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Organogenesis in *Drosophila melanogaster*: embryonic salivary gland determination is controlled by homeotic and dorsoventral patterning genes

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Summary

We have investigated *Drosophila* salivary gland determination by examining the effects of mutations in pattern forming genes on the salivary gland primordium. We find that the anterior-posterior extent of the primordium, a placode of columnar epithelial cells derived from parasegment 2, is established by the positive action of the homeotic gene *Sex combs reduced (Scr)*. Embryos mutant for *Scr* lack a detectable placode, while ectopic *Scr* expression leads to the formation of ectopic salivary glands. In contrast, the dorsal-ventral extent of the placode is regulated negatively. Functions dependent on the *decapentaplegic* product place a dorsal limit on the placode, while *dorsal*-dependent genes act to limit the placode ventrally. We propose a model in which these pattern forming genes act early to determine the salivary gland anlage by regulating the expression of salivary gland determining genes, which in turn control genes that are involved in salivary gland morphogenesis.

Key words: pattern formation, *Sex combs reduced, decapentaplegic, dorsal, fork head.*

Introduction

The analysis of embryonic development in *Drosophila melanogaster* has focused primarily on the establishment of the larval cuticular pattern. Genetic studies have led to the identification of many genes whose products interpret maternally provided positional information to form a prepattern which governs the differentiation of a segmented larva (Nüsslein-Volhard and Wieschaus, 1980; Lewis, 1978; for recent review see Ingham, 1988). It is largely unknown, however, how this pattern is decoded so that tissues form at their appropriate locations in the embryo. One approach to this problem has been to search for targets of patterning genes which mediate their effects on development (eg. Gould et al. 1990). A different approach is to study the development of a particular organ to learn how it forms in response to positional information. Here, we have applied this approach to the larval salivary glands, which are derived early in embryogenesis from placodes formed in the overtly segmented gnathal region of the head, where segmentation and homeotic genes are known to be important in pattern formation.

Until recently, genetic analysis has ignored the development of many internal organs because mutations affecting this process are not expected to result in easily detectable cuticular phenotypes. The recent discovery that some genes governing cuticle pattern are also involved in the development of internal organs has provided approaches for the further study of organogenesis. For example, the observation that the pair-rule genes *fushi tarazu (ftz)* and *even-skipped* are re-expressed during neurogenesis has led to the conclusion that these genes specify the identity of particular neurons (Doe et al. 1988b; Doe et al. 1988a). Similarly, the observation that homeotic, segment polarity, and dorsoventral patterning genes are later expressed in the gut has enabled the discovery of roles for these genes in the formation of gut annexes and constrictions, and has also established the importance of inductive interactions in proper gut development (Tremml and Bienz, 1989; Immerglück et al. 1990; Reuter and Scott, 1990; Reuter et al. 1990; Panganiban et al. 1990). There are several examples of genes required for early pattern formation that are re-expressed during salivary gland development. We have taken advantage of the expression of several of these genes to mark the salivary gland during its early developmental stages. The gene we have used most is *fork head (fkh)*, which is required early for proper development of the embryonic termini and is later redeployed in several internal tissues, including the salivary glands (Jürgens and Weigel, 1988; Weigel et al. 1989a; Weigel et al. 1989b). We have been able to use an antibody recognizing the *fkh* product to study the effects on salivary gland determination of mutations in pattern forming genes. Another gene that

is expressed in the salivary glands and placodes, but which was identified by its mutant cuticular phenotype, is *Toll (Tl)* (Gerttula et al. 1988; Hashimoto et al. 1991). We have used antibodies against its product to confirm our results using anti-*fkh*. In addition, we have used an antibody recognizing the product of the *crumbs (crb)* (Tepaß et al. 1990) gene to visualize the lumen of the salivary gland and its associated ducts.

Using these tools, we show here that homeotic genes and genes regulating dorsoventral pattern define the pattern elements that position and determine the salivary gland placode. Furthermore, we suggest the existence of a hierarchy of gene expression governing salivary gland determination and morphogenesis. Our results provide a genetic framework for studying the molecular events leading to salivary gland determination.

