity binding buffer (Simmonds *et al.* 1998). The GST beads were then pelleted and washed three times in 50 volumes of affinity binding buffer. Following washing, the protein bound to the GST and GST-protein fusions was eluted by resuspension in 40 μl of 1× SDS protein gel loading buffer (Amersham) and heating to 95° for 5 min. For each probe protein used, a 1/100 dilution of the original *in vitro* labeling reaction was added to the protein/gel loading buffer to a final volume of 40 μl. These samples were then separated on a 5–15% polyacrylamide gel, which was subsequently dried and exposed to X-ray film (Eastman Kodak, Rochester, NY) overnight.

RESULTS

Phenotypes associated with the scalloped lethal alleles: The five recessive lethal *sd* alleles studied herein were generated in a screen for mutations uncovered by *Df(1)* sd^{72b} and previously mapped to the C-terminal region of the SD protein (Campbell *et al.* 1991). They can be grouped in two categories on the basis of the developmental stage at which lethality occurs for each *sd* allele (Figure 1A) and the relative ability of the lethal allele to complement the wing phenotype of a weak *sd* allele, sd^{ETX4} (Figure 1B). The sd^{3L} and sd^{47M} alleles belong to the early lethal class because they die in the first larval instar. The sd^{31H}, sd^{68L}, and sd^{11L} alleles comprise the late lethal class and these mutants survive through the larval instars and die at the pupal stage. Pharate adults that are homozygous or hemizygous for these alleles also show gross head defects (not shown). In addition to recessive lethal phenotypes, the sd^{3L}, sd^{47M}, sd^{68L}, and sd^{31H} alleles display a *scalloped* wing phenotype when heterozygous with the sd^{ETX4} weak allele of *sd* (Figure 2, A–F). That is, they do not complement the wing phenotype of sd^{ETX4}. The failure to complement the phenotype of a homozygous viable *sd* allele indicates that these alleles all must compromise proper wing development in some way, either directly or indirectly. In contrast, when sd^{11L} is heterozygous with sd^{ETX} the outcome is a wing (data not shown) that is indistinguishable from the wild-type wing shown in Figure 2A. Even though sd^{11L} is also a recessive lethal, the ability to complement the wing phenotype of sd^{ETX} suggests that it does not compromise wing development. A positive complementation result has been used classically to indicate that two mutants affect different functions and this may be extended to alleles. Heterozygous combinations of the lethal alleles with sd^{ETX4} exhibit varying wing sizes and degrees of notching. The sd^{3L}, sd^{47M}, and sd^{31H} alleles exhibit an extreme wing phenotype when heterozygous with sd^{ETX4} (Figure 2, C–E) that involves loss of the majority of the wing blade tissue and erosion of the margin. In contrast, when sd^{68L} is heterozygous with sd^{ETX4} very little loss of wing blade tissue occurs but variable erosion of the margin is seen, ranging from

