Regulation of the *spalt/spalt-related* gene complex and its function during sensory organ development in the *Drosophila* thorax

José F. de Celis¹*, Rosa Barrio² and Fotis C. Kafatos²

¹Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK
²European Molecular Biology Laboratory, Meyerhofstrasse, 69117 Heidelberg, Germany

*Author for correspondence (e-mail: jdc@mole.bio.cam.ac.uk)

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SUMMARY

The nuclear proteins Spalt and Spalt-related belong to a conserved family of transcriptional regulators characterised by the presence of double zinc-finger domains. In the wing, they are regulated by the secreted protein Decapentaplegic and participate in the positioning of the wing veins. Here, we identify regulatory regions in the *spalt/spalt-related* gene complex that direct expression in the wing disc. The regulatory sequences are organised in independent modules, each of them responsible for expression in particular domains of the wing imaginal disc. In the thorax, *spalt* and *spalt-related* are expressed in a restricted domain that includes most proneural clusters of the developing sensory organs in the notum, and are regulated by the signalling molecules Wingless, Decapentaplegic and Hedgehog. We find that *spalt/spalt-related* participate in the development of sensory organs in the thorax, mainly in the positioning of specific proneural clusters. Later, the expression of at least *spalt* is eliminated from the sensory organ precursor cells and this is a requisite for the differentiation of these cells. We postulate that *spalt* and *spalt-related* belong to a category of transcriptional regulators that subdivide the thorax into expression domains (prepattern) required for the localised activation of proneural genes.

Key words: *spalt*, Gene regulation, Sensory organ development, *Drosophila*

INTRODUCTION

The *Drosophila* thorax is covered by two different types of sensory organs, macrochaetae and microchaetae, distributed in a characteristic pattern. They are formed by the progeny of epidermal cells specified during larval (macrochaetae) and pupal (microchaetae) development in the presumptive thorax of the wing disc (Campuzano and Modolell, 1992). The immediate sensory organ precursor (SOP) cells are singled out amongst a cluster of potential precursor cells by the activity of the proneural genes *achaete* and *scute*, two genes that encode basic helix-loop-helix proteins (bHLH; Campuzano and Modolell, 1992). Position-specific enhancers (Ruiz-Gómez and Modolell, 1987; Gómez-Skarmeta et al., 1995) direct expression of both *achaete* and *scute* in clusters of cells at positions that prefigure the pattern of sensory organs. The identification of genetic elements participating in the regulation of *achaete* and *scute* is therefore a critical step in understanding the generation of the pattern of sensory organs. It is thought that *achaete* and *scute* expression is regulated in individual proneural clusters by the combinatorial effect of several genes that constitute a ‘prepattern’ of transcriptional regulators (Stern, 1954; Modolell and Campuzano, 1998). Several genes, such as the *iroquios* gene complex and *pannier*, have been found to be members of this postulated prepattern. They are expressed in the thorax in restricted domains which, individually, are broader than the proneural clusters (Gómez-Skarmeta et al., 1996; Haenlin et al., 1997).

The overall growth of the wing disc is under the control of several signalling molecules encoded by *hedgehog* (*hh*), *wingless* (*wg*) and *decapentaplegic* (*dpp*) (Blair, 1995). These genes also play critical roles during the patterning of sensory organs (Phillips and Whittle, 1993; Mullor et al., 1997), although their effects on *achaete* and *scute* expression might be mediated by transcriptional regulators downstream of these signalling molecules. In the patterning of veins, Hh activates the expression of the homeobox genes of the *iroquios* gene complex in the presumptive region of the L3 vein (Gómez-Skarmeta and Modolell, 1996) and Dpp activates the expression of the structurally related genes *spalt* (*sal*) and *spalt-related* (*salr*) in a central domain in the wing (de Celis et al., 1996; Nellen et al., 1996; Lecuit et al., 1996). *sal* and *salr* encode transcription factors with spaced sets of double zinc-finger motifs (Kühnlein et al., 1994; Barrio et al., 1996) and participate in the correct spacing of the wing veins (de Celis et al., 1996). Elimination of both genes during development of the wing results in phenotypes that are stronger than those resulting from elimination of only *sal*, suggesting that the genes have complementary roles. *sal* and *salr* are also expressed in other regions of the wing disc that correspond to the presumptive thorax, hinge and pleura, but their roles there have not been yet established.
In this work, we identify regulatory regions that direct salr expression at specific regions of the wing disc, and show that hh, dpp and wg regulate these genes in the thorax. Furthermore, we implicate sal and salr in the patterning of sensory elements in the thorax. The expression of sal and salr here occurs in a restricted domain that includes the position of several macrochaetae proneural clusters. The analysis of genetic mosaics of cells lacking both sal and salr indicates that the function of these genes is required for the formation of a subset of macrochaetae. We also show that expression of Sal is specifically eliminated from the SOPs soon after they can be identified, and that ectopic expression of sal or salr in these SOPs prevents their differentiation.

