Multiple signaling pathways and a selector protein sequentially regulate *Drosophila* wing development

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Summary

***Drosophila* wing development is a useful model to study organogenesis, which requires the input of selector genes that specify the identity of various morphogenetic fields (Weatherbee, S. D. and Carroll, S. B. (1999) *Cell* 97, 283-286) and cell signaling molecules. In order to understand how the integration of multiple signaling pathways and selector proteins can be achieved during wing development, we studied the regulatory network that controls the expression of Serrate (Ser), a ligand for the Notch (N) signaling pathway, which is essential for the development of the *Drosophila* wing, as well as vertebrate limbs. Here, we show that a 794 bp cis-regulatory element located in the 3′ region of the *Ser* gene can recapitulate the dynamic patterns of endogenous *Ser* expression during wing development. Using this enhancer element, we demonstrate that Apterous (Ap, a selector protein), and the Notch and Wingless (Wg) signaling pathways, can sequentially control wing development through direct regulation of *Ser* expression in early, mid and late third instar stages, respectively. In addition, we show that later *Ser* expression in the presumptive vein cells is controlled by the Egfr pathway. Thus, a *cis*-regulatory element is sequentially regulated by multiple signaling pathways and a selector protein during *Drosophila* wing development. Such a mechanism is possibly conserved in the appendage outgrowth of other arthropods and vertebrates.**

Key words: *Drosophila*, Serrate, Notch, Apterous, Wing development

Introduction

Organogenesis requires the input of both cell signaling pathways and tissue specific selectors in multicellular organisms (Curtiss et al., 2002). Seven major cell-cell signaling pathways – Notch (N), Hedgehog (Hh), Wnt, receptor tyrosine kinases (RTKs), Tgfβ, JAK/STAT and nuclear receptors – regulate the majority of cell fates during animal development by activating specific target genes (Barolo and Posakony, 2002; Gerhart, 1999). For example, N signaling determines wing cell fate by upregulating *vestigial* (*vg*) during *Drosophila* development (Kim et al., 1996). However, cell fate decisions also require the input from selector proteins. For example, Vg is a selector protein that specifies wing cell fate during larval development (Kim et al., 1996). How selector and signaling molecules collaborate to promote appropriate organ development remains unresolved. Recent evidence shows that a selector protein complex, Vestigial-Scalloped (Vg-Sd), and various signaling pathways regulate the *Drosophila* wing target genes in a cooperative manner, providing a possible mechanism by which a selector protein and cell signaling pathways integrate to regulate spatial expression of tissue-specific enhancers (Guss et al., 2001). However, such a mechanism cannot easily explain the dynamic expression of many tissue-specific genes, such as *Ser*, which may additionally require temporal regulation by selectors and signaling cascades.

The *Drosophila* wing imaginal disc has provided one of the best experimental systems for studying the general principles of organogenesis. Patterning of the *Drosophila* wing imaginal disc is coordinated by organizers localized in the dorsoventral (DV) and anteroposterior (AP) boundaries (reviewed by Brook et al., 1996). Long-range signaling molecules, Wingless (Wg; the vertebrate homolog of which is Wnt) and Decapentaplegic (Dpp; the vertebrate homolog of which is Tgfβ), are induced by N signaling in the DV organizer, and by Hh signaling in the AP organizer, respectively. Wg and Dpp form gradients to regulate target genes in a dose-dependent fashion, thereby providing correct spatial information during development (Entchev et al., 2000; Strigini and Cohen, 2000; Teleman and Cohen, 2000). The organizers formed between the DV and the AP compartments also serve as barriers to prevent cells of different compartments from intermingling, thus preventing disorganized pattern formation (Milan et al., 2001). Both the N and Hh signaling pathways play important roles in building up compartment barriers (Dahmann and Basler, 2000; Micchelli and Blair, 1999; Rauskolb et al., 1999).

Apterous (Ap) is an essential selector protein in *Drosophila* wing development. It is expressed in the dorsal compartment of the wing disc, thereby conferring dorsal identity (Diaz-Benjumea and Cohen, 1993). Chicken Lmx1, a protein similar to Ap, is also expressed in the dorsal part of the limb bud. Lack

of Lmx1 or mouse Lmx1b leads to double ventral limbs, suggesting a conserved function of Ap homologs in specifying dorsal appendage development (Chen et al., 1998; Riddle et al., 1995; Vogel et al., 1995). In addition to its ability to specify dorsal identity, Ap is also required for growth and DV compartmentalization in flies, where it functions upstream of the N pathway, as N pathway activation is sufficient to rescue the growth defect of Ap mutants (Milan and Cohen, 1999a; O'Keefe and Thomas, 2001). Although it is generally agreed that N signaling plays an important role in DV compartmentalization, other unidentified molecules downstream of Ap may also participate (reviewed by Irvine and Rauskolb, 2001).

The ligands Serrate (Ser) and Delta (Dl) activate the N pathway at the developing DV boundary of the *Drosophila* wing. This activation is mediated by Fringe (Fng), which is expressed in the dorsal compartment, and which glycosylates N to inhibit its responsiveness to Ser dorsally, while potentiating its ability to respond to Dl in ventral cells. (Fleming et al., 1997; Moloney et al., 2000; Rauskolb et al., 1999). Indeed, the Ser-Fng-N signaling pathway is evolutionarily conserved in appendage development between insects and vertebrates (Rodriguez-Esteban et al., 1997). The vertebrate homologs of Ser, Fng and N are important for the outgrowth of the limb bud, as indicated by both functional analysis and their expression patterns in the apical ectodermal ridge (AER), a structure similar to the *Drosophila* DV border (Rodriguez-Esteban et al., 1997). Molecules involved in the establishment of proximodistal (PD) and AP polarities are also highly conserved, suggesting that arthropod and vertebrate appendages may use similar genetic circuitry to control their outgrowth (Shubin et al., 1997).

Here, we report the identification of a *Drosophila* wing enhancer at the *Ser* locus, which can be sequentially activated by selector and multiple signaling molecules during wing development. We show that *Ser* is temporally regulated by Ap, N, Wg and Egfr signals, and that the *Ser* enhancer can serve as a direct integration module for this selector protein and the extracellular signaling molecules. Our results suggest a possible mechanism by which selector(s) and signaling pathway(s) act in a sequential fashion to control the outgrowth of arthropod and vertebrate appendages.

Materials and methods

Transgene construction for functional rescue and expression studies

Constructs in Fig. 1 were constructed from *Ser* genomic DNA isolated by screening a *Drosophila melanogaster* λ phage library. Constructs 1-7 were constructed from pCaSpeR-hsGal4 or pCaSperR-Gal4. pCaSpeR-hsGal4 was adapted from pCaSpeR-hs (Thummel et al., 1988) by inserting a *Not*I/*Bam*HI fragment of the Gal4 coding sequence and the 3′ hsp70 polyadenylation signal from pGaTB (Brand and Perrimon, 1993). pCaSperR-Gal4 was modified from pCaspeR-hsGal4 by deleting the hsp70 promoter between the *Eco*RI and *Xho*I sites. Constructs 1 and 2 include 5 kb and 4 kb *Bam*HI fragments, respectively, inserted into the *Bam*HI site downstream of *Gal4* of pCaSperR-hsGal4. Construct 3 includes a 7.4 kb *Eco*RI/*Xba*I fragment inserted into the *Bam*HI site upstream of *Gal4* of pCaSperR-Gal4. Construct 4 includes an 8 kb *Bam*HI fragment inserted into the *Bgl*II site downstream of *Gal4* of pCaSperR-Gal4. Constructs 5, 6, and 7 were adapted from construct 3 by inserting 8 kb *Bam*HI, 5 kb *Bam*HI/*Hin*dIII, and 2.7 kb *Bam*HI/*Hin*dIII fragments, respectively, into the *Bgl*II site downstream of *Gal4*. pCaSpeR-hsp70-AUG-βgal was adapted from pCaSpeR-AUG-βgal (Thummel et al., 1988) by inserting a PCR fragment of a minimal hsp70 promoter at the *Kpn*I site. Constructs 8, 9 and 10 include 2.7 kb, 1.8 kb and 0.8 kb PCR fragments, respectively, inserted into pCaSper-hsp70-AUG-βgal between the *Bam*HI and *Eco*RI sites. PCR-based mutagenesis was used on the Ap, Su(H), and dTCF binding motifs. The primers used were as follows (corresponding sites in parentheses and mutated bases shown in small letters; *Eco*RI and *Bam*HI cloning sites are underlined):

mAp (A-E) sense *Eco*RI, 5′-AGAATTCCAAACGGATGGCGCATTtTTACTTTTCGAAGTGCTTttTCATAAGGTAaAATTCCAAATAAaATTTGGAGGACTTGGaAAaCAGAAC-3′;

mAp (F) sense, 5′-TTGAAATATGCGCTAtTTGAATGTG-3′;

mAp (F) antisense, 5′-CACATTCAAaTAGCGCATATTTCAA-3′;

mAp (G) sense, 5′-TTGTTCGGTTTCTAAaaAAAATACC-3′;

mAp (G) antisense, 5′-GGTATTTTttTTAGAAACCGAACAA-3′;

mAp (H-J) sense, 5′-GTGTGTTtTTttTaAAaaAGAAAATGCCAGGAATTTTCGCTTttTTGTGG-3′;

mAp (H-J) antisense, 5′-CCACAAaaAAGCGAAAATTCCTGGCATTTTCttTTtTAaaAAaAACACAC-3′;

mAp (K-N) antisense *Bam*HI, 5′-CGGGATCCAAGCTTAAaAATCTACGtTTGGGATTcTTGTAAGTTGTTTttTGAAA-3′;

mSu(H) sense, 5′-TTGAATaTaAAAACCAAATGCAAGTTaGaAACTAC-3′;

mSu(H) antisense, 5′-GTAGTTtCtAACTTGCATTTGGTTTTtAtATTCAA-3′;

mdTCF (A-B) sense, 5′-GTCCATCTggaGATGTAAGGCAGCTCTGGACTTttGGttTtTTTTTTttTtAGCATC-3′;

mdTCF (A-B) antisense, 5′-GATGCTaAaaaAAAAAAaAaaCCaAAAGTCCAGAGCTGCCTTACATCtccAGATGGA-3′;

mdTCF (C-D) sense, 5′-ATCGCAggaGAAATATGCGCTAggaGAATG-3′;

mdTCF (C-D) antisense, 5′-CATTCtccTAGCGCATATTTCtccTGCGAT-3′;

mdTCF (E-F) sense: 5′-AATACCtccAAGAGCTGGCCGAAACACACAAAACTAAGCTCAGCGGATGATCtccGCCCG-3′;

mdTCF (E-F) antisense, 5′-CGGGCggaGATCATCCGCTGAGCTTAGTTTTGTGTGTTTCGGCCAGCTCTTggaGGTATT-3′;

mdTCF (G-H) sense, 5′-GCGTAAAaaagGaaAaaGGTAAAaaCGGCTCGAACtccACGAAA-3′;

mdTCF (G-H) antisense, 5′-TTTCGTggaGTTCGAGCCGTTTTTACCttTttCctttTTTACGC-3′;

mdTCF (I) antisense *Bam*HI, 5′-CGGGATCCAAGCTTAATAATCTACGATTGGGATTATTaTAAGTTGTTTAATGAAATGTGTggaGATCTT-3′;