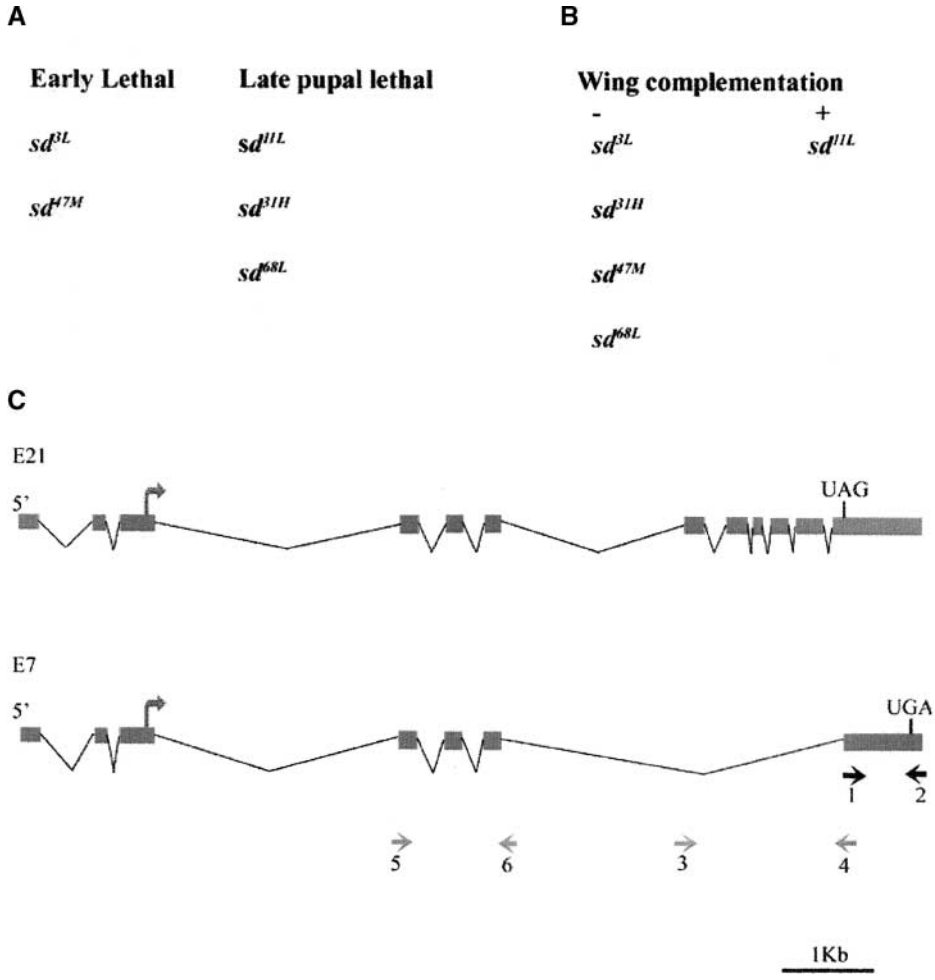

FIGURE 1.—Phenotypic classification of *sd* lethal alleles. (A) Classification of *sd* lethal alleles according to the stage of lethality and (B) the ability to complement the wing phenotype of a weak allele of *scalloped*, sd^{ETX4}. (C) PCR primers used to amplify regions bounded by each pair and collectively, covering the 3′ exons of the locus where the lethal alleles had been previously mapped (CAMPBELL *et al.* 1991). The gene structure corresponding to two alternatively spliced isoforms is shown. Solid boxes represent exons and lines represent introns. Details on primers are in MATERIALS AND METHODS.

very conspicuous to loss of only a few margin-specific bristles (Figure 2F). Heterozygous sd^{31H}/y^{+} *Binsn* females also show a dominant wing notching phenotype with a frequency of ~20% (8 of 39 heterozygous females) and this combination has also been reported to exhibit ectopic bristles (CAMPBELL *et al.* 1991).

Molecular lesions associated with the lethal alleles: While the E21 isoform of SD has been the most thoroughly characterized, another isoform, E7, could potentially produce a related protein that would retain part of the TEA domain but differ completely in the C-terminal half (Figure 1C). The lethal alleles were originally mapped by denaturing gradient gel electrophoretic analysis to the 3′ portion of the *sd* locus, a region where the E21 and E7 isoforms differ. Because the lethal alleles could be grouped into two phenotypic categories, it seemed possible that the wing complementing and wing noncomplementing alleles could be a result of distinct mutations in the two isoforms. Alternatively, the early and late lethal alleles could be due to mutations affecting different isoforms. To assess these possibilities, all of the lethal alleles were characterized by PCR amplification of the regions bounded by primers in Figure 1C followed by sequencing of the amplified products.

The molecular lesions associated with the different *sd* lethal alleles are described below and appear to affect only the protein encoded by the E21 isoform and not the E7 isoform. The characterized lesions were superimposed on the E21 amino acid sequence and are presented in Figure 3. The data for the allelic lesions are presented in the NH_2 to COOH direction with respect to how and where they mutate the SD protein.

sd^{31H}*:* This allele is associated with a G → A mutation, resulting in a codon change of AGA → AAA, which causes an arginine-to-lysine substitution at amino acid position 143 of the E21 isoform. This lesion is located within the TEA domain of SD.

sd^{3L}*:* This allele is associated with a T → A substitution resulting in a codon change of TTA → TAA. This introduces a stop codon in place of a leucine at amino acid position 232 in the E21 isoform.

sd^{47M}*:* In a previous study this allele was shown to be associated with a deletion of ~100 bp located between an *Eco*RI site at +12.2 and a *Bgl*II site at +12.6 of the genomic walk described in CAMPBELL *et al.* (1991). DNA sequencing has shown that this deletion is actually 157 bp in size and it removes the majority of intron 8 and also 22 nucleotides from exon 9. The result is the removal of