MATERIALS AND METHODS

Drosophila strains

Flies were raised on standard Drosophila medium at 25°C. Mutations not described in the text and balancer chromosomes can be found in Lindsley and Zimm (1992). We used the reporter lines ara-lacZ (irorF209; Gómez-Skarmeta et al., 1996), emc-lacZ (emcF3C; Garrell and Modolell, 1990) and neu-lacZ (newF101; Ghysen and O’Kane, 1989); the GAL4 lines GAL4-253, GAL4-MS209 and GAL4-765 (Gómez-Skarmeta and Modolell, 1996); and the UAS lines UAS-wg (Klein et al., 1998), UAS-hh (Guillén et al., 1995), UAS-ci (Dominguez et al., 1996), UAS-dpp (Staehling-Hampton and Hoffmann, 1994), UAS-lacZ (Brand and Perrimon, 1993), UAS-sal and UAS-salr (de Celis et al., 1996). The sal mutants used were Df(2L)32FP-5, a small deficiency that deletes both sal and salr (de Celis et al., 1996), and FCK-25, a translocation with a breakpoint 5’ to the salr gene (data not shown). The wingless mutations used were wgF07, wgCX3 and wgCX4. We also used the following recombinant chromosomes: tkvF125 FRT40A (a gift from I. Rodriguez), pkaB3 FRT40A (Li et al., 1995), pkaB3 dppF12 FRT40A (Pan and Rubin, 1995) and armLacZ FRT40A.

Identification of sal/salr regulatory regions in the wing disc

Individual EcoRI fragments originated from digestion of the phages located between the breakpoints of FCK-25 and FCK-68 mutations (except the ones covering the coding region – phage G3.1 described in Frei et al., 1988, Fig. 2) were subcloned into the same restriction site of the enhancer tester C4PLZ vector (Wharton and Crews, 1993). These fragments were placed in front of a weak P-element promoter attached to the nuclear lacZ gene. The vector also contains the mini-white gene as an eye colour marker. The constructs generated were introduced in the germline by P-element transformation as described (Rubin and Spradling, 1982). The β-gal expression was detected by immunostaining of 3rd instar larval tissues. For each construct, at least three independent lines were tested.

Generation of mosaics

Gal4-expressing clones

The expression of Gal4 was induced in individual cells using a combined Gal4-Flip out system (de Celis and Bray, 1997). Clones were labelled by the presence of β-gal, and the expression of sal was monitored in clones of cells that express the proteins Wg, Hh, Dpp and Ci using the corresponding UAS lines. The genotypes of the larvae were F6a hsFLP1.22; P[Ubx/abx]FRT40A FRT40A/Gal4-β-gal]UAS-wg (or UAS-hh, UAS-dpp, UAS-ci). Clones were induced by 7 minutes heat shock at 37°C: 48-72 hours after egg laying.

D(f2L)32FP5 clones

Mutant cells homozygous for a deficiency of sal and salr (D(f2L)32FP5) were induced by X-ray-mediated mitotic recombination in flies of genotype F6a; D(f2L)32FP5; M(2)30 In(f*)j35B. Clones were labeled by the homozygosity of the cell marker forked.

FLP/FRT clones

Mitotic recombination was induced by 1 hour heat shock at 37°C in larvae of the following genotypes: hsFLP1.22; tkvF125 FRT40A/ armLacZ FRT40A, hsFLP1.22; pkaB3 FRT40A/ armLacZ FRT40A and hsFLP1.22; pkaB3 dppF12 FRT40A/ armLacZ FRT40A. Mutant clones were visualised in third instar larvae by the absence of β-gal expression.