Materials and methods

Drosophila *stocks*

An Oregon-R lab stock was used as wild type. lab^{vd1}, lab^{f8}, $In(3R)lab^{a73}$, Dfd^{rR1}, Dfd^{rW21}, Scr^{eK6}, Scr^{eW17}, $Antp^{NS+R11}$, $Antp^{w10}$, ftz^{m13}, ftz^{w20}, hb^{14f}, and zen^{W36} were all obtained from the Indiana Stock Center. spi^{IIA14}, rho^{7M43}, and pnt^{9J31} were obtained from the Bowling Green Stock Center. sim^{H9}, dpp^{Hin48}, dl^{1}, tld^{B6}, and srw^{B2} were obtained from K. Anderson. The enhancer trap line N33, which expresses β-galactosidase in salivary placodes and glands, was a gift of C. Klämbt. HS3, a line bearing a fusion of the inducible *hsp70* promoter to the *Scr* structural gene (Gibson et al. 1990) was a gift of W. Gehring. P[ry^+, HNK] is a transformed line that carries a fragment of the *fkh* regulatory region containing a salivary gland enhancer element (Weigel et al. 1990) driving expression of *lacZ* (B. Zhou and S.K.B., unpublished data). Some mutations were placed in *trans* to one of the *lacZ*-marked balancer chromosomes TM3β and CyOβ (gifts of Y. Hiromi). Embryos from such balanced stocks that did not stain with anti-β-galactosidase (obtained from 5 Prime-3 Prime, Inc.) were presumed to be mutant. dpp^{Hin48} was balanced over the chromosome In(2LR)CyOP20, *P[*dpp^{Hin+}*]*, which covers the haploinsufficient lethality of this mutation (R. Padgett and W. Gelbart, personal communication). A chromosome bearing both dpp^{Hin48} and *dl* was generated by recombination and maintained as the balanced stock *Dp(2;1)G146 / +;* dpp^{Hin48} *dl / SM6B*. *Dp(2;1)G146* is an X-chromosomal duplication which covers the *dpp* haploinsufficiency (Irish and Gelbart, 1987).

Immunohistochemistry

Embryos collected on grape juice/agar plates were dechorionated in 50% Chlorox followed by 0.7% NaCl, 0.1% Triton X-100, and fixed in a 1:1:2 mixture of 1× PBS: 10% formaldehyde (E. M. Grade, Polysciences): heptane (HPLC grade, Aldrich). The embryos were devitellinized by shaking in the heptane with 90% methanol, 5 mM EGTA, and were washed extensively with methanol. Antibody staining was performed essentially according to Patel et al. (1989), using the Vectastain Elite kit (Vector) or secondary antibodies directly coupled to alkaline phosphatase (AP) or horseradish peroxidase (HRP) (Jackson Immunoresearch). Color was developed using Vector substrate kit III for AP or 0.5 mg/ml diaminobenzidine, 0.06% H_2O_2 for HRP. Embryos were cleared in methyl salicylate (HRP) or 70% glycerol, 1× PBS, and examined with Nomarski DIC optics on a Zeiss Axiophot. Except where noted, at least 50 mutant embryos were examined for each allele tested.

Anti-*Toll* (Hashimoto et al. 1991) was obtained from C. Hashimoto. Anti-*crumbs* (Tepaß et al. 1990) was obtained from E. Knust. Anti-*engrailed* (MAb 4D9, Patel et al. 1989) was obtained from J. Heemskerk and A. Brivanlou.

Heat shocks

3 hour embryo collections were aged to the desired time, dechorionated, and incubated in a 37°C water bath for 30 minutes. The embryos were then removed from the water bath and returned to 25°C until the embryos were 7-10 hours old, an age at which all embryos should have visible salivary glands or placodes. The embryos were then fixed and stained as described above.

