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

Shian-Jang Yan\(^1,2\), Yi Gu\(^1,\ast\), Willis X. Li\(^2,\dagger\) and Robert J. Fleming\(^1,\dagger,\ddagger\)

\(^1\)Department of Biology, University of Rochester, Rochester, NY 14627, USA
\(^2\)Department of Biomedical Genetics, University of Rochester Medical Center, Rochester, NY 14642, USA
*Present address: Division of Experimental Hematology, Cincinnati Children's Hospital Medical Center, Cincinnati, Ohio 45229, USA
†Present address: Biology Department, Trinity College, 238 Life Sciences Center, Hartford, CT 06106, USA
‡Authors for correspondence (e-mail: willis_li@urmc.rochester.edu and Robert.Fleming@trincoll.edu)

Accepted 20 October 2003

Development 131, 285-298
Published by The Company of Biologists 2004
doi:10.1242/dev.00934

**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; Telemann 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 ventralizer 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 pCaSpeR-R-Gal4. pCaSpeR-R-hsGal4 was adapted from pCaSpeR-hsGal4 (Thummel et al., 1988) by inserting a NotI/BamHI fragment of the Gal4 coding sequence and the 3′ hsp70 polyadenylation signal from pGaTB (Brand and Perrimon, 1993). pCaSpeR-R-Gal4 was modified from pCaspeR-hsGal4 by deleting the hsp70 promoter between the EcoRI and Xhol sites. Constructs 1 and 2 include 5 kb and 4 kb BamHI fragments, respectively, inserted into the BamHI site downstream of Gal4 of pCaSpeR-R-Gal4. Construct 3 includes a 7.4 kb EcoRI/Xhol fragment inserted into the BamHI site upstream of Gal4 of pCaSpeR-R-Gal4. Construct 4 includes an 8 kb BamHI fragment inserted into the BglII site downstream of Gal4 of pCaSpeR-R-Gal4. Constructs 5, 6, and 7 were adapted from construct 3 by inserting 8 kb BamHI, 5 kb BamHI/HindIII, and 2.7 kb BamHI/HindIII fragments, respectively, into the BglII 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 KpnI 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 BamHI and EcoRI 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; EcoRI and BamHI cloning sites were indicated):

- mAp (A-E) sense EcoRI, 5′-GAATTCACACACTTTGCGGCGTCTTCTCTATTTTGAATGTGTTTACG-3′
- mAp (F) sense, 5′-TTGGGATCTCAACTTGAAGCTTGGCTTCTC-3′
- mAp (G) sense, 5′-TTGGCTGAATTTTTCACAATGTG-3′
- mAp (H) sense, 5′-GGTGTATTTTGAACCGGAAACATGAC-3′
- mAp (I) sense, 5′-CCACACAAACGGAATTTTCACTGCGG-3′
- mSu(h) sense, 5′-TCTAATACATTTGCGCTTTGTTTACATC-3′
- mSu(h) antisense, 5′-GTAGTTGCAAATTTCTCGATTTTATTCA-3′
- mdTCF (A-B) sense, 5′-GTCCCATCTGCGGTATGTTAGAGCCTCGAGTCTCTTCGAATTTGCTAG-3′
- mdTCF (A-B) antisense, 5′-GATGCTAaaaAAACAAAACAAAGCTAGCCTCCTACCATCCAGAGTGA-3′
- mdTCF (C-D) sense, 5′-ATCGCAaggAATAATTCGCTAGaggAAATGATG-3′
- mdTCF (C-D) antisense, 5′-CATTCCATGCGCCTATTTCCCTGCGAG-3′
- mdTCF (E-F) sense, 5′-AATACCCCAAAGACAGTGGCGGCA-3′
- mdTCF (E-F) antisense, 5′-CGGCGGCGATCGATCCTCGAGCAGTGGGTTTAC-3′
- mdTCF (G-H) sense, 5′-GGTTAAAGAAAGAAATGGTAAATACGACGTCGAGACACGAGAA-3′
- mdTCF (G-H) antisense, 5′-TTTTGGGTAATTAGCGCCAGCTTGCGGGTCGCCCCAGG-3′
- Ser-lacZ sense EcoRI, 5′-GAATTCACACACTTTGCGGCGTCTTCTC-3′
- Ser-lacZ antisense BamHI, 5′-TCCCTGTAATCTTTCCCAACTTGTGGGATC-3′

Mutant Ser enhancer constructs were cloned into pCaSpeR-hs70-AUG-βgal between the BamHI and EcoRI sites. All constructs were verified by DNA sequencing before being introduced into w118 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 rat anti-Ser (1:1000, provided by K. Irvine) and monoclonal mouse anti-Dl Mab202 (1:250, provided by M. Muskavitch). The following

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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, BamHI; E, EcoRI; H, HindIII; X, XbaI. 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 (BdG/Ser+83k) 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) BdG/Ser+83k animals display little wing tissue compared with wild type (B). (D) Ser expression under construct 3 can partially restore 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 BdG/Ser/+ r83k mutant wing phenotype, we crossed transgenic flies carrying constructs 1-7 in a BdG/TM6B, Tb Ser +/+ genetic 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-DMO (Milan and Cohen, 1999b), UAS-N (provided by S. Artavanis-Tsakonas), UAS-arm p10 (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 fly stocks were used: v1118, UAS-rho (+) (Xiao et al., 1996) and v118, v118 (rho’v118) (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-32P]ATP by T4 DNA polynucleotide kinase. DNA sequencing products were labeled with α-32P-ATP using Thermo Sequenase Cycle Sequencing Kit (Amersham Pharmacia Biotech). 6xHis-tagged ApdLIM (Benveniste et al., 1998) and 6xHis-tagged TcFL-EN (Hallon 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 MgCl2. 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 BdG [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 BdG/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 BdG/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 BdG/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 BdG/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 region of the wing disc.
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 (Micheilli 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.

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\(^6\)/Ser\(^{+794}\) wing phenotype and to direct lacZ expression in wing discs. Constructs 6 and 7 were able to rescue the Bd\(^6\)/Ser\(^{+794}\) 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 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 (Irving 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.
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 (Fig. 3W). The (mAp)Ser-lacZ construct, which included mutations in all 14
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\(^3\)) 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 (RTGRGAR to RTARAAR) (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\(^{10}\) (Arm\(^{10}\)) (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 (CCTTTGATCTT and WTTGTG to CCGGAGATCTT and GGAGGWW, 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 pathway.
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.

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 (1· mg 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 CCGTGAATCTTTTCT 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 rhovl² (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. 3I,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 BamHI/BamHI 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)sSer-lacZ displayed no activity, and (mSu(H))sSer-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.
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 DI 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 CTTTTGATCTT and the HMG consensus sequence WTTGGWW, 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 proein cells by Egfr signaling**

We have demonstrated that Ser is expressed in proein 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 proein 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 proein 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 (Lauffer 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.

We wish to thank Drs S. Artavanis-Tsakonas, J. de Celis, S. Cohen, K. Irvine, A. Michelson, M. Muskavitch, J. Nambu, J. Pasakony, N. Perrimon, P. Taghert and C. Thummel, and the Bloomington Stock Center for fly stocks and reagents. We thank H. Lyon for help with confocal microscopy; Dr C. Benyajati for lending electrophoresis apparatus to perform DNase I footprinting; and C. Sommers for embryo injection. We thank Dr L. Silver-Morse for comments on the manuscript, and the two anonymous referees for the helpful suggestions. This study was supported by a National Science Foundation grant (IBN-9727951) to R.J.F. and a National Institutes of Health grant (R01 GM65774) to W.X.L.

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