Generation of antisera

Two fragments of spal cDNA (from amino acid 403 to 515 and from 803 to 936; Kühlein et al., 1994) subcloned in frame into the pRSET C vector (Invitrogen; these subclones were kindly provided by R. Shih) were expressed in BL21 cells. The resulting truncated Sal proteins were purified according to the manufacturer instructions and injected together in rats and rabbits following standard protocols.

Immunocytochemistry

Imaginal discs were stained with anti-β-gal antibodies following standard procedures. Rabbit anti-β-gal from Cappell was used at a final 1/5000 dilution in PBT-BSA (PBS, 0.5% Triton X-100, 1% BSA). For double staining, rabbit anti-β-gal (1/1000) and rat anti-Sal (1/200) antibodies were used. We also used mouse anti-Ac (1/20), mouse anti-En (1/20), mouse anti-Sal (1/500), mouse anti-Wg (1/5000), rabbit anti-Sal (1/200) and rat anti-Ci (1/100) antibodies. Secondary antibodies were from Jackson Immunological Laboratories and were used at a final 1/200 dilution. Samples were analysed with a Zeiss Axiohot for HRP staining or a Leika TCS confocal microscope for double staining with secondary fluorescence antibodies.

RESULTS

Molecular analysis of sal and salr regulation in the wing disc

The genes sal and salr are expressed in identical patterns in the wing disc (de Celis et al., 1996). The territory of expression includes a central stripe in the wing blade, anterior and posterior pleural regions and a subset of the proximal hinge and thorax (Fig. 1B). The region necessary to drive both sal and salr expression in the thorax and some domains of the wing is approximately 60 kb in size, and is located between the breakpoints of two translocations, FCK-25 and FCK-68 (Fig. 1 and data not shown). We cloned this DNA in fragments of 0.3 to 10 kb in front of the reporter lacZ gene to uncover the relevant regulatory elements in the wing disc. Several fragments directly express β-gal in places where sal and salr are present in the thorax (AS, ABO, ABI and LA), hinge (EME, AL, LA), pleura (BE, EME, AK, BI) and wing blade (BE, EME, BO, LA, BI, AK; Fig. 1). In most cases, the expression of β-gal occurs both in subdomains where sal and salr are normally expressed and in specific ectopic domains (Fig. 1). For each construct, β-gal expression patterns were identical in (at least) three independent transformant lines, demonstrating that the complexity of each pattern is generated by the driving DNA, and is not due to insertion position effects (data not shown). For instance, the constructs ABO and ABI, which overlap by approximately 2.2 kb, reproduce the
endogenous expression of sal/salr in the posterior compartment of the thorax (Fig. 1); they also express β-gal in the anterior pleura (Fig. 1), a place where these genes are not normally transcribed. Similarly, the construct LA is expressed in a pattern that includes part of the domain of endogenous sal/salr expression in the thorax and also in an anterior ectopic domain in the wing blade (Fig. 1). Several constructs located 5’ to the salr gene or in the large intron upstream of its coding region (BE, EME) direct generalised expression of β-gal in the wing blade, in a pattern that includes the stripe of normal sal/salr expression but also adjacent ectopic anterior and posterior regions (Fig. 1). The observation that consistent expression of β-gal is driven by several constructs in places where sal and salr are not expressed suggests that the regulation of these genes involves the interplay of both activating and repressing regulatory sequences. The combined analysis of these β-gal constructs, in a total of 92 independent transgenic lines, allows a broad localisation of multiple DNA sequences responsible for sal and salr expression in the wing disc (Fig. 1). It also suggests that the expression of sal/salr in the different parts of the wing disc is regulated in an independent manner. We have shown previously that, in the wing blade, these two genes are regulated by Dpp (de Celis et al., 1996), and the present molecular analysis predicts that the expression in thorax, hinge or pleura is achieved by a different set of factors (see below).

Wg, Dpp and Hh are implicated in the regulation of sal/salr expression in the wing thoracic region

The sal and salr genes are expressed in only part of the thorax, in three domains (1, 2 and 3 in Fig. 1A), which were defined with reference to en, wg and ci: the thoracic posterior compartment marked by En (Figs 1A, 2A), an adjacent stripe anterior to the anteroposterior compartment boundary corresponding to the stripe of maximal accumulation of Ci (Figs 1A, 2B), and a zone between the stripe of wg expression and the hinge (Figs 1A, 2C). A fourth domain (4 in Fig. 1A), the central thorax from where only microchaetae develop, does...
not express *sal/salr*. To explore the regulatory mechanisms that localise *sal/salr* expression with respect to the antero-posterior compartment boundary and *wg*, we first performed experiments in which genes that function in developmental signalling were expressed ectopically using the Gal4 system (Brand and Perrimon, 1993; see Materials and Methods).