Results

Origin of the salivary glands

The development of embryonic salivary glands has been described by Campos-Ortega and Hartenstein (1985), and here we summarize this process. The salivary gland primordia are flat, disc shaped placodes of columnar epithelial cells which form during stage 11 (germ band extension) on the ventral side of the embryo, in the labial lobe, a gnathal head segment. At the end of stage 11, the placodes begin to invaginate near their posterior edges (Fig. 1A). Invagination continues during germ band retraction (Fig. 1B) until, at approximately the end of stage 12, all of the gland cells have been internalized (Fig. 1C). During stages 13-15 duct formation occurs and the glands come to lie horizontally within the embryo (1D,E). As illustrated in Fig. 1F, each gland is connected by ducts which empty into the pharynx. The duct cells appear to originate from the epidermis between (ventral to) the placodes.

To determine the exact location of the placode with respect to parasegmental borders, we double-stained embryos with anti-*fkh* and anti-*engrailed (en)*. Expression of the *fkh* product in the uninvaginated placodes is uniform in some embryos (Fig. 2B). Other embryos have nonuniform expression, in which staining is more intense near the posterior edge of the placodes (Fig. 2A). Because *fkh* expression in placodes that have just begun to invaginate is uniform (data not shown), we infer that embryos with nonuniformly staining placodes are slightly younger than those with uniform expression and are just beginning to express *fkh*. The placodes in these younger embryos frequently appear more irregular in shape than those of older embryos. This change in the shape of the placode might occur by changes in the expression of *fkh* in cells at the edge of the placode or by increases in adhesion among the placode cells as they become determined. Because the placode cells become columnar as opposed to the cuboidal shape of the surrounding cells (Sonnenblick, 1950), and because they express cell surface adhesion molecules such as *Notch* (Fehon et al. 1990; Johansen et al. 1989; Kidd et al. 1989) and *Toll* (Keith and Gay, 1990; Gerttula et al. 1988; Hashimoto et al. 1991), we believe that increasing adhesion is at least partially