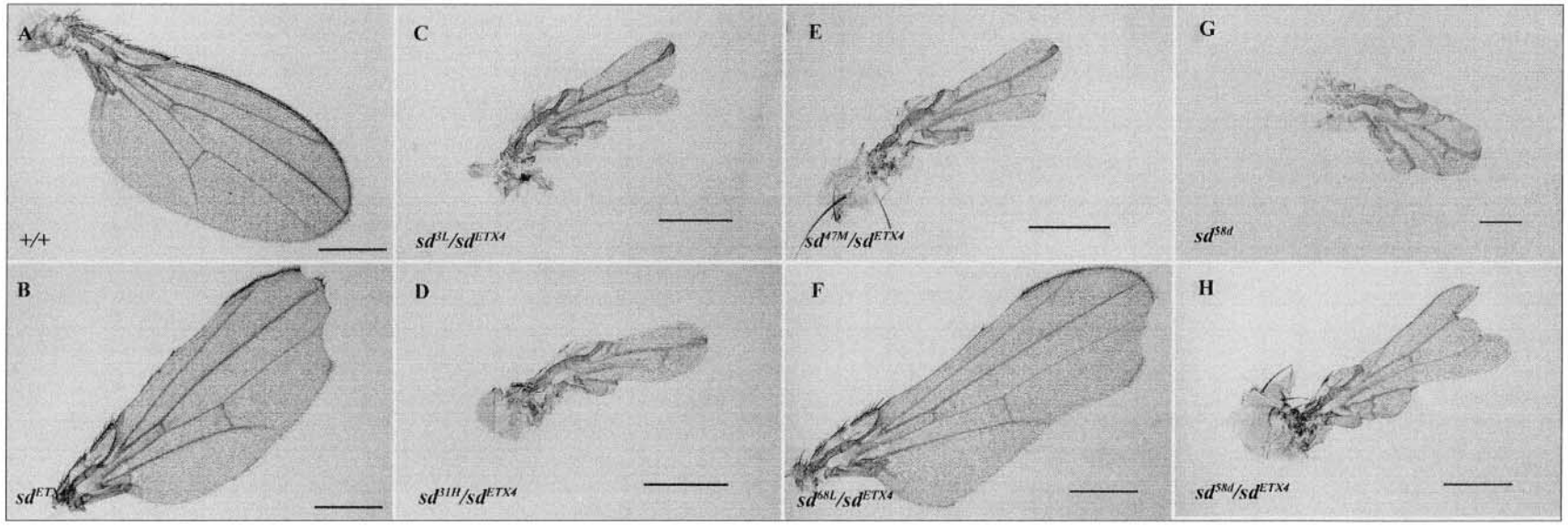

FIGURE 2.—Whole mount of wings from various *sd* allelic combinations illustrating the heterozygous wing phenotype of recessive lethal and viable *sd* alleles. (A) Wild-type wing showing an intact wing margin. (B) Wing from an sd^{ETX4} female showing notches in the wing margin. (C–F) Wings from heterozygotes between the *sd* lethal allele indicated and sd^{ETX4}. Note the variability in the wing phenotype in the different heterozygous combinations. (G) Wing from an sd^{58d} fly showing a more extreme wing phenotype compared to the sd^{ETX4} phenotype. (H) Wing from a heterozygote between sd^{ETX4} and sd^{58d}. The sd^{11L}/sd^{ETX4} combination is not shown, as in this combination the wings are indistinguishable from the wild-type wing in A.

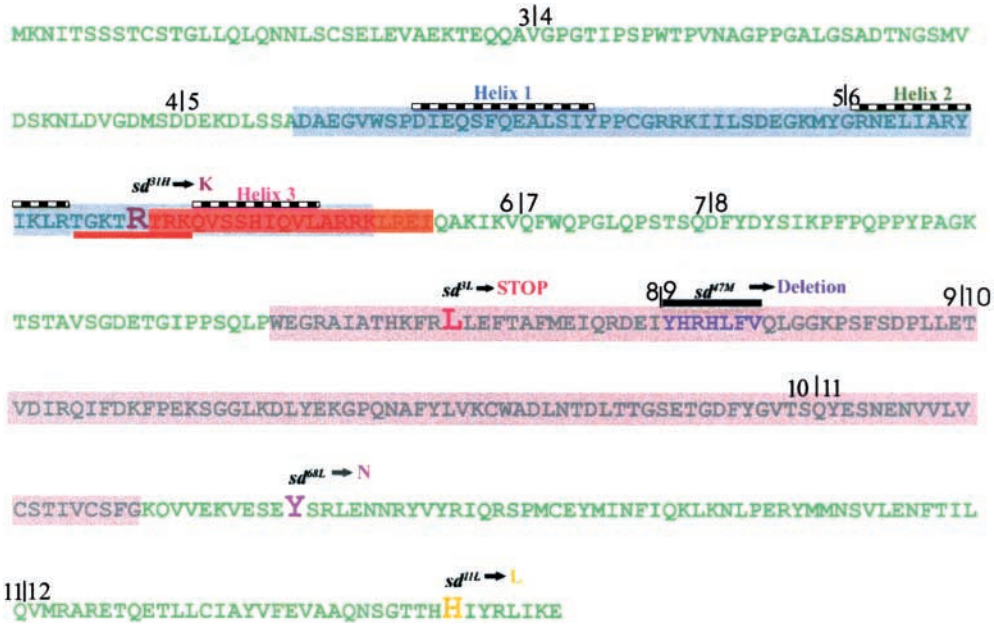

FIGURE 3.—Molecular lesions associated with the *sd* lethal alleles. The specific lesions are superimposed over the wild-type SD E21 isoform sequence (CAMPBELL *et al.* 1992). The wild-type amino acid affected by the various lethal alleles is depicted with an oversized letter and the mutated amino acid substitutions are shown over the wild-type sequence for the various alleles, as indicated. The colored boxes represent known and predicted domains in the SD protein. The blue box corresponds to the TEA DNA-binding domain (BURGLIN 1991; CAMPBELL *et al.* 1992) whereas a predicted VBD (VAUDIN *et al.* 1999) is shown as a magenta box. The three α-helices in the DNA-binding domain (BURGLIN 1991) are located as indicated by the hatched line over the respective amino acids. The nuclear localization signal overlapping the TEA domain is depicted with a red box. A potential protein phosphorylation site is marked with a red line under the respective amino acids. The encoded amino acids deleted in the sd^{47M} allele are shown with a black line over the affected amino acids. While sd^{3L} and sd^{47M} affect the predicted VBD, sd^{31H} affects the TEA DNA-binding domain and sd^{11L} does not affect either domain. Similarly, the sd^{68L} mutation does not affect either domain but does influence VG localization *in vivo*. The exon boundaries of the expressed portion of the *sd* mRNA (part of exon 3 through part of exon 12) are also indicated either above or between the respective amino acids with a vertical bar between the exon designations. If above, this indicates those cases in which that amino acid codon is encoded by adjoining exons.