A series of experiments led to the conclusion that, in the thorax, dual *hh* signalling is required to induce *sal/salr* expression: signalling through *dpp* and signalling that is *dpp* independent. Thus, expression of *hh* in clones within the central thorax (presumably accompanied by induction of *dpp*) leads to ectopic expression of *sal/salr*; interestingly, this ectopic expression is observed both in *hh*-expressing cells and in adjacent cells (Fig. 2E). In contrast, ectopic expression of *dpp* does not result in activation of *sal* transcription in the thorax (Fig. 2G) or in the hinge (Fig. 2F), but it does so in the wing blade (Fig. 2F). We also mis-expressed in clones of cells the transcription factor Cubitus interruptus (Ci), a key mediator of Hedgehog signalling (Ruiz i Altaba, 1997). We find that Ci is only able to activate *sal* ectopically in the wing blade (Fig. 2H), a place where ectopic expression of Ci results in novel expression of *dpp* (Dominguez et al., 1996), but not in the central thorax or wing hinge (Fig. 2H, I). In any other tissue studied to date, Hh signalling depends on Ci; since *hh* positively regulates *sal* in the thorax, the failure of ectopic Ci to activate *sal* expression there may be ascribed to the presence of countermanding repressors (see below).

However, even though *dpp* is not sufficient to induce *sal/salr* in the thorax, it is required. Thus, mitotic clones of *Pka* (corresponding to constitutive activation of hh signalling; Li et al., 1995; Pan and Rubin, 1995) show cell autonomous expression of Sal in a *Pka* clone localised in the central thorax (arrow). Sal expression remains unchanged (arrowhead) in a mutant clone generated in thoracic region 2 (see Fig. 1). (B-B") Expression of Sal in the thorax in *Pka, dpp* double mutant clones. Due to the absence of *dpp*, *Pka* mutant clones are now incapable of inducing *sal* ectopic expression in the central thorax (arrowhead). In other thoracic regions, the double mutant cells show a reduction in the level of expression of *Sal/Salr* (arrow). (C-C") Example of *tkv*5/w124 clone that causes a reduction in Sal expression in region 2 (arrow), but do not modify this expression in other areas (arrowhead). (D-D") The reduction of Tkv levels does not modify the *sal* expression levels in the central thorax or in region 3 (arrowheads).
The requirement of dpp function for induction of sal differs in different parts of the thorax. In the central thorax, where sal is not expressed normally, tkv clones have no effect (Fig. 3D, arrowhead). In region 2 (Fig. 1A), where dpp is expressed normally, tkv clones result in reduced expression of sal (Fig. 3C, arrow). In other regions of the thorax, expression of sal is unaffected by the reduction of tkv (Fig. 3CD, arrowheads).

A prominent stripe of wg expression is seen in the thorax, within the sal non-expressing region 4 and close to the border of the sal-expressing region 3 (Figs 1A, 4A,C). This observation raised the possibility that wg may act as a repressor of sal expression in the thorax, and possibly in other regions of the wing disc. Indeed, in thoracic region 3, sal expression is repressed in and around clones of cells that overexpress wg (Fig. 4E,F). Furthermore, wg overexpression in the hinge region (in Gal4-MS209/UAS-wg) results in a reduction of sal expression (Fig. 4E), whereas a reduction of wg expression in the hinge of imaginal discs as a result of the regulatory mutation spade flag (wg<sup>med</sup>) results in a consistent increase in sal expression (Fig. 4B). Reduction of wg expression in the thorax in the heteroallelic combination wg<sup>CX3</sup>/*<sub>wg</sub>CX4 results in the expansion of Sal expression (Fig. 4D). However, Sal is not expressed in all region 4, indicating that a repressor other than wg is responsible for the exclusion of Sal in this region.