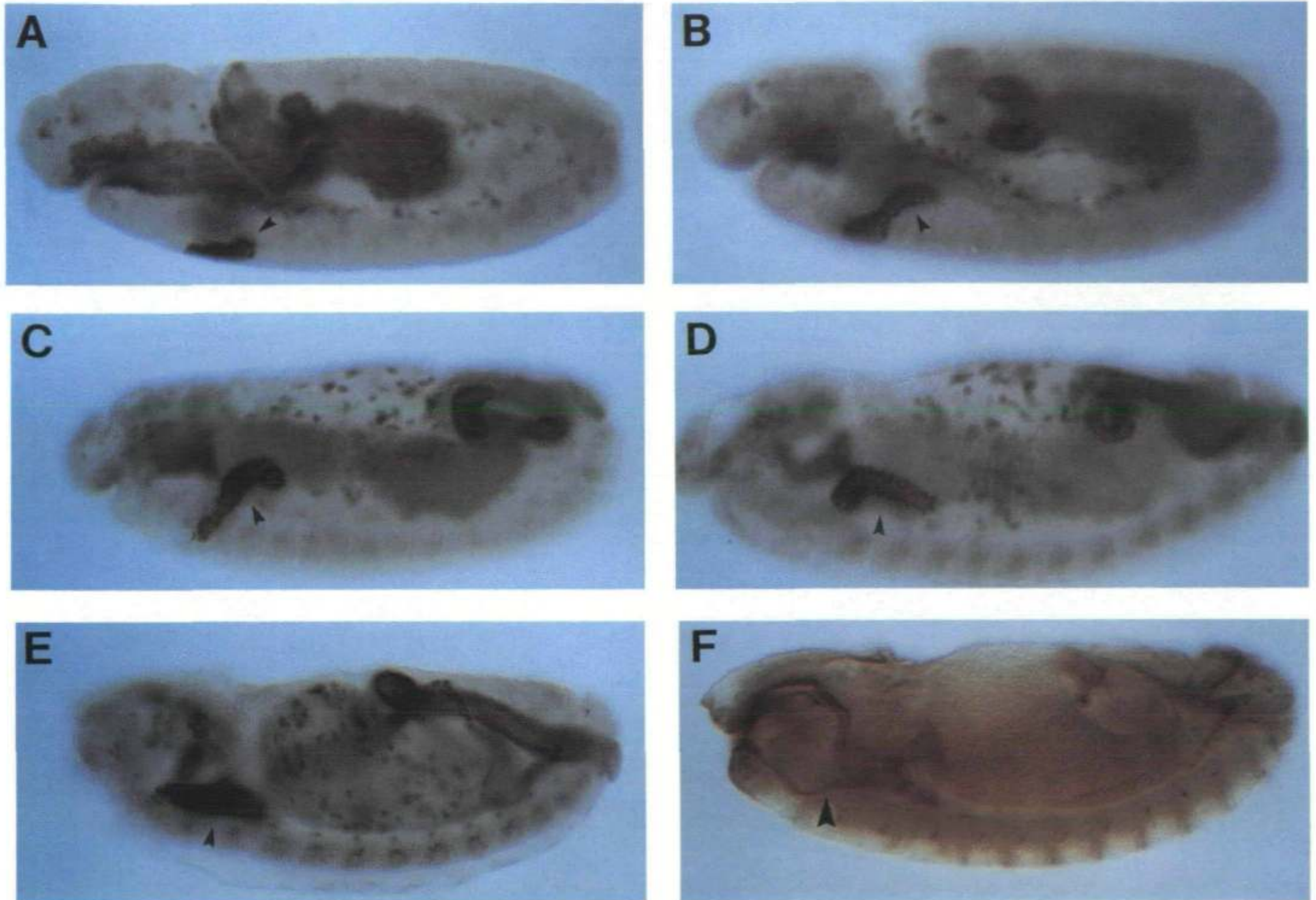

Fig. 1. Development of the salivary glands. Immunohistochemical staining of wild-type embryos. (A-E) Anti-*fkh* (Weigel et al., 1989b) staining. The salivary placode and gland are indicated by small arrowheads. (A) Late stage 11/early stage 12 embryo. Invagination has just begun near the posterior edge of the placode (small arrowhead). (B) Stage 12. (C) Stage 12/13. The last *fkh*-expressing cells in the salivary gland have left the surface of the embryo (D) Stage 14. (E) Stage 15. (F) Stage 14/15, stained with anti-*crb*, which preferentially stains the lumen of the glands and ducts (Tepaß et al., 1990). Large arrowhead indicates junction of a gland and its duct. The end of the duct is being involuted into the pharynx. All panels are lateral views. In this and all subsequent figures, dorsal is up and anterior is to the left for lateral views, and for ventral views anterior is to the left.