seven amino acids as well as the splice site at the intron 8/exon 9 boundary, possibly generating a frameshifted protein beyond this point.

sd^{68L}: This allele is associated with a T → A substitution, producing a codon change of TAC → AAC. The mutation introduces an asparagine in place of a tyrosine at amino acid position 355 in the E21 isoform.

sd^{11L}: This allele is associated with an A → T mutation, resulting in a codon change of CAC → CTC, which causes a histidine-to-leucine substitution at amino acid position 433 of the E21 isoform.

Amino acid residues mutated in the *sd* lethal alleles are evolutionarily conserved: If the lesions associated with the lethal alleles affect the structural integrity and thereby important functions of the protein, then the respective wild-type residues are likely to be evolutionarily conserved. Therefore, the amino acid positions mutated in the lethal alleles were examined and compared across phyla. The SD amino acid sequences from various organisms were aligned using the ClustalW software and the results for four of them are shown in Figure 4. The alignment data indicate that the wild-type amino acids altered by the *sd* lethal mutations are, in fact, conserved across different phyla, implying that these amino acid positions are likely important for the structural and functional integrity of the protein. Furthermore, even discounting the possibility of a splicing defect and/or frameshift in sd^{47M}, 5/7 of the amino acids deleted are also conserved across phyla.

The molecular lesions associated with all but the sd^{31H} allele appear to be located in the C-terminal region of the SD protein (Figure 3) whereas the sd^{31H} allele affects the TEA domain. The molecular lesion associated with the sd^{11L} allele (the only wing complementing allele) occupies the most C-terminal position of all the alleles and is spatially distant from the other alleles in the C-terminal domain as shown in Figure 3. On the basis of the data, we conclude that the wing noncomplementing alleles, other than sd^{31H}, affect a domain responsible for some function involved in wing development as well as a vital function. Previous *in vitro* binding experiments support this interpretation by showing that the VG protein binds to the C-terminal region of the SD E21 isoform (SIMMONDS *et al.* 1998). In another study, VAUDIN *et al.* (1999) identified a domain in TEF-1 that binds a human *vestigial* ortholog that they called TONDU. Protein sequence comparisons between the TONDU binding domain of TEF-1 with the amino acid sequence of SD led to the prediction of a domain in SD that binds VG. Two of the wing noncomplementing lethal alleles of *sd* lie in the C-terminal half of the protein, where they appear to overlap with the above predicted domain and a third lies just outside of this domain. It is likely that the lesions associated with the sd^{3L} and sd^{47M} alleles help to define the molecular boundaries of a domain responsible for binding VG *in vivo*.

Localization of VG in the wing discs of larvae harboring various *sd* lethal alleles: VG is a nuclear protein that