It is likely that both sal activation by hh and dpp and its repression by wg are mediated by the regulatory regions that we identified as responsible for direct sal/salr expression in the thorax (Fig. 1). As described before, the expression of β-gal driven by the sal/salr regulatory regions occurs both in the normal domain of sal/salr expression and also in some ectopic territories (Figs 1, 5A,C). The expression of β-gal in the transgenic line LA is included in the territory of sal/salr expression in the thorax (Fig. 5D). Much of this expression is removed when wg is expressed ectopically (Fig. 5E,F). Wu et al. used two Gal4 drivers, Gal4-253, which is expressed in all proneural clusters and in the SOPs (not shown), and Gal4-756, which is expressed homogeneously at a lower level throughout the thorax (Gómez-Skarmeta and Modolell, 1996; data not shown). When wg overexpression is generalised and at low level (Gal4-756/UAS-wg), the expression of the LA enhancer is limited to the dorsal part of thoracic region 2 (Fig. 5E). When wg overexpression is stronger but focused in the region of macrochaetae precursors (Gal4-253/UAS-wg; see below), the expression of the LA enhancer is only seen in a ventral part of region 2 (Fig. 5F). These results confirm the repressive action of wg and indicate that it is mediated by sequences contained within the LA fragment. However, in the same experiments, the endogenous sal expression is unaffected by wg overexpression (Fig. 5E,F), suggesting that the levels or time of expression of ectopic wg are ineffective in repressing sal/salr expression. Therefore, we suggest that, in these experiments, the inhibitory action of wg can be counteracted by sequences of the endogenous regulatory region located outside the LA fragment. Consistent with a requirement for both hh and dpp signalling to activate sal/salr in the thorax, the expression of β-gal in the line LA is not modified when dpp is expressed ectopically (data not shown).

**Relationships between sal expression and sensory organ patterning in the thorax**

The domain of sal/salr expression in the thorax was mapped with more precision with respect to the emerging Sensory Organ Precursor cells (SOP), which can be identified in the disc using the reporter line neuralised-lacZ (neu-lacZ; Ghysen and O’Kane, 1989). The sensory organs included in the sal/salr domain are most of the lateral macrochaetae (ANP, PNP, ASA, PSA, APA, PPA) and also the ASC, PSC and PDC macrochaetae (Fig. 6E,F; see figure legend for abbreviations); the PS and ADC macrochaetae arise outside this domain. As shown above, sal and salr are not expressed in the central domain of the thorax, the region from which most of the microchaetae will develop during pupal development. This was confirmed by demonstrating that extramacrochaetae (emc), a negative regulator of the ac and sc genes that marks the microchaetae territory, is expressed in a domain nearly

![Image](image.png)

**Fig. 4.** Effects of wg on Sal expression in the thorax. In all panels, Wg is shown in red and Sal in green. (A) Expression of Wg and Sal in wild-type late third instar wing disc. The limit of Sal expression is slightly more lateral than the Wg expression region in the central thorax. (B) Expression of Wg and Sal in wg<sup>med</sup> mutant wing disc. In this genotype, wg expression is strongly reduced in the hinge, where sal expression is now enhanced (arrow). (C) Expression of Wg and Sal in a wild-type thorax. (D) Expression of Wg is almost absent in the heteroallelic combination wg<sup>CX3</sup>/*<sub>wg</sub>CX4 and Sal expression extends into more lateral regions. (E) Expression of Wg and Sal in Gal4-MS209/UAS-wg wing disc, showing a strong reduction of Sal expression in the expanded hinge region (arrow). (F) Sal expression is reduced at and around clones of cells that express wg ectopically (labelled in red, see Material and Methods). This repression is clearly evident in the lateral thorax (arrowheads), whereas within the Sal expression domain in the wing pouch ectopic wg does not repress sal (arrow; yellow). (G) Higher magnification of the thoracic region of a disc carrying wg-expressing clones. Below, single green channel showing the absence of Sal in the clones, and its reduction in immediately surrounding cells.
complementary to sal/salr (Fig. 6C). Sal is expressed in the domain that encompasses most of the macrochaetae proneural cell clusters (Fig. 6A,B). Interestingly, when specific cells of the proneural clusters are ‘singly out’ to form the SOP, sal expression is eliminated from these cells and their descendants (Fig. 6G,H). This is a requisite for SOP development, because when sal expression is experimentally maintained in SOP cells they do not differentiate (see below).