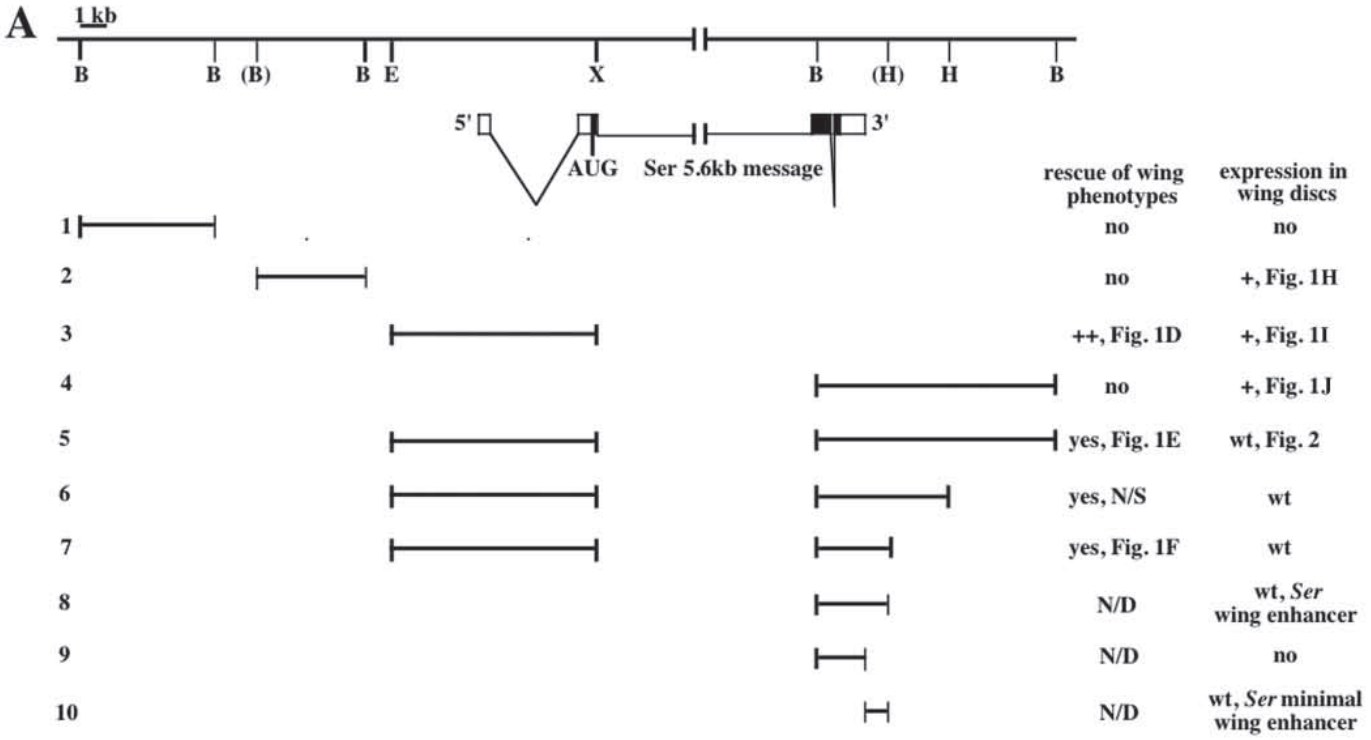
Ser-lacZ sense *Eco*RI, 5′-AGAATTCCAAACGGATGGCGCA-3′; and

Ser-lacZ antisense *Bam*HI, 5′-TCCTGTGAATCCTCCCAACTTGCATTTGGT-3′.

Mutant *Ser* enhancer constructs were cloned into pCaSper-hsp70-AUG-βgal between the *Bam*HI and *Eco*RI sites. All constructs were verified by DNA sequencing before being introduced into w^{1118} flies by standard methods of P element-mediated transformation (Spradling, 1986). At least three independent lines were analyzed for each construct.

In situ hybridization, immunostaining and X-Gal staining

In situ hybridization was performed as described by Fleming et al. (Fleming et al., 1990) with modifications, including the use of a digoxigenin-UTP-labeled *Ser* RNA probe (Boehringer Mannheim), omission of proteinase K treatment, and a hybridization temperature of 55°C. The following primary antibodies were used: monoclonal mouse anti-βgal (1:1000, Promega), rabbit anti-βgal (1:4000, Cappel), rat anti-Ser (1:1000, provided by K. Irvine) and monoclonal mouse anti-Dl Mab202 (1:250, provided by M. Muskavitch). The following



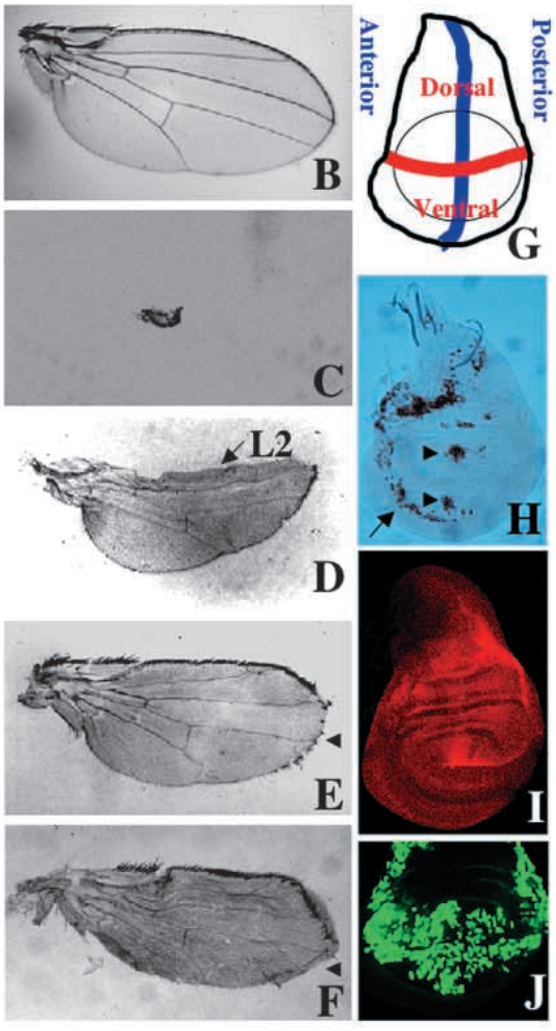


Fig. 1. Identification of the *Ser* minimal wing enhancer. (A) Molecular map of the *Ser* region. Top horizontal line shows a restriction map of the *Ser* locus. B, *Bam*HI; E, *Eco*RI; H, *Hin*dIII; X, *Xba*I. Polymorphic restriction sites are placed in parentheses. White boxes represent UTRs; black boxes represent exons in the transcription unit. For simplicity, the middle regions of the restriction map and the *Ser* transcript are omitted. The rescue experiments were carried out by expressing UAS-*Ser* under the control of Gal4 fused to constructs 1 to 7 in a *Ser* mutant (Bd^{G}/Ser^{+r83k}) background. The rescue efficiencies are indicated on the right (++, partial rescue; N/S, not shown; N/D, not determined). (B-F) Adult wing cuticle preparation; (G) schematic representation of the wing imaginal disc; (H,I) β-galactosidase antibody staining; (J) GFP expression in late third instar wing imaginal discs. Dorsal is up, anterior to the left for all imaginal wing discs in this paper, if not indicated otherwise. (C) Bd^{G}/Ser^{+r83k} animals display little wing tissue compared with wild type (B). (D) *Ser* expression under construct 3 can partially restore the wing from the posterior to around L2 (L, longitudinal vein) in Bd^{G}/Ser^{+r83k} animals. Constructs 5 and 7 can fully rescue the wing phenotype with margin defects (arrowheads in E and F, respectively), which is identical to the phenotype when UAS-*Ser* is expressed under constructs 5 and 7 in a wild-type background (not shown). (G) The DV border (red band) is located between the dorsal and ventral compartments; the AP border (blue band) is located between the anterior and posterior compartments. The wing pouch, which gives rise to the adult wing blade, is demarcated by an oval. (H) UAS-*nuc-lacZ* expression under construct 2 is observed near the AP border (arrowheads) as well as in the pleura (arrow). (I) UAS-*lacZ* under construct 3 is expressed exclusively in the dorsal compartment, and mostly in the posterior. (J) UAS-*GFP* expression under construct 4 was detected at the DV border and in the cells flanking the DV border. Construct 5 recapitulates endogenous *Ser* expression during larval development (see Fig. 2 for details).

secondary antibodies were used: goat anti-mouse TR, donkey anti-mouse TR, donkey anti-rabbit Cy5 (1:250, Jackson Immunological Laboratories) and goat anti-mouse HRP (1:250, Promega). All discs were dissected in PBS, fixed in 4% paraformaldehyde/PBS for 10 minutes, and rinsed four times in PBT (0.3% Triton X-100/PBS). They were then incubated at 4°C overnight with primary antibodies in 5% normal goat or donkey serum/PBT (depending on the choice of secondary antibodies). The discs were washed three times in PBT for 20 minutes, and then incubated at room temperature for 2 hours, or at 4°C overnight, with secondary antibodies in 5% normal goat or donkey serum/PBT. The discs were then washed three times in PBT for 20 minutes, further dissected, and mounted in 2%DABCO/70% glycerol. All steps were performed at room temperature except those mentioned specifically. HRP detection was performed by standard protocols. Fluorescent images were obtained using a Leica confocal microscope. X-Gal staining was performed as described (O'Kane, 1998) with the following modifications. Larvae were dissected in PBS, fixed in 4% paraformaldehyde/PBS for 3 minutes and stained in an Eppendorf Thermomixer at 600 rpm. *Ser-lacZ* middle and late third instar discs were stained for 12 minutes at 37°C; *Ser-lacZ* early third instar discs, all *(mAp)Ser-lacZ* and *(mdTCF)Ser-lacZ* discs were stained at 37°C overnight.