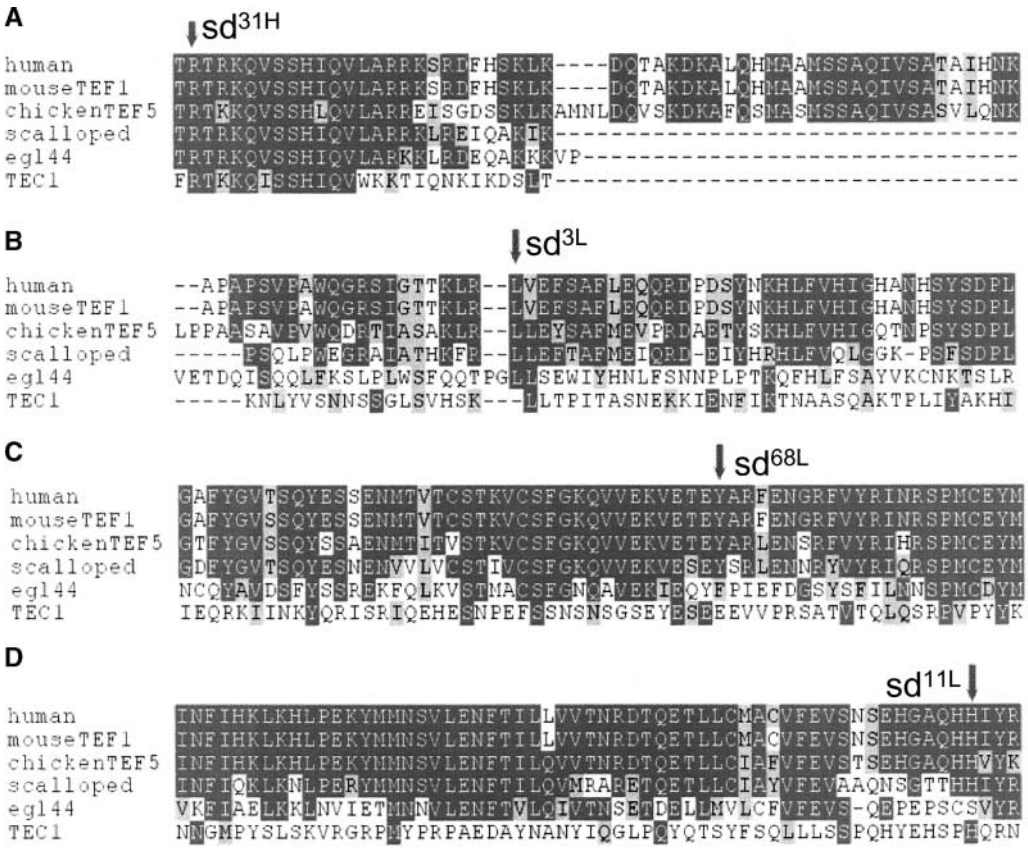

FIGURE 4.—Evolutionary conservation of the residues affected by various *sd* lethal alleles. (A–D) Portions of the wild-type Drosophila SD sequence aligned with homologs from human TEF-1, mouse TEF-1, chicken TEF-5, *Caenorhabditis elegans egl-44*, and yeast TEC-1 showing the sequence identity in dark gray and similarity in light gray. The amino acid affected in each lethal *sd* allele is marked by an arrow over the respective wild-type amino acid and the respective *sd* allele is designated beside the arrow. Wild-type amino acid is mutated (A) in sd^{31H} from an arginine to lysine, (B) in sd^{3L} from a leucine to a stop codon, (C) in sd^{68L} from a tyrosine to an asparagine, and (D) in sd^{11L} from a histidine to a leucine. In addition to the possibility of a splicing defect in sd^{47M}, 5/7 of the deleted amino acids (residue positions 248–254) are also highly conserved across phyla.

has no recognized nuclear localization signal. A VG protein lacking the SD interaction domain fails to enter the nucleus, suggesting that the binding of VG to SD is necessary for VG to get into the nucleus (SIMMONDS *et al.* 1998). More recently, it was shown that the VG/SD complex likely uses a putative nuclear localization signal (NLS) contained within the SD TEA domain for this function (SRIVASTAVA *et al.* 2002). To assess whether some of the wing noncomplementing alleles may have lesions that affect the VBD, VG localization was examined in mutant wing discs from larvae carrying each of the *sd* pupal lethal alleles (sd^{31H}, sd^{68L}, and sd^{11L}). Because *sd* is on the X chromosome, 50% of the male larvae from the relevant crosses are mutant and easily identified using a *y* marker. If an allele affects the VBD, the prediction is that VG would be predominantly cytoplasmic in wing discs and nuclear if it does not affect the VBD. The data generally support these predictions, as discs from larvae carrying the sd^{68L} allele show diffuse VG localization that is partially nuclear and partially cytoplasmic (Figure 5, D–E″). There is variability in this localization in the sense that in some discs the VG protein appears very diffuse and cytoplasmic while in the others it appears that some VG protein could be in the nucleus. On the other hand, sequence analysis of the sd^{11L} allele indicates that the lesion is unlikely to affect the VBD since it is very near the C terminus of the protein. Thus, not surprisingly, VG localization in discs from larvae harboring this allele is punctate and nuclear. This is comparable to VG localization in wild-type discs (compare Figure 5C″ to the wild-type VG localization in Figure 5A″ and to VG localization in sd^{68L} in Figure 5, D″ and E″). Further support for the VBD being affected in *sd* wing noncomplementing lethal alleles comes from the fact that mitotic clones of the sd^{47M} allele in wing discs exhibit diffuse localization of VG (HALDER *et al.* 1998) in accordance with the proposal that the lesion in this allele affects the VBD.

While the above data on VG localization in an sd^{68L} background lend support to the notion that this allele affects the VBD of SD, the partial mislocalization results could also result from other causes. Therefore, it was necessary to test directly whether or not the SD protein from sd^{68L} was defective in binding to VG. A GST pull-down experiment was performed (Figure 6) comparing wild-type SD protein and SD from sd^{68L} larvae with respect to their relative ability to bind VG as well as SD. Although these data are not quantitative and could easily mask important kinetic differences in binding, the results indicate that under the *in vitro* conditions used the mutant SD^{68L} protein appears to bind VG as well as wild-type SD can. Further, the results also show that the two SD proteins can also self-bind to wild-type SD protein under these conditions but not to luciferase. Notably, SD appears to have significant affinity for GST-tagged SD and SD^{68L} protein in this assay, compared to