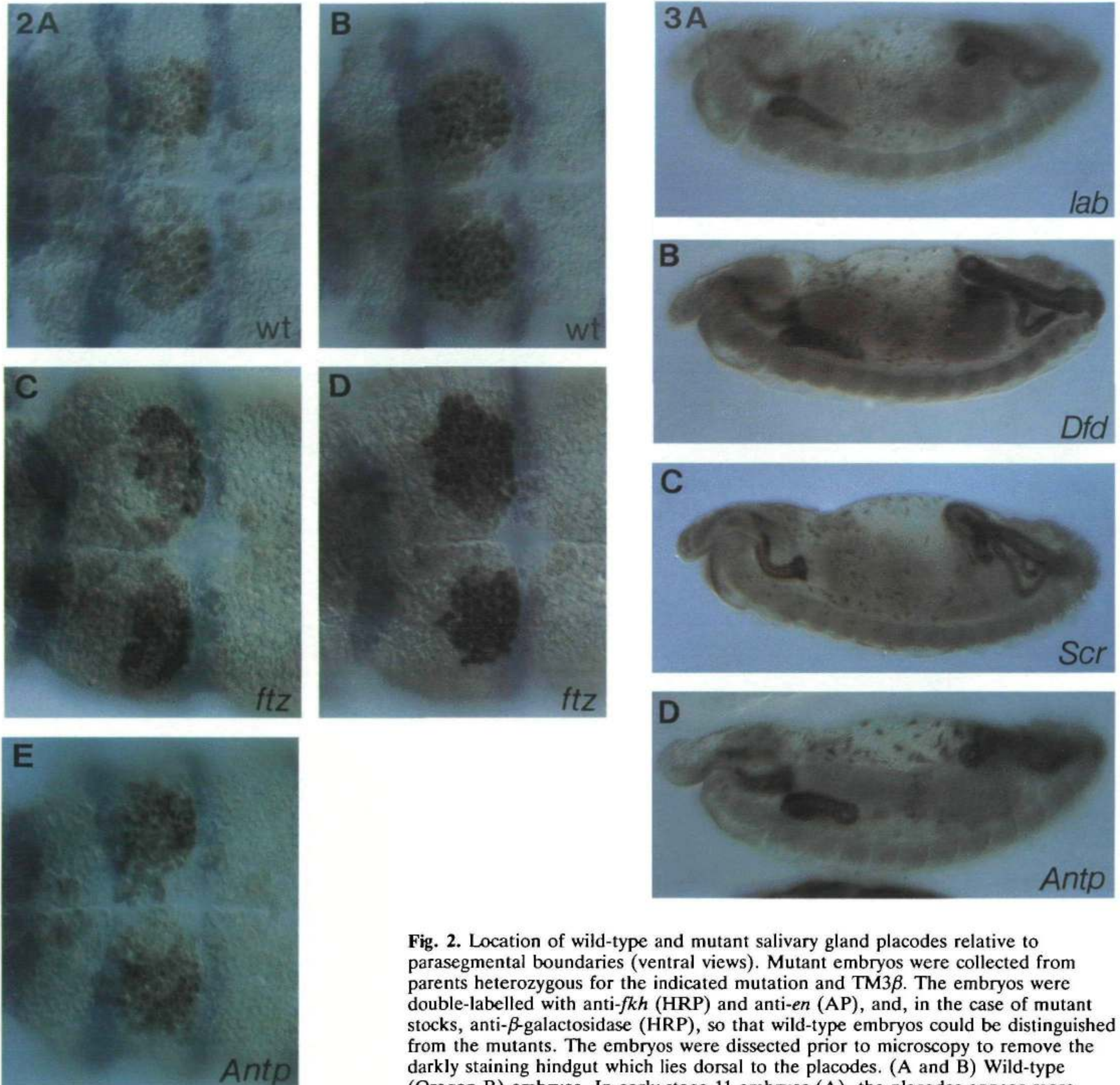

Fig. 2. Location of wild-type and mutant salivary gland placodes relative to parasegmental boundaries (ventral views). Mutant embryos were collected from parents heterozygous for the indicated mutation and TM3β. The embryos were double-labelled with anti-*fkh* (HRP) and anti-*en* (AP), and, in the case of mutant stocks, anti-β-galactosidase (HRP), so that wild-type embryos could be distinguished from the mutants. The embryos were dissected prior to microscopy to remove the darkly staining hindgut which lies dorsal to the placodes. (A and B) Wild-type (Oregon-R) embryos. In early stage 11 embryos (A), the placodes appear more ragged and the intensity of *fkh* staining is less uniform than in slightly older embryos (B). In both early and late stage 11 embryos, the placodes overlap the PS2 *engrailed* stripe, extending anteriorly to the PS1/2 border, while they do not overlap the PS3 stripe but abut the PS2/3 border. (C and D) ftz^{w20} mutant embryos. Again, early embryos (C) have more ragged placodes than older embryos (D). In both cases, the placodes abut the PS2/3 border but extend anteriorly only about half way across the fused PS1-PS2. This allele of *ftz* is a protein null (Weiner et al., 1984; Carroll and Scott, 1985). (E) $Antp^{w10}$ mutant embryos have placodes that are not distinguishable from those of wild-type embryos.

Fig. 3. Salivary gland development in homeotic mutants. Lateral views of embryos collected from parents heterozygous for the indicated mutation and TM3β, stained with anti-*fkh* and anti-β-galactosidase. (A) lab^{f8}. (B) Dfd^{rR1}. (C) Scr^{eW17}. (D) $Antp^{w10}$. In each case except for *Scr*, salivary gland development was completely normal. In the case of *Scr*, no salivary gland or placode was ever detectable with anti-*fkh*, anti-*Tl*, or salivary gland specific enhancer traps. Each of these mutations is null or presumably null (Merrill et al., 1989; Merrill et al., 1987; Riley et al., 1987; Carroll et al., 1986), and similar results were obtained using the other alleles mentioned in Materials and methods.