Genetics and phenotypic examination

The Gal4/UAS system (Brand and Perrimon, 1993) was used for the following experiments. To rescue the Bd^G/Ser^{+r83k} mutant wing phenotype, we crossed transgenic flies carrying constructs 1-7 in a $Bd^G/TM6B$, *Tb* background to *UAS-Ser/UAS-Ser*; Ser^{+r83k}/Ser^{+r83k} flies. Experimental flies died in the late pupal stage, and the adult wings were dissected out of non-Tubby pupae and examined. To study expression patterns of constructs 1 to 7, the following fly stocks were used: UAS-*nuc-lacZ*, UAS-*lacZ*, UAS-*GFP* and *ap-lacZ*. To study constructs 8 and 10, the following fly stocks were used: dpp-Gal4, ptc-Gal4, en-Gal4 (e116e), UAS-*ChAp* (Milan and Cohen, 1999b), UAS-*dLMO* (Milan and Cohen, 1999b), UAS-N^i (provided by S. Artavanis-Tsakonas), UAS-arm^{S10} (Pai et al., 1997) and UAS-*DN-TCF* (van de Wetering et al., 1997). Ectopic expression was also achieved using a flip-out technique under control of the *actin* promoter (Ito et al., 1997). *hs-flp* was used to generate the random clones; heat shock was performed in a water bath (30 minutes at 37°C) in late second to early third instar. The clones were marked by the presence of GFP. To study *Ser* mRNA expression, the following stocks were used: w^{1118}, UAS-*rho** (Xiao et al., 1996) and ve^1vn^1 (rho^1vn^1) (de Celis et al., 1997). For separating third instar larvae into early, middle and late stages, second instar larvae (with closed openings at the end of anterior spiracles) were collected and transferred to apple juice plates. The third instar larvae were selected and transferred to new apple juice plates every hour, and staged (as hours after the second/third instar (L2/L3) molt) by measuring the incubation time of the third instar larvae at 25°C. The beginning of the third instar was characterized by the presence of finger-like anterior spiracles, and molting (Bodenstein, 1994). Each period of the third instar early, middle and late lasts for about 24 hours (0-24 hours, 25-48 hours and 49-72 hours after the L2/L3 molt, respectively).

DNase I footprinting and electrophoretic mobility shift assays

Footprinting assays were performed using the Core Footprinting System (Promega) with a minor modification in preparation of the probes. The 794 bp *Ser* minimal enhancer was divided into two overlapping fragments (75 bp overlap) by PCR, and cloned into pBluescript II SK+ (Stratagene). To generate single-end-labeled probes, DNA fragments were amplified by PCR with 5′-phosphorylated T7 or T3 primers. Only one unphosphorylated 5′ end of the PCR DNA fragments could be labeled with [γ-^{32}P]ATP by T4 DNA polynucleotide kinase. DNA sequencing products were labeled with α-^{35}S-ATP using Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech). 6×His-tagged ApΔLIM (Benveniste et al., 1998) and 6×His-tagged dTCF-HMG domain (Halfon et al., 2000) were purified using QIAexpressionist (Qiagen). GST-Su(H) was purified, and electrophoretic mobility shift assays were performed as described by Bailey and Pasakony (Bailey and Pasakony, 1995). The proteins were dialyzed and recovered in 27.5 mM HEPES (pH 7.5), 55 mM KCl and 5.5 mM $MgCl_2$. 1.1 mM DTT was also present in the GST-Su(H) protein mixture. One-tenth volume of glycerol was added, and aliquots were frozen in liquid nitrogen and stored at –80°C.

Results

Identification of a minimal *Ser* enhancer for wing development

We investigated *Ser* gene regulation during *Drosophila* wing disc development as a model of both temporal and spatial gene regulation during appendage development. Consistent with an essential role of *Ser* in wing outgrowth (Speicher et al., 1994), we found that *Ser* mutants heterozygous for Ser^{+r83k} (a *Ser* hypomorphic allele; R.J.F., unpublished) (Gu et al., 1995) and Bd^G [a *Ser* dominant-negative allele (Hukriede and Fleming, 1997)] developed little wing tissue (Fig. 1B,C).

To study *Ser* gene regulation during wing development, we first identified wing regulatory elements in the *Ser* gene by attaching various 5′ and 3′ flanking sequences to a yeast *Gal4* gene, and then tested their ability to rescue the Bd^G/Ser^{+r83k} mutant wing phenotype by using the Gal4/UAS system to direct expression of a *Ser* cDNA (Fig. 1A) (Brand and Perrimon, 1993). As indicated by rescue efficiencies shown in Fig. 1A, constructs 1, 2 and 4, containing sequences located far from the coding region, either 5′ or 3′, showed no rescue of the mutant phenotype. Construct 3, containing 7.4 kb of the 5′ UTR and putative promoter sequences, was able to partially rescue the Bd^G/Ser^{+r83k} mutant wing phenotype from the posterior end up to L2 (Fig. 1D). We also examined the expression patterns of a UAS-*nuc-lacZ* or UAS-*lacZ* reporter gene driven by constructs 1-4. Consistent with the rescue experiments, constructs 1 and 2 showed little or no expression in the wing disc (Fig. 1H; data not shown). Construct 3 was mostly expressed in the dorsal compartment, preferentially in the posterior region (Fig. 1I), also in line with the rescue experiment. Although construct 4, containing 8 kb of the 3′ end of the *Ser* transcript and flanking region was expressed in wing discs, its expression pattern was less defined and did not completely recapitulate the endogenous *Ser* pattern (Fig. 1J, Fig. 2). Thus, constructs 1-4, containing individual regulatory regions of 20 kb 5′ and 8 kb 3′ flanking sequences, are not sufficient to fully rescue the Bd^G/Ser^{+r83k} mutant wing phenotype.

To assess possible cooperation between the regulatory elements of 5′ and 3′ flanking regions, we tested combinations of different constructs. Construct 5, which combined both flanking sequences from constructs 3 and 4, was capable of mimicking the endogenous *Ser* expression pattern in the wing disc (Fig. 2; see below). More importantly, construct 5 was able to almost completely rescue the Bd^G/Ser^{+r83k} mutant wing phenotype, with normal size, bristles, and margin development except for minor defects in the distal margin (Fig. 1E). Several lines of evidence suggest that this margin defect phenotype was not due to the inability of construct 5 to rescue, but rather resulted from the overexpression of *Ser* in the distal

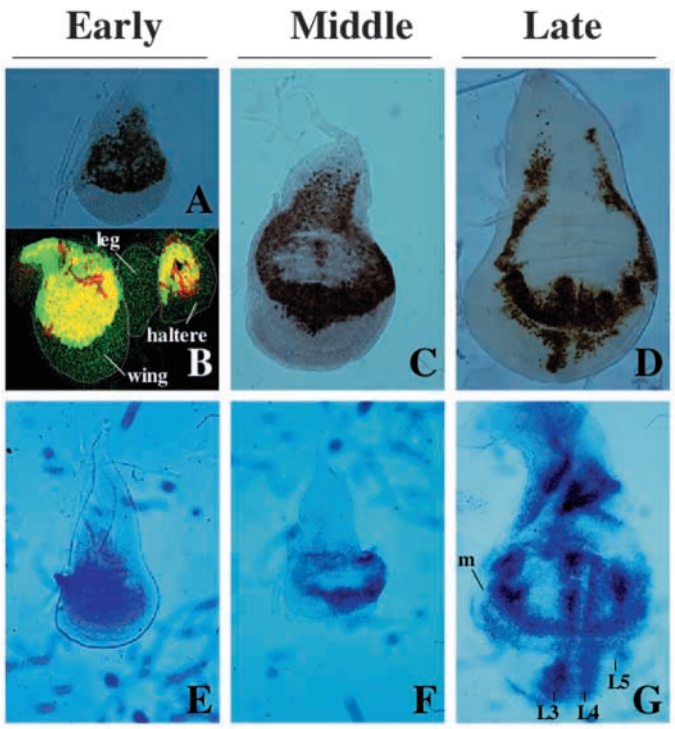


Fig. 2. Expression patterns of *Ser* and a *Ser* wing enhancer in wing imaginal discs. (A-G) Wing imaginal discs. UAS-*nuc-lacZ* expression driven by the *Ser* wing enhancer construct 5 (Gal4) was visualized by β-galactosidase antibody staining (A,C,D). These patterns are identical to those of the endogenous Ser protein (not shown). *Ser* mRNA was detected by in situ hybridization in developing wing discs (E-G). (A,B,E) *Ser* mRNA and construct 5 are expressed in dorsal cells in early third instar. (B) The expression patterns of construct 5 (Gal4)/UAS-*GFP* (green) and *apterous-lacZ* (red), are co-localized (yellow) in dorsal cells of wing and haltere discs. Dorsal is to the left for the haltere disc. White dashed circles outline the discs. (C,F) By the mid third instar, construct 5 (Gal4)/UAS-*nuc-lacZ* and Ser mRNA are preferentially expressed along the DV border. (D,G) At the end of the third instar, they are expressed in two stripes flanking the DV boundary, with higher expression dorsally. They are also expressed in presumptive veins (L3, L4 and L5). m, margin.

presumptive wing margin (the space between extensions of L3 and L4 in the DV boundary) under construct 5 control (Fig. 2C,D). First, a *Ser* mutant allele, Ser^{D}, which has a higher *Ser* expression level in the same distal presumptive margin, showed a similar distal wing-nicking phenotype (Thomas et al., 1995). Second, it has been reported that N ligands Ser and Dl inhibit N signaling cell-autonomously (Micchelli et al., 1997); the loss of N signaling would result in a margin defect. Third, the same wing margin defect was also observed when *Ser* cDNA was expressed under construct 5 control in a wild-type background (data not shown). Thus, we conclude that the combination of the 7.4 kb 5′ sequence and the 8 kb 3′ flanking region is sufficient to fully rescue the Bd^{G}/Ser^{+r83k} wing phenotype, and that wing-specific regulatory elements reside in the 5′ and 3′ flanking regions.