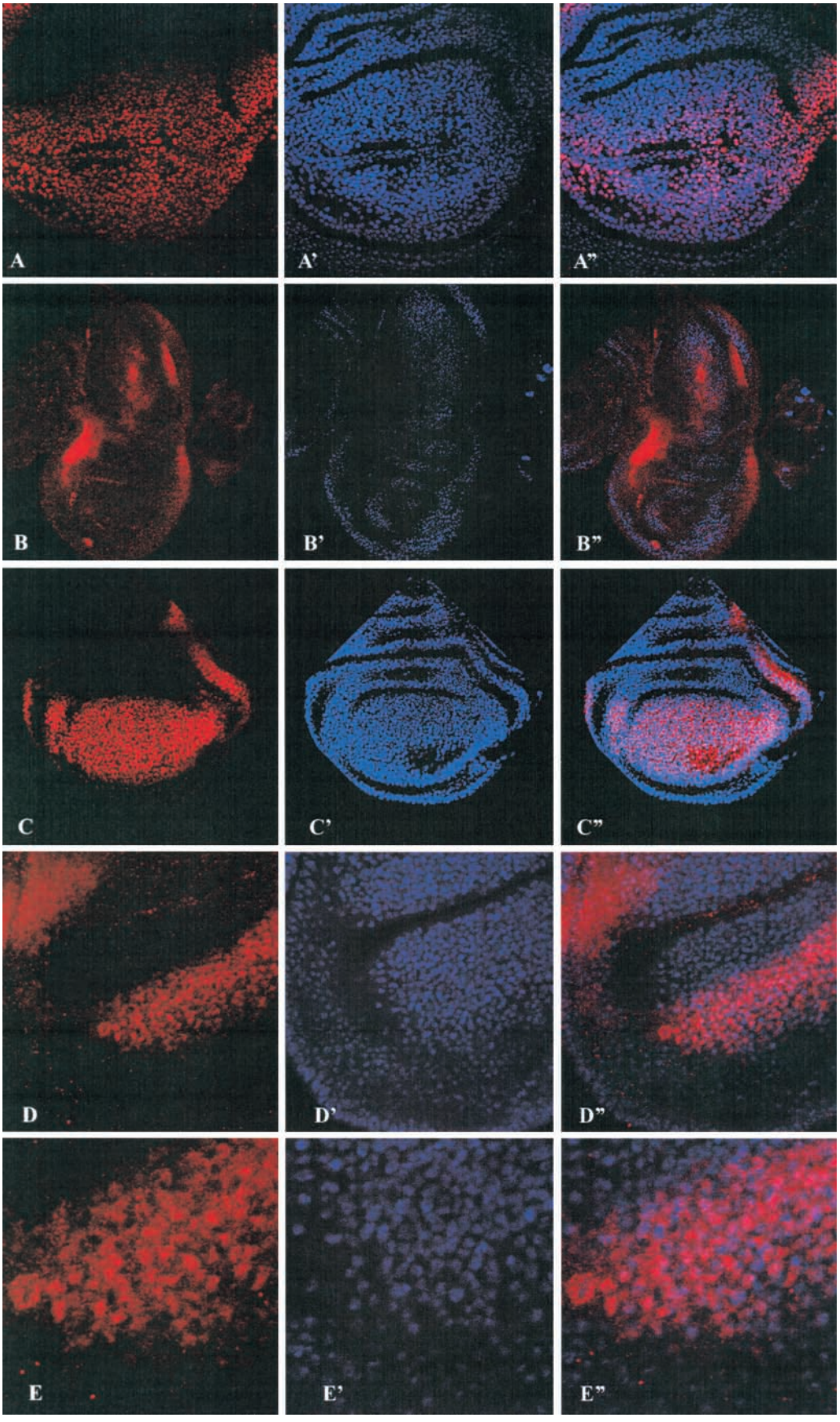

FIGURE 5.—VG localization in wing discs from various pupal lethal allelic backgrounds. The wing discs are derived from larvae harboring various pupal lethal *sd* alleles and stained for VG protein (red channel). Nuclei are stained with 4′,6-diamidino-2-phenylindole, which gives off a blue fluorescence (marked with a prime in all genotypes), and the merge between the two channels is shown with a double prime. (A–A″) Wild-type wing disc exhibiting the nuclear localization of VG. (B–B″) Wing disc derived from an sd^{31H} hemizygous larva. This disc is at a magnification different from the others to highlight the fact that the hinge and notum expression of VG is not affected, even though the wing pouch expression of VG is absent. Note the loss of any wing pouch-specific VG localization. (C–C″) Wing disc from an sd^{11L} hemizygous larva showing the nuclear localization of VG as judged by the punctate pattern and the magenta color in the merge. (D–D″) Wing disc from an sd^{68L} hemizygous larva showing the diffuse and cytoplasmic localization of VG, seen more clearly in the magnified regions shown in E–E″.

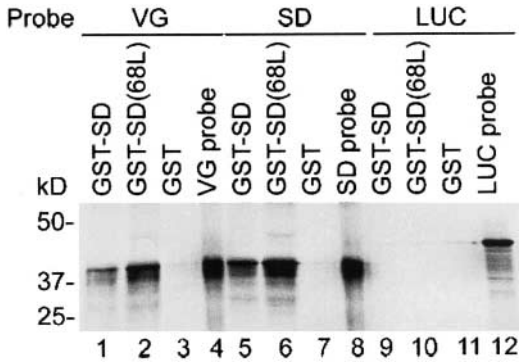

FIGURE 6.—VG binds to both SD and SD^{68L} in a GST-pull-down assay. Lanes 1–3 contain: (1) GST-SD, (2) GST-SD^{68L}, and (3) GST probed with labeled VG protein. VG binds to bacterially expressed wild-type SD and mutant SD^{68L} proteins but not to GST alone. Lane 4 contains 1/100 dilution of the radiolabeled VG protein used in the pull-down assays in lanes 1–3. Lanes 5–8 contain: (5) GST-SD, (6) GST-SD^{68L}, and (7) GST probed with labeled SD protein. SD binds to bacterially expressed wild-type SD and mutant SD^{68L} proteins but not to GST alone. Lane 8 contains 1/100 dilution of the radiolabeled SD protein used in lanes 5–7. Lanes 9–11 are controls using (9) GST-SD, (10) GST-SD^{68L}, and (11) GST probed with radiolabeled luciferase (Luc) protein. The luciferase does not bind to either of the test proteins or to GST alone. Lane 12 contains 1/100 dilution of the radiolabeled Luc protein used in lanes 9–11.