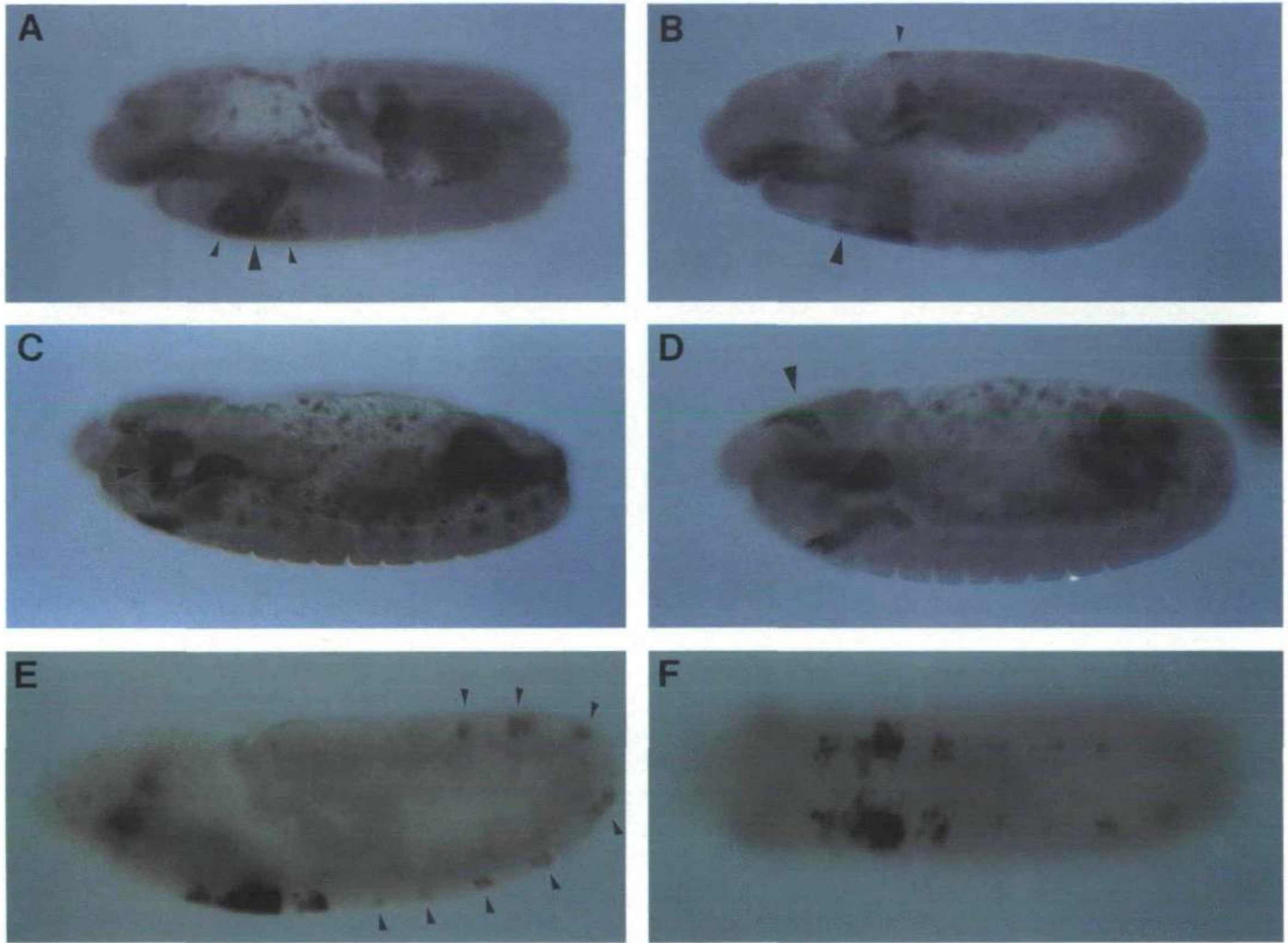

Fig. 4. Global expression of *Scr* induces the formation of ectopic salivary gland tissue. (A-D) Lateral views of embryos homozygous for the heat shock-*Scr* construct HS3 (Gibson et al., 1990). Embryos were heat shocked at 37°C for 30 minutes and allowed to recover at 25°C until aged 7-10 hours. The embryos were then fixed and stained with anti-*fkh*. (A) Stage 12 embryo. 3 invaginated salivary glands are visible in this plane of focus. The one in the center (large arrowhead) arises from PS2, while the smaller ectopic structures arise from PS1 and PS3 (small arrowheads). (B) Stage 11 embryo. A small patch of ectopic *fkh* expression is observed in PS1 (large arrowhead) and in A8 (small arrowhead). (C) Stage 12-13 embryo. An ectopic placode in PS1 has invaginated and fused with the main salivary gland, forming a small anterior branch (arrowhead). This embryo also had an invaginated salivary gland arising from the procephalon, which is difficult to see in this photograph. (D) A late stage 12 embryo with a prominent invaginated salivary gland arising from the procephalon (arrowhead). (E) Lateral and (F) ventral views of a heat shocked stage 11 embryo heterozygous for HS3 and the *fkh* enhancer-*lacZ* construct P[ry$^+$, HNK] which normally expresses β-galactosidase only in salivary glands and placodes, and in the anal pads (B. Zhou, personal communication). In addition to the normal staining observed in PS2, and the ectopic staining in PS1, PS3, and two patches in the procephalon (out of this plane of focus), we observed small patches of β-galactosidase expression in PS4-11, which are not typically observed by anti-*fkh* staining of HS3 embryos. These patches occupy the same dorsoventral position as the wild-type placodes and do not correspond to the CNS neurons which express *fkh* (Weigel et al., 1989a; Weigel et al., 1989b). We attribute our ability to detect these patches to the high stability of β-galactosidase.