The localisation of most macrochaetae SOPs within the territory of sal/salr expression in the thorax, and the dynamics of sal expression associated with proneural clusters (Sal+), and SOPs (Sal−), raise the possibility of a function for the sal/salr genes in sensory organ patterning. The requirement of sal and salr was studied by inducing clones of cells homozygous for a deficiency, Df(2L)32FP5, that includes both genes. Mutant clones in the thorax, marked with forked, were viable and of normal size, indicating that sal and salr are not required for the viability of thoracic cells (data not shown). Loss of salr caused the absence of two macrochaetae, the ANP and PNP, and also the anterior displacement of the PSC and PDC, which differentiated abnormally close to their anterior counterparts; the remaining seven macrochaetae were unaffected (Fig. 7B).

A similar, albeit weaker, phenotype was observed in some different UAS lines, named UAS-salr1 and UAS-salr2, which produce low and high levels of Salr expression, respectively (Fig. 7D,H and Gómez-Skarmeta et al., 1996). As expected from the expression pattern of sal and salr, the differentiation of the macrochaetae was not affected either in heteroallelic combinations or in homozygous Df(2L)32FP5 clones (Fig. 7B).

The influence of sal and salr on macrochaetae pattern formation was also studied in experiments in which either of these genes was ectopically expressed in the thorax using the Gal4 system. In the case of salr, we also used two different UAS lines, named UAS-salr1 and UAS-salr2, which produce low and high levels of Salr expression, respectively, in combination with any Gal4 driver (not shown). When sal or salr were expressed at low levels in all thoracic cells (combinations between Gal4-756 with UAS-sal or UAS-salr1) several extra macrochaetae differentiated in ectopic positions (Fig. 7E,F and data not shown), indicating that sal and salr have the capability to promote SOP development. In addition, stronger ectopic expression of salr using the UAS line UAS-salr2 caused the absence or size reduction of some macrochaetae (Fig. 7F).

These contradictory effects on the pattern of macrochaetae when sal/salr are ectopically expressed were associated with defective localisation of proneural clusters in the imaginal disc. Thus, generalised low expression of salr (UAS-salr1) causes a weak expansion of the APA proneural cluster (Fig. 7I), whereas higher levels of generalised salr expression (UAS-salr2) result in a broader rearrangement of proneural clusters, including a larger expansion of the DC and NP (which now appear fused), and the apparent loss of the PA proneural cluster (Fig. 7J). These results indicate that the restriction and levels of sal/salr expression participate in the positioning of proneural clusters in the thorax.

Combinations between Gal4-253 (which strongly targets expression to all proneural clusters and SOPs) with UAS-sal, UAS-salr1 or UAS-salr2 have dramatic effects on the macrochaetae (Fig. 7G and data not shown). Only APA formed normally; the others either disappeared or differentiated as extremely small bristles (ADC, PDC, PPA and PSC; Fig. 7G). The effects of ectopic sal or salr overexpression specifically in the macrochaetae SOPs were further investigated by following directly the formation of each SOP and its progeny in the wing disc. We found that, in Gal4-253/UAS-sal wing discs, all proneural clusters, where Sal is overexpressed, appear in their normal positions, and that later the SOPs are singled out normally

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**Fig. 5.** Comparison of Sal and β-gal protein localisation in several reporter lines and effects of wg on LA reporter expression. Expression of endogenous Sal (green) and transgenic β-gal (red) in the reporter lines BE (A), AK (B), ABI (C) and LA (D-F). Expression of β-gal in each line occurs in places where Sal and Salr are normally expressed (thorax in the lines LA and ABI; pleura in AK; wing blade in BE) and, in some cases, also in positions where Sal/Salr are not expressed (anterior and posterior domains in the wing blade in BE and vein L5 in AK). (E,E,F) From LA discs where wg is ectopically expressed (Gal4 lines 756 and 253), respectively, resulting in reduced β-gal expression. E′ and inset in F present the single red channels, which shows β-gal expression restricted to the most dorsal and lateral domains, respectively, of LA β-gal pattern.
but either do not divide or divide later than normal (Fig. 7K). These observations indicate that the elimination of sal expression from the SOPs observed in normal development is functionally significant, being a requisite for later SOP differentiation.