To determine a minimal sequence requirement in the 8 kb of the 3′ flanking region, two smaller enhancer fragments, 4 kb and 2.7 kb, respectively, were combined with the 7.4 kb 5′ flanking sequence to make constructs 6 and 7. They were then tested for their ability to rescue the Bd^{G}/Ser^{+r83k} wing phenotype and to direct *lacZ* expression in wing discs. Constructs 6 and 7 were able to rescue the Bd^{G}/Ser^{+r83k} wing phenotype as well as construct 5 (Fig. 1F); their expression patterns were also indistinguishable from that of construct 5 (data not shown). These results suggest that regulatory elements important for correct *Ser* expression during wing development reside in the 2.7 kb 3′ flanking region. This hypothesis was confirmed by a fusion of the 2.7 kb sequence and a *lacZ* reporter gene (construct 8), which recapitulated *Ser* expression patterns in wing discs (Fig. 5F). We refer to construct 8 as the *Ser* wing enhancer.

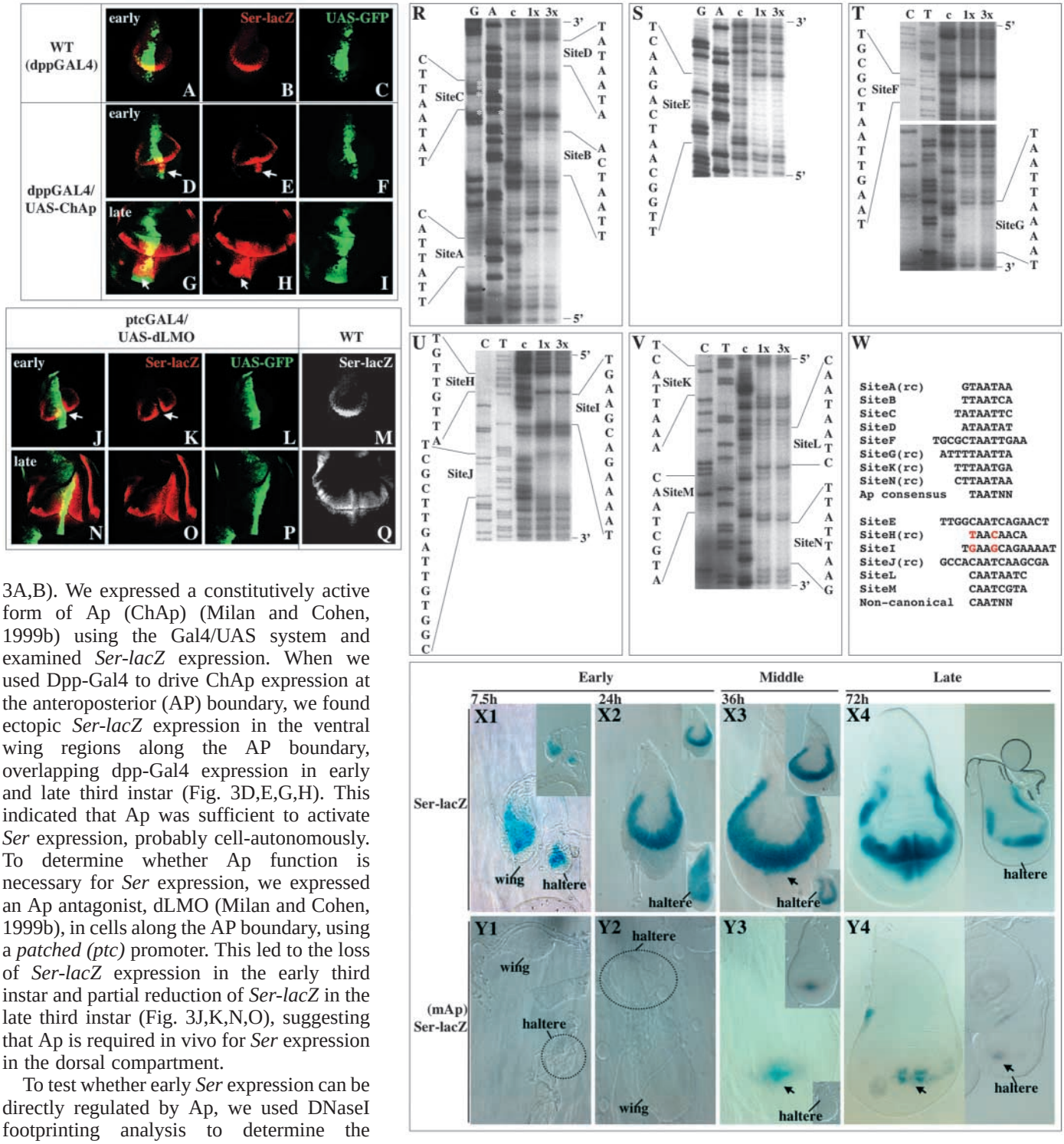
To determine the minimal enhancer sequence in this 2.7 kb region, we divided it into a 1.9 kb fragment that includes the Ser 3′ coding region and UTR (not only the UTR), and a 0.8 kb genomic sequence fragment, and attached these sequences to a *lacZ* reporter gene to make constructs 9 and 10, respectively. Construct 10 recapitulated endogenous *Ser* wing expression in the third instar (Fig. 3M,Q,X1-4), whereas construct 9 was not expressed at all in wing discs. We conclude that the 0.8 kb (794 bp) fragment of construct 10 represents a minimal wing enhancer and is henceforth referred to as the *Ser* minimal wing enhancer.

Dynamic *Ser* expression is regulated at the transcriptional level

It has been shown by immunostaining that Ser protein exhibits dynamic expression patterns in the wing disc (data not shown) (de Celis and Bray, 1997; Panin et al., 1997). To determine at what level the Ser patterns are regulated, we examined the expression patterns of *Ser* mRNA by in situ hybridization. We found that in situ hybridization detects *Ser* mRNA expression patterns in the wing comparable to immunostaining of Ser protein. *Ser* mRNA was detected exclusively in the dorsal compartment of the early third instar wing disc (Fig. 2E). By the mid third instar, *Ser* is expressed at the DV boundary (Fig. 2F). In late third instar, *Ser* is expressed in two stripes flanking the DV boundary with higher expression dorsally; *Ser* is also expressed in the presumptive veins (Fig. 2G). These results suggest that dynamic *Ser* expression patterns are regulated at the transcriptional level during wing development. Indeed, the recapitulation of *Ser* expression patterns by construct 5 is consistent with transcriptional control being the primary mechanism of *Ser* regulation.

Ap regulates *Ser* expression in early third instar

Ser is expressed in the dorsal compartment during the early stages of wing disc development (Fig. 2E). This expression pattern is identical to that of the selector gene of the dorsal compartment, *ap*, which encodes a homeodomain transcription factor (Cohen et al., 1992). It has been hypothesized that early *Ser* expression in the dorsal compartment is under the direct control of Ap (Irvine and Vogt, 1997). However, no direct evidence has been shown to support this hypothesis. To determine whether *Ser* is a direct target gene of Ap, we tested whether the 794 bp *Ser* minimal wing enhancer is regulated by Ap using both in vivo and in vitro methods. Construct 10, *Ser-lacZ* containing the 794 bp *Ser* minimal enhancer, is expressed in a stripe in the dorsal compartment flanking the DV boundary at 24 hours after the L2/L3 molt in early third instar (Fig.



3A,B). We expressed a constitutively active form of Ap (ChAp) (Milan and Cohen, 1999b) using the Gal4/UAS system and examined *Ser-lacZ* expression. When we used Dpp-Gal4 to drive ChAp expression at the anteroposterior (AP) boundary, we found ectopic *Ser-lacZ* expression in the ventral wing regions along the AP boundary, overlapping dpp-Gal4 expression in early and late third instar (Fig. 3D,E,G,H). This indicated that Ap was sufficient to activate *Ser* expression, probably cell-autonomously. To determine whether Ap function is necessary for *Ser* expression, we expressed an Ap antagonist, dLMO (Milan and Cohen, 1999b), in cells along the AP boundary, using a *patched (ptc)* promoter. This led to the loss of *Ser-lacZ* expression in the early third instar and partial reduction of *Ser-lacZ* in the late third instar (Fig. 3J,K,N,O), suggesting that Ap is required in vivo for *Ser* expression in the dorsal compartment.

To test whether early *Ser* expression can be directly regulated by Ap, we used DNaseI footprinting analysis to determine the interaction sites between the 794 bp DNA sequence and Ap. A total of 14 protected Ap binding sites were detected spanning the 794 bp element (Fig. 3R-V, Fig. 7A). The binding of Ap to this *Ser* minimal wing enhancer is sequence specific with two major binding sequences, TAATNN and CAATNN (Fig. 3W). The TAATNN consensus sequence matches the six-nucleotide consensus binding sequence for homeodomain proteins (Gehring et al., 1994). There is also the non-canonical CAATNN consensus sequence derived from the aligned sequences, which matches the consensus binding sites for some homeodomain proteins, such as murine S8 (de Jong et al., 1993). The existence of four CAATNN sites suggests that Ap may bind the CAATNN sequences specifically, in addition to the canonical TAATNN sites.