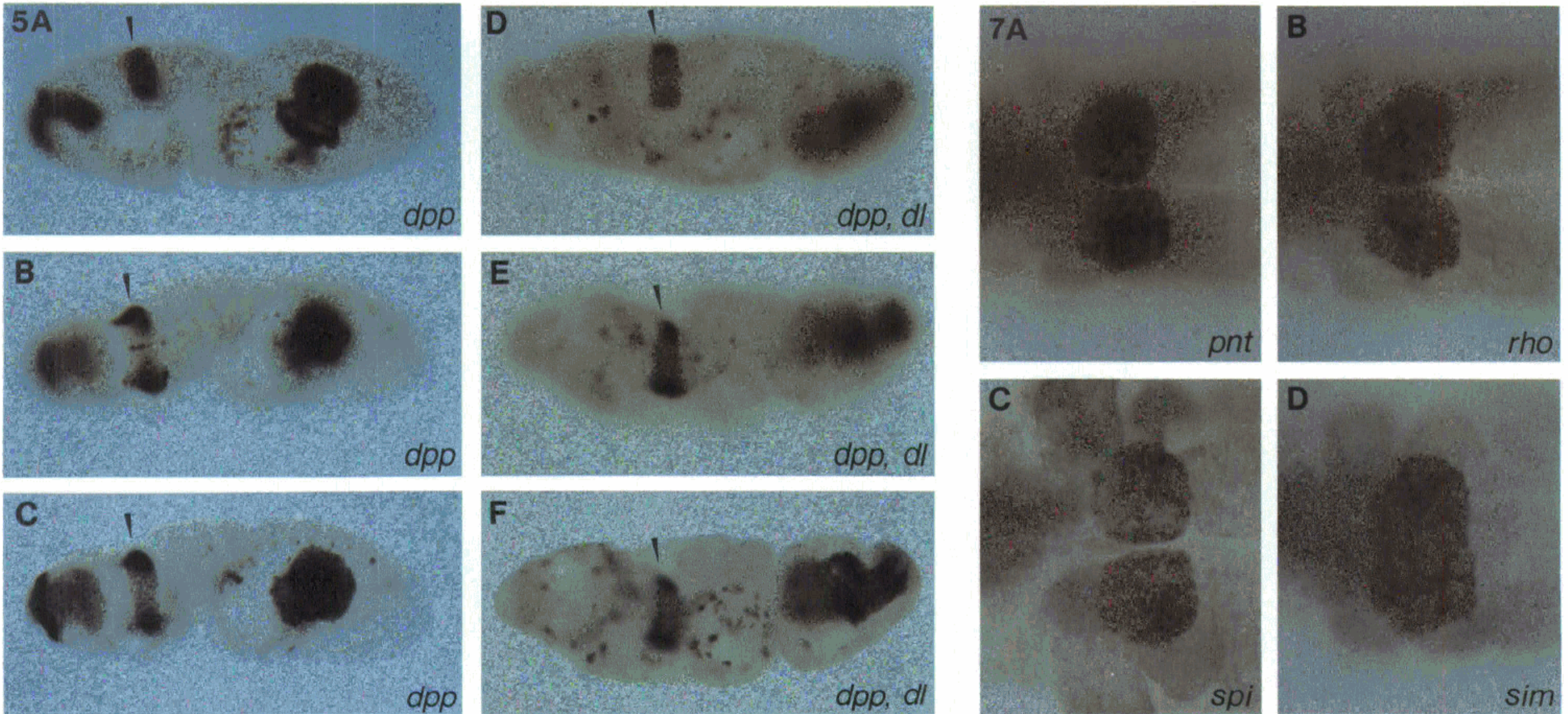

Fig. 5. Dorsoventral patterning genes negatively regulate placode determination. All embryos were stained with anti-*fkh*. The planes of focus shown in (A-C,D-F) are illustrated schematically in Fig. 6. (A) Lateral and (B, C) frontal views of an embryo homozygous for dpp^{Hin48}, a null allele (Irish and Gelbart, 1987). The mutant embryos were distinguished from their heterozygous siblings by their twisted morphology. The expanded salivary placode is indicated by the arrowhead. (B) A tangential plane of focus shows the ventral portion of PS2. The placode is discontinuous ventrally. (C) A plane of focus through the middle of the embryo reveals the dorsal part of parasegment 2. The *fkh*-stained placode is continuous across this region. (D) Lateral and (E,F) frontal views of a *dpp dl* double mutant embryo, stained with anti-*fkh*. Such embryos were recovered among the progeny of *Dp(2;1)G146/+; dl* dpp^{Hin48}/*dl* females mated to dpp^{Hin48}/In(2LR)CyOP20, *P[*dpp^{Hin+}*]* males. Two phenotypic classes of embryos were obtained from this cross. One class was indistinguishable from the *dl* embryos shown in G. Members of the second class (approximately 25 out of 200-300 stained embryos) were strikingly different and were distinguished by the prominent ring of *fkh*-expressing cells similar to those observed