DISCUSSION

The *Drosophila* thorax appears to be divided into domains of expression of prepattern genes (Modolell and Campuzano, 1998; Calleja et al., 1996), which might be related with the patterning of sensory organs. In this work, we have identified the gene complex *spalt/spalt-related* as a member of this category of genes. sal and salr encode proteins characterised by paired zinc-finger domains that have been conserved in structure and sequence during evolution. So far, only the DNA-binding affinity of *Drosophila* Salr has been tested; the central fingers of Salr show affinity for double-stranded A/T-rich sequences of DNA (Barrio et al., 1996). The structural and sequence conservation suggests that proteins of the Sal family recognise similar DNA sequences, although no downstream gene for any Sal protein has yet been identified.

Regulation of sal/salr expression

The expression of sal and salr in the thorax is restricted to a broad domain that includes the positions where most proneural clusters appear. As in many regulatory *Drosophila* genes, the genomic regions directing sal and salr expression are organised in modules, and we have been able to identify within a 60 kb region distinct regulatory domains responsible for distinct aspects of sal/salr expression in the thorax and other parts of the wing disc. A surprising observation is that many of the constructs consistently drive reporter gene expression in some places where sal/salr are not normally expressed. Most likely the genomic DNA used in such constructs contains enhancer sequences able to activate sal/salr expression and lacks other sequences that normally restrict spatially this activation. The observed ‘ectopic’ expression is frequently combined with...
expression in regions where the endogenous sal/salr genes are expressed. Thus, we envision the regulation of sal and salr as a complex process in which both transcriptional activators and repressors interact with particular regulatory regions to generate the normal restricted expression domains.

We do not know what genes participate directly in the regulation of sal and salr, although we expect that future refined characterisation of the putative sal/salr enhancers will help to identify conserved binding sites and consequently suggest candidate transcription factors implicated in sal/salr regulation. At this stage, it is clear that several signalling molecules play critical roles in defining the territories of sal/salr expression. Previous analysis showed that the stripe of sal/salr expression in the wing is under direct regulation by the TGF-β molecule Dpp (de Celis et al., 1996; Nellen et al., 1996; J. F. de Celis, R. Barrio and F. C. Katatos).
Lecuit et al., 1996). We have been able to identify fragments of DNA directing expression of the reporter gene in the wing blade, but none of them reproduces the restricted pattern of endogenous expression of sal/salr. A 6.7 kb long DNA fragment 5' to the sal gene reproduces the expression of sal in the wing blade, but smaller subfragments of this region do not (Kühnlein et al., 1997). The fragments that we have analysed in the equivalent region and in the first intron of salr are of considerably smaller size, and consistently result in expression of the reporter gene in ectopic positions of the wing blade. Thus, it seems that a large 5' regulatory region is responsible for the generation of the restricted central domain of expression of both sal and salr in the wing blade, and that both regions include a combination of Dpp-responsive positive elements as well as sites that mediate repression. Our analysis also shows that most enhancers responsible for sal and salr expression in other regions of the wing disc are located 3' to each gene. The level of resolution of the present analysis does not allow us to address whether there are common enhancers for both sal and salr or whether, alternatively, they are duplicated.

Some of the localised enhancers are regulated by factors other than Dpp, and we identify Wingless and Hedgehog as two additional signalling molecules that appear to participate in regulating sal/salr in the thorax and wing hinge. sal is expressed throughout the posterior thorax and in an adjacent sickle-shaped anterior region that excludes the central thorax (Fig. 8). The activation of sal by hh in the thorax requires the presence of dpp, because sal is only ectopically expressed in Pka mutant cells, but not in Pka, dpp double mutant cells. However, ectopic expression of dpp is not enough to activate sal/salr outside of its normal expression domain. We suggest that the requirement for both a short-range signal (Hh) and a long-range signal (Dpp) to activate sal/salr in the thorax contributes to the observed restriction of sal/salr expression within the thorax. A collaboration of hh and dpp signalling has also been observed in the activation of the iro gene complex expression in the wing blade (Gómez-Skarmeta and Modolell, 1996). We have shown that wg overexpression in thoracic region 3 can repress sal expression within a short range. Thus, the restricted pattern of thoracic sal/salr expression may depend not only on positive regulation by hh/dpp, but also on repression mediated by wg signalling. It is notable that Wg is expressed in a stripe of cells parallel to the central border of sal/salr expression (Fig. 8). However, the position of this stripe of wg-expressing cells with respect to the sal/salr expression domain is asymmetrical. Furthermore, strong reductions in wg function do not result in a complete expansion of sal expression in region 4. Therefore, we suggest that additional factors present here help repress sal/salr expression independently of wg (Fig. 8).