To test whether these Ap binding sites were functionally important in vivo, we mutagenized nucleotides in the Ap-binding sequences of *Ser-lacZ* construct 10, from TAATNN and CAATNN to AAAANN or TTTTNN, in most cases. The *(mAp)Ser-lacZ* construct, which included mutations in all 14

Fig. 3. *Ser* is directly regulated by Apterous (Ap). (A-I) The *Ser-lacZ* fusion gene (construct 10) is upregulated by Ap. (A,B) *Ser-lacZ* (red) is expressed in a stripe in the dorsal compartment around 24 hours after the L2/L3 molt in third instar in a wild-type background. (A,C) UAS-*GFP* (green) under dpp-Gal4 control reveals the wild-type *dpp* expression pattern at the AP border. ChAp is expressed under dpp-Gal4, which induces ectopic *Ser-lacZ* expression more ventrally along AP border in early third instar (arrows; D,E) and late third instar (arrows; G,H), as compared with the same stage discs in a wild-type background (B,Q). The ChAp expressing cells are marked by co-overexpression of GFP (green) under *dpp*-Gal4 (D,F,G,I). (J-Q) *Ser-lacZ* (construct 10) is downregulated by dLMO. UAS-*dLMO* and UAS-*GFP* (green, to mark dLMO expressing cells) are co-expressed at the AP border under *ptc*-Gal4. Note that *ptc*-Gal4 has a stronger expression level more posteriorly. *Ser-lacZ* expression is downregulated in dLMO expressing cells, particularly in the posterior compartment in early third instar (arrows; J,K). The reduction of *Ser-lacZ* in dLMO expressing cells is less dramatic in late third instar (N,O). The expression patterns of *Ser-lacZ* in a wild-type background are also shown for comparison (M, early third instar; Q, late third instar). (R-W) Binding of the Ap homeodomain (ApΔLIM, 6×HIS-tagged) to the 794 bp *Ser* minimal wing enhancer is direct and sequence specific. (R-V) DNase I footprinting analysis of the Ap homeodomain bound to the 794 bp *Ser* wing enhancer. Autoradiograms of denaturing polyacrylamide gels show the separated products after DNase I digestion of ApΔLIM/794 bp *Ser* wing enhancer complexes, and the relative amounts of ApΔLIM protein (1×, ~20ng protein; 3×, protein increased threefold) or no ApΔLIM protein (lanes 'c' for control). The DNase I-sensitive sequences protected by ApΔLIM are marked (site A-site N). The DNA sequencing products of the 794 bp *Ser* wing enhancer are shown here with G (ddGTP) and A (ddATP), or C (ddCTP) and T (ddTTP), in the first two lanes. (W) Alignment of sequences that bind ApΔLIM (from R-V). The sequences of sites H and I do not match either the TAATNN or the CAATNN consensus and are shown with non-matched nucleotides in red; they are determined arbitrarily. rc, reverse complementary sequence. (X1-Y4) X-Gal staining to reveal in vivo activity of the wild-type *Ser-lacZ* (construct 10; X1-X4) and *(mAp)Ser-lacZ* transgenes (Y1-Y4). The *(mAp)Ser-lacZ* construct contains mutations in all fourteen Ap-binding elements. *Ser-lacZ* expression was detected in dorsal cells of wing and haltere discs at 7.5 hours after the L2/L3 molt (X1). At 24 hours after the L2/L3 molt, expression was restricted to a stripe in the dorsal compartment of the wing disc, and in the entire dorsal compartment of the haltere disc (X2). At the corresponding stages, *(mAp)Ser-lacZ* displayed no enhancer activity in the wing and haltere discs (Y1,Y2). By 36 hours after the L2/L3 molt, in mid third instar, *Ser-lacZ* was expressed along the DV border, and at a low level in the ventral compartment of the wing disc (arrow) (X3); *(mAp)Ser-lacZ* expression was much reduced (arrow) in the wing disc and was not detected in the haltere disc (Y3). At the end of third instar, *Ser-lacZ* expression in the wing and haltere discs was evident (X4); *(mAp)Ser-lacZ* expression was reduced and restricted (arrows; Y4). Note that in Y1, dorsal is to the left for the wing disc. The insets in the upper right hand corner of (X1-3,Y3), and in the lower right hand corner of (X3,Y3) show the wing and haltere discs, at lower magnification.

Ap-binding sites, showed no enhancer activity in the wing and haltere discs in early third instar (Fig. 3Y1-2), as compared with *Ser-lacZ* expression, which was first detected in much of the dorsal compartment and then as a dorsal stripe (Fig. 3X1-2). In mid and late third instar, *(mAp)Ser-lacZ* expression was reduced or eliminated (Fig. 3Y3-4). These results show that the Ap-binding sites identified in vitro are crucial for the activity of the 794 bp *Ser* minimal wing enhancer in vivo. In summary, *Ser* expression is mediated by direct Ap interaction with the 794 bp wing enhancer during the early third instar stage.

A positive-feedback loop through the N pathway regulates Ser expression in mid third instar

Ser is expressed along the DV boundary in the mid third instar. It has been shown that a constitutively active N expressed under control of the *ptc* promoter causes ectopic *Ser* expression along the AP border (Panin et al., 1997). However, it is not clear whether the N pathway directly regulates *Ser* through its downstream transcription factor, Suppressor of Hairless [Su(H)] (reviewed by Artavanis-Tsakonas et al., 1999). To test whether the *Ser* enhancer is directly regulated by the N pathway, we first tested the responsiveness of the *Ser* wing enhancer (in construct 8, which is identical to Construct 10 in terms of expression patterns and levels; see Fig. 1) to N signaling. Using the flip-out system (Ito et al., 1997), we generated random clones expressing constitutively active N (N^i) in the wing disc. As shown in Fig. 4A-D, the ectopic expression of construct 8 *Ser-lacZ* was detected in the clones expressing constitutively active N. Thus, the *Ser* enhancer contains cis elements responsive to the N pathway.

To investigate whether N signaling exerts a direct effect on *Ser* transcription, we used gel mobility shift assays to test whether Su(H) could bind specifically to the *Ser* minimal wing enhancer. Computer-based searches for Su(H) binding consensus sequences identified two putative Su(H) binding sites, which were conserved in *D. melanogaster* and *D. pseudoobscura*, in the *Ser* minimal wing enhancer (Fig. 4G and Fig. 7A). Gel-shift analysis confirmed that the two putative sites actually bind GST-Su(H) (Fig. 4E). A competition assay suggested that these two sites are weaker in binding Su(H) than a strong binding site in the *Enhancer of split* (*E(spl)*) locus (Fig. 4F) (Bailey and Posakony, 1995).

To test whether the two Su(H)-binding sites were functional in vivo, we synthesized a mutant *Ser-lacZ* construct, *(mSu(H))Ser-lacZ*, carrying mutations in two nucleotides of both Su(H)-binding consensus sequences (RT**G**R**G**AR to RT**A**R**A**AR) (Nellesen et al., 1999). This construct showed significantly reduced activity in the wing disc in mid third instar (Fig. 4I), as compared with the *Ser-lacZ* disc at the same stage (Fig. 4H). These data show that at least two Su(H)-binding elements are involved in determining the activity of the *Ser* minimal wing enhancer in vivo. We conclude that N signaling directly regulates *Ser* gene expression by binding of Su(H) to the *Ser* minimal wing enhancer.

Wg signaling regulates *Ser* expression in late third instar

In late third instar, *Ser* is expressed in cells flanking the DV boundary. It has been shown that Wg signaling can regulate *Ser* expression in these flanking cells (de Celis and Bray, 1997; Micchelli et al., 1997). However, it is not known how Wg signaling controls *Ser* expression at the molecular level. To assess the possibility that *Ser* may be directly regulated by the Wg pathway through the *Ser* wing enhancer, we first tested

whether construct 8 *Ser-lacZ* responds to the Wg pathway. Using the flip-out system, we found that a constitutively active component of the Wg pathway, Armadillo^{S10} (Arm^{S10}) (Pai et al., 1997), can upregulate *Ser-lacZ* expression cell autonomously in the wing pouch territory, which is consistent with a previous study demonstrating that Wg signaling induces *Ser* expression in that area, but not in the thorax or hinge (Fig. 5A-D) (de Celis and Bray, 1997). This result demonstrated that Wg signaling is sufficient to upregulate *Ser* enhancer expression. To further test whether Wg signaling is required for *Ser-lacZ* expression, we expressed a suppressor of Wg signaling, dominant-negative TCF (DN-TCF), in the posterior wing compartment, driven by the *engrailed* (*en*) promoter. Expression of DN-TCF greatly diminished *Ser-lacZ* expression in posterior cells of the ventral compartment, and significantly reduced *Ser-lacZ* levels in the posterior dorsal compartment, as compared with wild-type *Ser-lacZ* expression (Fig. 5F,G). Thus, Wg signaling is necessary for expression of the *Ser* enhancer in cells flanking the DV boundary. Taken together, we conclude that Wg signaling contributes to activation of the *Ser* enhancer in these cells.

To test whether *Ser* enhancer expression could be directly regulated by Wg signaling through its downstream transcription factor dTCF, we performed DNase I footprinting to look for binding sites for dTCF-HMG (DNA binding domain) (Halfon et al., 2000). dTCF-HMG is able to bind nine sites within the 794 bp Ser enhancer. Three of these sites conform to a class of canonical dTCF binding sites, CCTTTGATCTT. Interestingly, consistent with a recent report (Lee and Frasch, 2000), we also found that four other sites match a motif bound by HMG proteins. There are two non-canonical binding sites, which do not conform to either the dTCF or HMG canonical class (Fig. 5L and Fig. 6A).