previous assays using 6xHIS or immobilized SD protein (SIMMONDS *et al.* 1998). Thus, the inefficient *in vivo* nuclear localization of VG in an sd^{68L} background does not appear to be due to any defect in the ability of that mutant SD to bind VG, at least as assessed by an *in vitro* GST pull-down assay.

The lesion associated with the sd^{31H} allele affects the TEA DNA-binding domain and it also appears to be very close to a previously reported NLS (SRIVASTAVA *et al.* 2002), as well as a putative phosphorylation site (underlined in Figure 3). Thus, it is possible that this mutation also affects the NLS. It has been reported that this NLS is likely utilized by VG to get into the nucleus (SRIVASTAVA *et al.* 2002) by virtue of its ability to bind SD. To test whether the sd^{31H} mutation affects the NLS, nuclear localization of VG was examined in discs derived from sd^{31H} larvae. If this lesion affects some aspect of the nuclear localization signal, then VG in sd^{31H}-derived wing discs should be diffuse and cytoplasmic. In fact, there is no detectable VG within the wing pouch of these discs even though VG protein is still seen in the hinge and notum areas (Figure 5, B–B″). In addition, the wing pouch appears to be reduced in size. This could simply be because sd^{31H} may compromise the SD/VG interaction more severely than the other *sd* lethal alleles characterized herein.

DISCUSSION

Four of the five lethal alleles studied affect the wing phenotype and the physical lesions associated with three of these four are within the C-terminal half of SD and are localized between amino acids 232 and 355. Two of these mutations help define a VBD *in vivo* that overlaps a domain previously predicted by *in vitro* experiments to be responsible for binding VG (VAUDIN *et al.* 1999). The sd^{3L} and sd^{47M} lesions are within this predicted domain and, due to the molecular nature of these mutations, are predicted to abolish the VBD completely. The sd^{68L} lesion is located just outside and 3′ to this domain but also affects VG localization *in vivo* to some extent. This conclusion is supported by the observation that the wing phenotype produced from these two alleles when heterozygous with sd^{ETX4} is more severe than the sd^{68L}/sd^{ETX4} phenotype (Figure 2, C–E compared to F). Since the lesions in sd^{3L} and sd^{47M} would also be expected to abolish all aspects of SD function C terminal to the respective lesion, this could also account for the early recessive lethal phenotype of these two alleles. The sd^{68L} lesion is only a missense mutation so it would not be surprising if some *sd* function is retained. Although the sd^{68L} lesion is located just outside and 3′ to the predicted VBD (Figure 3), it is associated with the mutation of tyrosine, an amino acid that is often subject to phosphorylation and dephosphorylation. Phosphorylation-based mechanisms are known to play a role in a great many interactions between proteins (DARNELL 1997). For example, the sd^{68L} lesion is in the vicinity of a domain where phosphorylation is known to modulate RTEF-1 function in cardiac muscle (UEYAMA *et al.* 2000). Therefore, it initially seemed that the simplest interpretation of the results for the sd^{68L} allele was that it also directly affected the VBD. However, given the results presented in Figure 6, this now seems unlikely. This partial mislocalization of VG could also result from reduced SD levels due to protein instability or even from mislocalization of SD itself. Figure 5 data are *in vivo* results and Figure 6 data are *in vitro* results, so it is also possible that other protein factors present *in vivo* are important in regulating the kinetics of SD binding to VG or play a vital role in SD stability. In an $sd^{68L}/+$ heterozygote, regulation of this binding or SD stability could be inefficient and result in the observed variability of the wing complementation phenotype with this allele. The wing phenotype of sd^{68L} *in trans* with the more severe but phenotypically stable sd^{58} allele is nonvariable, but still less severe than that produced by sd^{3L} or sd^{47M} over sd^{58d} (results not shown). Thus, it appears that the sd^{68L} allele provides some wild-type function, with respect to wing development, in a genetic background shared with the sd^{ETX4} or sd^{58} alleles. However, since sd^{68L} is a recessive lethal, the lesion also compromises some as yet unknown vital function as well. The most likely reason for complementation of the wing phenotype with sd^{11L} is that the lesion does not affect the VBD because it is more distally located: 78 amino acids from the sd^{68L} mutation and only eight residues from the C-terminal end of SD (Figure 3). Moreover, VAUDIN *et al.* (1999) have reported that the TEF-1 sequence (SD homolog) from residue 329 to the