Both Hh and Wg signalling have dramatic effects on chaetae pattern formation (Phillips and Whittle, 1993; Mullor et al., 1997) and it is possible that some of these effects are mediated by sal and salr. Interestingly, the expression of a vertebrate sal homologue appears to be regulated by a hedgehog homologue (Köster et al., 1997), raising the possibility that, not only the structure, but also some aspects of the regulation of this family of transcription factors has been conserved. In conclusion, the regulatory sequences of sal/salr in the wing appear to integrate the action of multiple signalling pathways and transcription factors to generate the restricted expression of these genes. A similar situation occurs during embryonic development, where expression of sal is regulated by several maternal and gaps genes (Kühnlein et al., 1997).

A role for sal/salr in macrochaetae patterning and differentiation

The positioning and differentiation of sensory organs in the thorax is a multistep process that is initiated by the activation of proneural genes in clusters of cells named proneural clusters. Within each proneural cluster, a characteristic number of SOP cells are then singled out and undergo two differential divisions each to generate the four cells that form each sensory organ (Campuzano and Modolell, 1992). The normal expression pattern of sal/salr and the results of manipulating their activity indicate that these genes play significant roles in both the generation of particular proneural clusters and the differentiation of the SOPs. First, salr expression is present in the domain from which most proneural clusters will form. Second, elimination of salr activity in genetic mosaics shows that the function of these genes is required for the development of two notal macrochaetae (ANP, PNP) as well as the fine positioning of two other macrochaetae (PDC, PSC). Furthermore, some weak salr allelic combinations cause a reduction in the expression of the proneural gene ac in the proneural cluster corresponding to the affected macrochaetae. Third, weak but generalised ectopic expression of either sal or salr results in ectopic macrochaetae, and in considerable reorganisation of the corresponding proneural clusters, which is aggravated by higher expression levels of salr. Interestingly, ectopic expression of wg in the thorax represses or leads to the misplacement of a set of macrochaetae that are mostly unaffected by overexpression of Sal/Salr (Fig. 7C versus E,F).

Many macrochaetae, corresponding to proneural clusters and SOPs that are included in the domain of sal/salr expression, differentiate normally in the absence of these genes. This indicates that at these positions sal/salr are not essential for regulation of proneural genes, possibly because other activators of proneural genes can provide a redundant function. In summary, our results place Sal/Salr in the group of transcription factors implicated in the spatial activation of the proneural genes. This group also includes the proteins encoded by the iroquois gene complex (Gómez-Skarmeta et al., 1996) and Pannier/U-shaped (Cubadda et al., 1997). These transcription factors are expressed in a complex landscape of overlapping domains in the thorax, which could be part of the postulated prepatterning that directs SOPs positioning (Stern, 1954). It is not clear whether these overlapping expression territories are generated independently of each other, or whether, alternatively, some cross-regulatory interactions between these transcription factors participate in their refinement.

In contrast to its earlier expression throughout the pertinent proneural clusters, we find that Sal disappears from the SOPs from the moment that SOPs can be identified using the enhancer trap neu-lacZ. The elimination of Sal from the SOP and its progeny is of critical importance for differentiation of these cells, because strong ectopic expression of either sal or salr in the SOPs prevents or delays their differential divisions and leads to the disappearance of most of the macrochaetae or their replacement by very small bristles. SOP cells express a characteristic class of genes named pan-neural, such as asense (Dominguez and Campuzano, 1993) and deadpan (Bier et al., 1992), which participate in SOP differentiation and confer on
them specific characteristics. It is likely that future experiments might reveal that sal/salr interferes with the expression or activity of some of the pan-neural genes, resulting in the failure of SOP differentiation.

There are some parallels between the role of sal/salr in the patterning of veins and sensory organs in the wing and thorax, respectively. In both cases, the expression of these genes is under the regulation of signalling molecules (Dpp and Wg/Hh/Dpp respectively) and occurs in restricted domains. In both processes, sal and salr participate in the positioning of pattern elements, and they may do so by determining the places where cell differentiation promoting genes are expressed. The identification of sal/salr regulators and target genes is therefore of critical importance for deepening our understanding of the development of the wing disc, and for revealing the molecular mechanisms that link the activity of signalling molecules such as Dpp, Wg and Hh with the final pattern of differentiation.

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