We then asked whether these dTCF-binding sites were functionally important in vivo. We synthesized a mutant *Ser-lacZ* construct with a change of three nucleotides in the dTCF and HMG binding consensus sequences (CC**TTT**GATCTT and **WTT**GWW to CC**GGA**GATCTT and **GGA**GWW, respectively), in most cases (Lee and Frasch, 2000). In late third instar, the *(mdTCF)Ser-lacZ* construct, which contained mutations in all nine dTCF-binding sequences, showed strongly reduced X-gal activity in the wing disc (Fig. 5M), as compared with the *Ser-lacZ* disc at the same stage (Fig. 3X4). These data show that these dTCF-binding elements are crucial for the activity of the *Ser* minimal wing enhancer in vivo. Altogether, these results suggest that dTCF regulates *Ser* through direct binding to the *Ser* minimal wing enhancer.

Ser is regulated by the Egfr pathway in presumptive wing veins

Ser is expressed in presumptive wing veins in late third instar, as well as at the pupal stage (Fig. 2G and Fig. 6A). As Egfr signaling is required for vein development (Diaz-Benjumea and Garcia-Bellido, 1990; Guichard et al., 1999), we analyzed whether *Ser* expression in provein cells is regulated by the Egfr

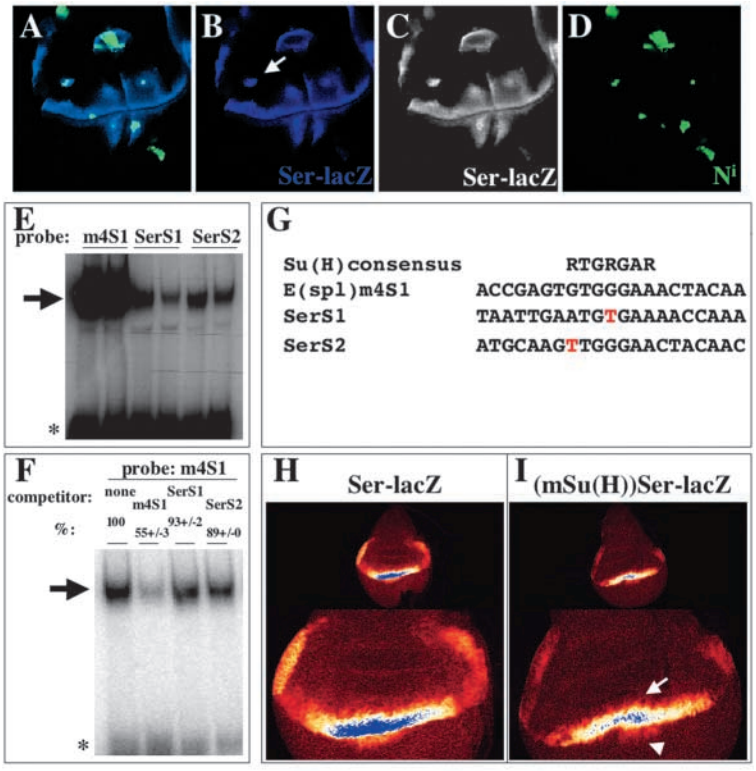


Fig. 4. *Ser* is directly regulated by the Notch pathway. (A-D) The *Ser-lacZ* fusion gene (construct 8) is upregulated by N signaling. Flip-out clones expressing constitutively active Notch (N^i) are shown in green. *Ser-lacZ* expression in blue (B) or in white (C) is upregulated in the N^{act} clones; one example is indicated by the arrow in B. (E-G) The 794 bp *Ser* minimal wing enhancer contains binding sites for Su(H). (E) Gel mobility shift assays with GST-Su(H) and oligonucleotides m4S1 [a strong Su(H) binding site of the E(spl)m4 locus as a control] and SerS1 and SerS2 [two putative Su(H) binding sites in the 794 bp minimal *Ser* wing enhancer, also shown in G and Fig. 7A]. Autoradiograms of native polyacrylamide gels show the separated products of GST-Su(H)-oligonucleotide complexes (arrow) with various amounts of GST-Su(H) protein (144 ng in lanes 1, 3 and 5; 68 ng in lanes 2, 4 and 6) and the same amount of ^{32}P-labeled oligonucleotides (10^7 cpm). Asterisk marks position of free probe. (F) Competition assay using a ^{32}P-labeled m4S1 probe with a 30-fold molar excess of unlabeled competitors; as quantified using a phosphoimager, SerS1 and SerS2 compete about one-fifth as well as m4S1. Three independent sets of experiments produced similar results. (G) Alignment of sequences to which Su(H) binds. SerS1 and SerS2 match the Su(H) RTGRGAR consensus defined by previous studies (Nellesen et al., 1999), except for one unmatched nucleotide in each case (red). (H,I) In vivo activity of the wild-type *Ser-lacZ* (construct 10) and *m(Su(H)Ser-lacZ*. The *m(Su(H)Ser-lacZ* construct contains mutations in two Su(H)-binding elements. *lacZ* expression is shown by immunostaining, using the glowover mode (confocal artificial coloring), where blue color indicates the highest expression level and brightness also indicates a higher expression level. (I) At 36 hours after the L2/L3 molt, in mid third instar, *m(Su(H)Ser-lacZ* expression was significantly reduced both in the D (arrow; less blue) and V (arrowhead; less bright and more diffused) compartments, as compared with a *Ser-lacZ* wing disc at the same stage (H). *m(Su(H)Ser-lacZ* expression levels from eight independent transgenic lines appear to be more sensitive to position effects than the wild-type *Ser-lacZ* transgenic lines. Both images in H are of one *Ser-lacZ* disc; both images in I are of one *m(Su(H)Ser-lacZ* disc.

pathway. We examined *Ser* expression in both gain-of-function (gof) and loss-of-function (lof) Egfr signaling-mutant backgrounds. First, in a *rho* gof mutant (UAS-*rho**) (Xiao et al., 1996), we observed that *Ser* appeared to be ectopically expressed between L3 and L4 (Fig. 6C,D), exactly where ectopic *rho* activity was localized (data not shown). We next observed that *Ser* expression in the proveins was eliminated in a *rho* and *vein* (*vn*, encoding a Egfr ligand) double-mutant (Egfr lof) background, in which vein formation is completely abolished (Fig. 6E,F) (de Celis et al., 1997). These results suggest that the Egfr pathway may regulate *Ser* expression during vein development at the pupal stage.