C terminus is dispensable with respect to its ability to interact with the TDU protein (VG homolog) even though high sequence conservation exists throughout this region. Further support for the mutations in sd^{3L} and sd^{47M} affecting the VBD and sd^{11L} not affecting this domain comes from observing VG localization data from wing discs derived from sd^{11L} hemizygous larvae (Figure 5C″) as well as from VG localization in mitotic clones of the sd^{47M} allele. VG localization in sd^{68L} wing discs (Figure 5) and in *sd* mutant clones harboring the sd^{47M} allele (Halder *et al.* 1998) is diffuse rather than nuclear. This is a clear indication that the VBD (in sd^{47L}) or a related role (as in sd^{68L}) must not be fully functional, even though our *in vitro* data indicate that sd^{68L} is not defective in binding VG. However, VG in sd^{11L} wing discs is entirely nuclear, supporting the conclusion that in this allele the VBD is unaffected, while still implicating the region in an alternative function that is essential for viability.

To date, our knowledge about the TEA DNA-binding domain has been based primarily on *in vitro* mutational analysis (Burglin 1991; Hwang *et al.* 1993; Berger *et al.* 1996; Jiang *et al.* 2001). However, extrapolation from *in vitro* observations to *in vivo* functions is not always valid. We have identified a mutation within the conserved TEA DNA-binding domain that affects both the essential and the wing-specific functions of *sd*. The TEA DNA-binding domain has been previously predicted to have three α-helices. However, the limits of the third helix within the domain are not very well defined (Burglin 1991). The mutation associated with the sd^{31H} allele (Arg to Lys) is located in the TEA DNA-binding domain between the second and third predicted helices (Figure 3). This lesion also lies between two putative phosphorylation sites, and the role of phosphorylation in regulation of DNA binding by the TEA domain from organisms other than Drosophila has been well documented (Gupta *et al.* 2000; Jiang *et al.* 2001). The cause of the observed heterozygous wing and homozygous lethal phenotypes associated with this allele can be explained in at least two ways. The phenotypes could simply be the result of a defect induced by this mutation in the regulation of DNA binding by phosphorylation. The second and the third helices of the TEA DNA-binding domain may actually be responsible for contacting the DNA (Burglin 1991) so this mutation could directly affect the ability of SD to contact DNA, thereby preventing transcription of essential and wing-specific genes controlled by *sd*. Alternatively, it is also possible that the mutation in sd^{31H} affects the nuclear localization signal that overlaps the TEA DNA-binding domain of SD (Srivastava *et al.* 2002), so that SD is prevented from entering the nucleus. In the absence of an SD antibody, we cannot determine if the mutation prevents SD from entering the nucleus or simply results in inefficient binding of the protein to its targets in the nucleus. Either of the above putative defects could explain the recessive lethality caused by sd^{31H}. The late pupal lethality associated with this allele is consistent with an argument that this mutation results in inefficient transport of SD to the nucleus. The mutant animal is able to survive until the pupal stage, beyond which the level of SD in the nucleus would be unable to sustain the level of transcription needed for survival. However, VG localization data from sd^{31H} mutant discs argue against a defect in nuclear localization of SD. Because SD is needed for maintenance of *vg* and *sd* expression (Halder *et al.* 1998; Simmonds *et al.* 1998), one would expect to see some VG in the wing pouch of the mutant discs if the mutation was simply causing inefficient nuclear localization of SD. The absence of any noticeable VG in the wing pouch (Figure 5, B–B″) favors the hypothesis that the mutation affects the DNA-binding ability of the TEA domain. So, this mutant SD would then not bind its cognate regulatory DNA elements and, as a result, VG expression in the wing pouch would not be maintained. It is also possible that the absence of VG in the wing pouch area of the sd^{31H} disc is merely a consequence of autoregulation in this system.

While we have identified specific lesions in each of the *sd* lethal alleles and have been able to correlate these with phenotypic consequences for the organism, we have not fully solved the molecular reason for lethality in every case. Since sd^{31H} has a lesion in the TEA domain it is relatively easy to understand why this may result in lethality. Similarly, the lethality associated with sd^{3L} and sd^{47M} is explicable because of the molecular nature of the lesions, in that all SD function downstream of the respective lesion would be abolished. The reasons for the lethality associated with sd^{68L} and sd^{11L} are still not obvious. We have provided evidence that the lesion in sd^{68L} affects wing development and also compromises a vital function, while sd^{11L} does not appear to affect wing development but does compromise a vital function. The current hypothesis is that these two *sd* lethal alleles likely affect residues within a domain that is necessary for binding cofactors involved in other critical developmental functions of SD. Future efforts will concentrate on attempting to identify these putative cofactors.

The data presented herein are relevant to several aspects of SD function. We have reported for the first time the molecular characterization of lethal alleles of *sd* and this analysis has enabled us to associate specific conserved residues within the SD protein sequence with specific mutant phenotypes. The results have helped to define a VBD in SD by *in vivo* criteria. We also show for the first time that a mutation within the SD-TEA DNA-binding domain is important for both wing development and viability of the fly. Because the residues affected in the *sd* lethal alleles are conserved across species and phyla, this study could also have important implications for understanding the properties of the vertebrate homolog TEF-1.

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