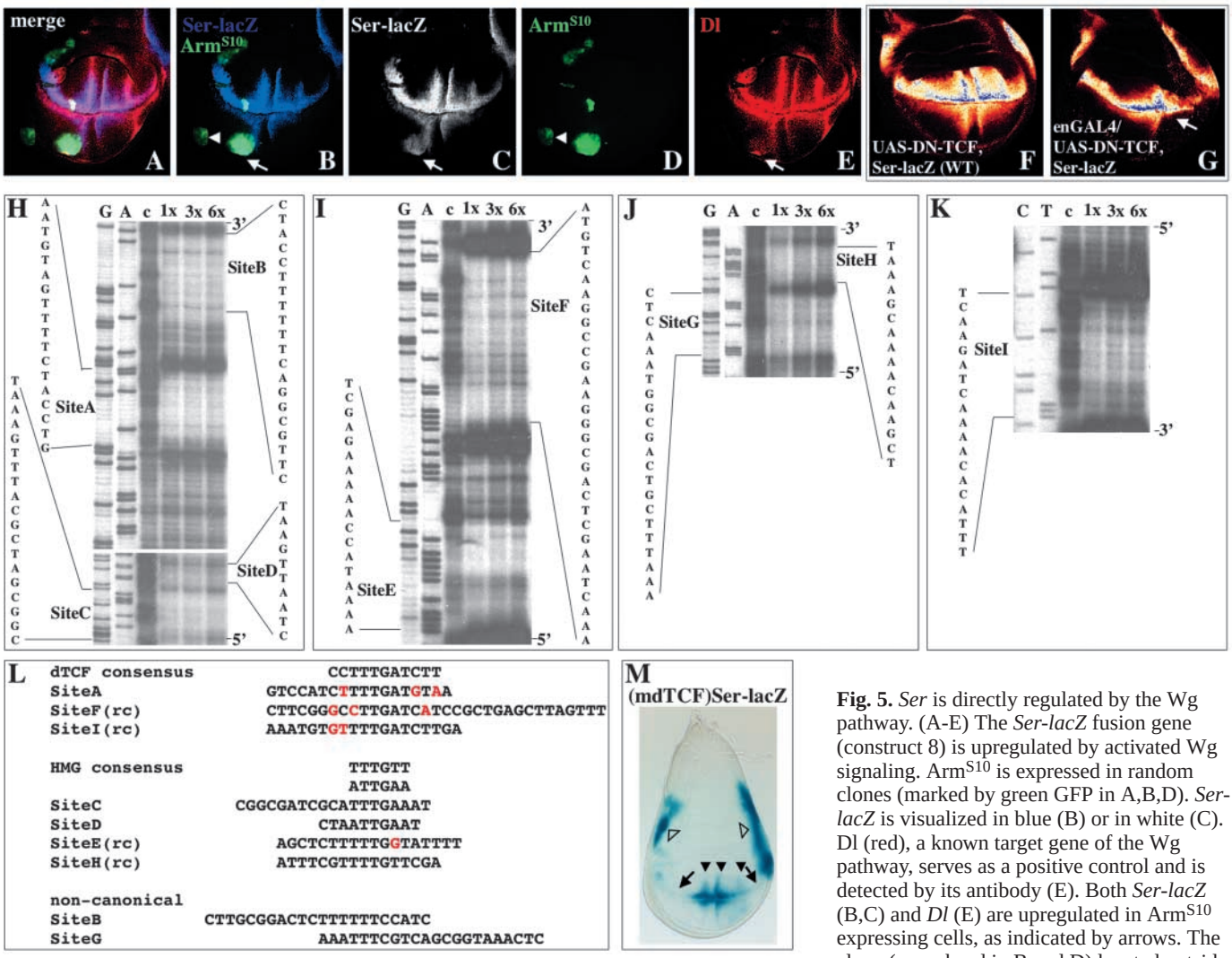


Fig. 5. *Ser* is directly regulated by the Wg pathway. (A-E) The *Ser-lacZ* fusion gene (construct 8) is upregulated by activated Wg signaling. Arm^{S10} is expressed in random clones (marked by green GFP in A,B,D). *Ser-lacZ* is visualized in blue (B) or in white (C). Dl (red), a known target gene of the Wg pathway, serves as a positive control and is detected by its antibody (E). Both *Ser-lacZ* (B,C) and *Dl* (E) are upregulated in Arm^{S10} expressing cells, as indicated by arrows. The clone (arrowhead in B and D) located outside of the wing pouch has no effect on *Ser-lacZ* and *Dl* expression. (F-G) *Ser-lacZ* is downregulated by DN-dTCF. (F) *Ser-lacZ* expression in a wild-type background without *en*-Gal4 but with UAS-*DN-dTCF*. Expression is shown in the glowover mode (see legend for Fig. 4H,I); *Ser-lacZ* is expressed at higher levels dorsally. (G) UAS-*DN-dTCF* is expressed in the posterior compartment of the wing disc under the control of *en*-Gal4. Note that *Ser-lacZ* expression is eliminated in the ventral posterior compartment. The reduction of *lacZ* expression in the dorsal posterior compartment is significant, when compared with *Ser-lacZ* expression in a wild-type background. (H-K) DNase I footprinting analysis of the dTCF-HMG protein bound to the 794 bp *Ser* wing enhancer. Autoradiograms of denatured polyacrylamide gels show the separated products of DNase I digestion of dTCF-HMG/794 bp *Ser* wing enhancer complexes with relative amounts of dTCF-HMG protein (1×, about 2 μg protein; 3× and 6×, protein increased threefold and sixfold, respectively), or no dTCF-HMG (lanes 'c' for control). The DNase I-sensitive bases protected by dTCF-HMG are marked, and their corresponding DNA sequences are shown (site A-site I). The DNA sequencing products of the 794 bp *Ser* wing enhancer are shown here with G (ddGTP) and A (ddATP), or C (ddCTP) and T (ddTTP), in the first two lanes. (L) Alignment of sequences that are bound by dTCF-HMG (from H-K). Sites A, F and I match the dTCF CCTTTGATCTT consensus, except for the unmatched nucleotides shown in red. Sites C, D, E and H are a good match for the HMG consensus, except for an unmatched guanine at site E. The non-canonical sequences at sites B and G show no obvious homology to either dTCF or HMG binding consensus sequences, except for a stretch of three thymidine residues in the middle. (M) Expression of the *(mdTCF)Ser-lacZ* transgene. The *(mdTCF)Ser-lacZ* construct contains mutations in all nine dTCF-binding elements. In the late third instar, *(mdTCF)Ser-lacZ* expression was greatly reduced in cells flanking the DV boundary (arrows), as compared to a wild-type *Ser-lacZ* disc (Fig. 3X4). Note that *lacZ* expression levels were higher in the notum (open arrowheads), where *Ser* expression is regulated independently of the Wg/dTCF pathway; *lacZ* expression in presumptive veins L3, L4 and L5 (arrowheads) was also detected.

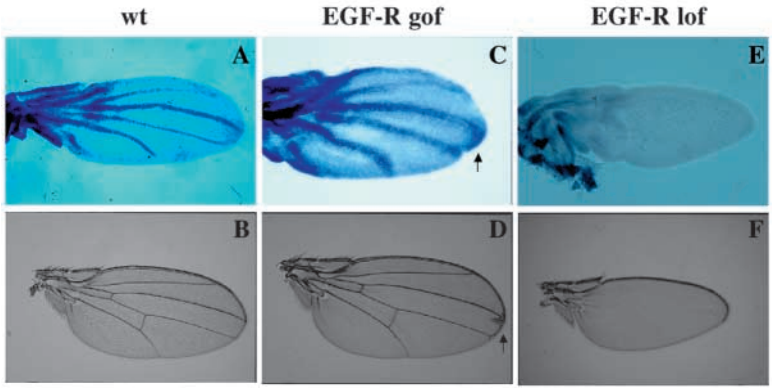


Fig. 6. *Ser* is regulated by the Egfr pathway. Wings of wild type (A), UAS-*rho** (Egfr gof; C), and *rho*^{1}*vn*^{1} (Egfr lof; E) at 28 hours after puparium formation, with their corresponding adult wings (B,D,F). *Ser* mRNA was detected by in situ hybridization (A,C,E). (A) *Ser* expression in the wild-type provein cells. (C) *Ser* is ectopically expressed between L3 and L4 (arrow), where the ectopic veins are developed (arrow in D). (E) *Ser* expression is not seen in the wing without veins (F).

Discussion

How do tissue-specific selectors collaborate with cell signaling pathways during organogenesis? In this paper, we show that the selector protein Ap and multiple signaling pathways, including the N, Wg and Egfr pathways, collaborate to regulate *Ser* in a sequential manner during *Drosophila* wing development.

Sequential regulation of *Ser* by a selector gene and multiple signaling pathways

The results reported here demonstrate that a 794 bp cis-acting regulatory module in the *Ser* locus can be temporally regulated by three distinct mechanisms that are employed for the proper establishment of the DV organizer during wing development. First, the selector protein Ap directly activates *Ser* expression in the dorsal compartment during the early third instar, which sets up N activation for the next stage. Second, by the middle of the third instar, the N pathway maintains *Ser* expression by a positive-feedback loop along the DV boundary. This feedback loop maintains Ser and Dl expression, leading to the activation of N signaling at the DV boundary, which is essential for establishing the DV organizer (Panin et al., 1997). Third, at the end of the third instar, as a result of Wg signaling, *Ser* is expressed in two stripes flanking the DV boundary, which limits N activation to the DV border (Fig. 7B). In addition, we have demonstrated that *Ser* expression in provein cells is dependent on input from the Egfr pathway. Our results indicate how tissue-specific selector and signaling molecules can work sequentially to achieve a complex developmental process, such as organogenesis, which involves a complex temporal and spatial regulation of genes. However, our conclusion that the *Ser* minimal wing enhancer is sequentially regulated by Ap, Notch, Wg and Egfr does not exclude the possibility that these molecules/signaling pathways may cooperate and synergistically stimulate gene expression at certain stages. In this case, mutations that specifically impair response to the intended factor would affect *Ser-lacZ* expression in other phases of disc development.

Ap regulates *Ser* directly in early third instar

Here, we provide evidence that *Ser* is indeed a direct target gene for Ap, thus forming a link between Ap, which specifies dorsal identity, and the signaling pathways that organize the DV boundary (Diaz-Benjumea and Cohen, 1993). Specifically, we show that Ap regulates the *Ser* minimal wing enhancer in vivo, and binds the enhancer in vitro through two major DNA sequences, TAATNN and CAATNN (Fig. 3W). Ap may regulate the *Drosophila* FMRFa neuropeptide gene and a mouse glycoprotein hormone α-subunit gene enhancer by binding to TAATNN sequences (Benveniste et al., 1998; Rincon-Limas et al., 2000; Roberson et al., 1994). Thus, TAATNN sequences may regulate most, if not all, Ap target genes.

The 794 bp *Ser* minimal wing enhancer is regulated by Ap, and is expressed in the dorsal compartment of wing and haltere discs at 7.5 hours after the L2/L3 molt in early third instar (Fig. 3X1,Y1). The 7.4 kb 5′ flanking sequence and the 8 kb 3′ flanking sequence can also direct reporter gene expression in all of the dorsal compartment during wing development (Fig. 1I,J; Fig. 2A,B; data not shown). A 9.5 kb Ap cis-response element was also isolated ~7.5 kb upstream of the Ser translational initiation site (it contains most sequences in construct 1 and construct 2, and a 2 kb *Bam*HI/*Bam*HI fragment in between the two constructs; Fig. 1A), although it is not clear whether Ap directly regulates this element. Further dissection of this element into smaller fragments did not succeed in recapitulating the dorsal anlage expression pattern (Bachmann and Knust, 1998). These results suggest that crosstalk between different cis-elements is required to regulate *Ser* dorsal expression, and that there is more than one Ap response element at the *Ser* locus (Fig. 1A, Fig. 2A,B) (Bachmann and Knust, 1998). Given the importance of Ap-regulated *Ser* expression, multiple Ap response elements might be expected. Enhancer redundancy has been observed in many genes and may have evolved as a protection against loss of gene activity when mutations occur in regulatory sequences (reviewed by Arnosti, 2003).

Around 24 hours after the L2/L3 molt, a transition occurs in *Ser* minimal enhancer expression from all dorsal cells to dorsal cells near the DV boundary [24 hours after the L2/L3 molt is defined as early third instar because 48-72 hours AEL (after egg laying) is generally taken as the early third instar, which is equal to 0-24 h after the L2/L3 molt] (Fig. 3X1-2). During this transition, *Ser* expression in dorsal cells flanking the DV boundary may be regulated by Ap, as well as by the N pathway (Klein and Arias, 1998; Klein et al., 2000). At 24 hours after the L2/L3 molt, *(mAp)Ser-lacZ* displayed no activity, and *(mSu(H))Ser-lacZ* expression was evident in dorsal cells near the DV boundary (Fig. 3Y2; data not shown). Although these data suggest that Ap regulates *Ser* expression in dorsal cells near the DV boundary, they do not exclude the possibility that Notch may still be involved in directly regulating *Ser*

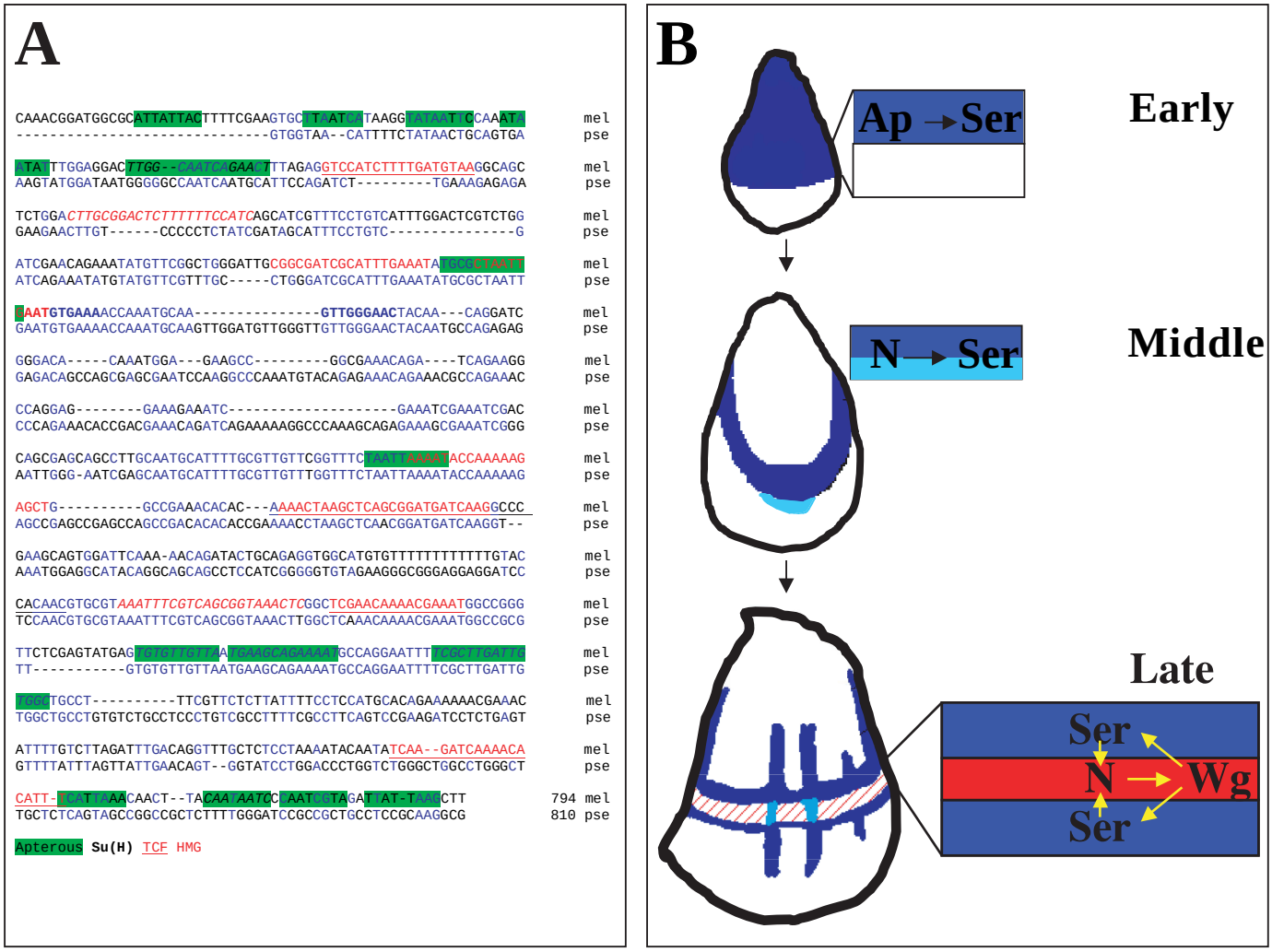


Fig. 7. *Ser* is sequentially regulated by the Ap, N and Wg pathways during larval development. (A) Alignment of the *Ser* minimal wing enhancer regions in *D. melanogaster* (mel; 794 bp) and *D. pseudoobscura* (pse; 810 bp). Both enhancers are located within 1 kb downstream of the *Ser* 3′UTR. Locations of Ap (green), Su(H) (bold type) and dTCF (red) binding sites are indicated, which are defined based on the in vitro DNA-protein binding data in this study. Non-canonical sites are italicized. Conserved nucleotides of the two species are denoted in blue. (B) Sequential regulation of *Ser* by the Ap, N and Wg pathways represents an integration point for proper wing development. The selector protein Ap directly activates *Ser* expression in the dorsal compartment during early third instar, which induces N activation at the DV border in mid third instar. The N pathway then regulates *Ser* expression by a positive-feedback loop along the DV boundary. Finally, as a result of Wg signaling, at the end of the third instar, *Ser* is expressed in two stripes flanking the DV boundary.

expression during this transition, as Su(H) may still be able to bind to and activate *(mSu(H))Ser-lacZ* (also see below).

Notch signaling in the formation of the DV boundary

Activation of N signaling at the nascent DV boundary is essential for the formation of the DV boundary (de Celis et al., 1996; Micchelli and Blair, 1999; Rauskolb et al., 1999; Sturtevant and Bier, 1995). Ser and Dl are highly expressed at the DV border in mid-third instar and their expression can be ectopically activated by a constitutively active form of N, which suggests a positive-feedback loop between N ligands and the receptor (de Celis and Bray, 1997; Panin et al., 1997). The activation of such a feedback loop between N and its ligands is likely to be among the earliest events in the formation of the DV boundary. Our finding that the *Ser* wing enhancer is regulated by the N pathway, and that two Su(H)-binding sites are required for the in vivo activity of this enhancer in the mid third instar, suggests that N signaling can directly regulate *Ser* expression through Su(H). Although these results are consistent with direct activation of the *Ser* gene by Su(H), they do not preclude the possibility that N signaling may regulate *Ser* through other transcription factors, possibly downstream of Su(H). This would explain why *(mSu(H))Ser-lacZ* showed a significant, but not dramatic, loss of enhancer activity (Fig. 4H,I). Alternatively, it remains possible that Su(H) can still bind to and activate at least one of the two mutant Su(H) binding sites in *(mSu(H))Ser-lacZ.*

Wg signaling directly regulates *Ser* in late third instar

Our in vitro and in vivo results suggest that the regulation of *Ser* by Wg signaling occurs directly through dTCF. Using

DNase I footprinting, we found two major classes of dTCF binding sequences: the dTCF consensus sequence CCTTTGATCTT and the HMG consensus sequence WTTGWW, which are consistent with previously identified dTCF binding sequences (Lee and Frasch, 2000; Riese et al., 1997; van de Wetering et al., 1997). Interestingly, the presence of dTCF/HMG binding sites in the *Ser* minimal wing enhancer may explain the crosstalk observed between the 3′ *Ser* enhancer and the 5′ *Ser* promoter (Fig. 1). HMG proteins can bend DNA, and could therefore bring the 3′ enhancer close enough to interact with the transcriptional machinery binding at the 5′ promoter (reviewed by Thomas, 2001).

In late third instar, Wg signaling is maintained in the DV organizer by the N pathway (Micchelli et al., 1997). Wg signaling activates *Ser* and *Dl* expression in the cells flanking the DV boundary, which in turn activates N signaling to maintain a positive-feedback loop between N and Wg signals (Fig. 7B) (de Celis and Bray, 1997; Micchelli et al., 1997). Because of an autonomous repression effect of N ligands on their receptor, *Ser* and *Dl* expression in the flanking cells also prevents N signaling from spreading out of the DV border. N signaling then turns off *Ser* and *Dl* expression by inducing *cut* at the border (de Celis and Bray, 1997). Although the molecular nature of the dominant-negative effects of N ligands, and the repression of Ser and Dl by N signaling remains unknown, these mechanisms may play important roles in keeping the boundary sharp (Micchelli et al., 1997). Interestingly, the *Ser* minimal wing enhancer is also repressed at the DV border, suggesting that it is possible to study the molecular mechanism of *Ser* repression at the border using this 794 bp enhancer.

Regulation of *Ser* in provein cells by Egfr signaling

We have demonstrated that *Ser* is expressed in provein cells and that its expression is regulated by Egfr signaling at the pupal stage. N signaling also plays an important role in determining vein cell fate (de Celis et al., 1997; Huppert et al., 1997). Our data on *Ser* expression in provein cells is consistent with a report on *Ser* function during vein development (Zeng et al., 1998). Thus, in addition to its essential role in development of the *Drosophila* leg and vertebrate limbs, Egfr/Fgf signaling also plays a role in *Drosophila* wing development, suggesting a conserved role of Egfr signaling in ‘appendage’ development (Campbell, 2002; Diaz-Benjumea and Garcia-Bellido, 1990; Galindo et al., 2002; Guichard et al., 1999). Interestingly, the *Ser* minimal wing enhancer is expressed in provein cells at both larval and pupal stages (S.-J.Y., W.X.L. and R.J.F., unpublished). Further investigation of this element may shed light on how Egfr signaling regulates vein differentiation.

The *Ser* minimal wing enhancer, an evolutionarily conserved element

Given that the Ser-Fng-N pathway is evolutionarily conserved in appendage development between insects and vertebrates (Laufer et al., 1997; Rodriguez-Esteban et al., 1997), the mechanism by which *Ser* is sequentially regulated by Ap, N, Wg and Egfr may also be conserved in appendage outgrowth of other arthropods and vertebrates. Consistent with this hypothesis, the Ap, Wg/Wnt and Egfr/Fgf pathways are also involved in appendage development in vertebrates, as well as *D. melanogaster* (Kawakami et al., 2001; Shubin et al., 1997). Indeed, a BLAST search of the *Drosophila pseudoobscura* genome identified a putative homolog of the *Ser* minimal wing enhancer. Interestingly, this enhancer region is also located less than 1 kb downstream of the putative *D. pseudoobscura Ser* 3′UTR. Sequence comparisons between the *Ser* minimal wing enhancer from *D. melanogaster* and the putative *D. pseudoobscura* enhancer show a significant degree of similarity, whereas the similarities in the 5′ and 3′ flanking regions are lower (Fig. 7A). Importantly, sequences of putative Ap, Su(H) and dTCF binding sites are highly conserved in *D. pseudoobscura* and *D. melanogaster.* Although the strong conservation of sequence and location suggests that the putative *D. pseudoobscura Ser* enhancer may be a functional homolog of the *D. melanogaster Ser* minimal wing enhancer, it remains to be tested whether this enhancer drives reporter gene expression at the identical time and location in the *D. melanogaster* wing discs.

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