in *dpp* embryos. Because of the twist of the germ band in this embryo, the optical sections shown do not correspond precisely to lateral, ventral, or dorsal views of parasegment 2. However, they do demonstrate that, in contrast to the phenotype of *dpp* embryos, the placode material is continuous around parasegment 2 in *dpp dl* embryos. (G) An embryo laid by a female homozygous for the maternal effect mutation *dl* (Nüsslein-Volhard, 1979). No *fkh*-staining region corresponding to salivary placodes is observed. Since it is not possible to stage these embryos by morphology, they were aged at least 7 hours before fixation to ensure that they had progressed beyond a stage of development equivalent to germ band extension, at which time the placode is first visible.

Fig. 7. In *spitz* group mutants, the placodes meet or are fused at the ventral midline. Ventral views of embryos from parents heterozygous for TM3β or CyOβ, stained with anti-*fkh* and anti-β-galactosidase and dissected. (A) pnt^{9J31}. (B) rho^{7M43}. This allele is similar in strength to a small deletion removing several hundred bases from the *rho* promoter region, and thus, may represent the null phenotype (Bier et al., 1990). (C) spi^{IIA14}. (D) sim^{H9}, a protein null (Nambu et al., 1990). In contrast to wild-type placodes (compare with figure 2A and B), the placodes in *pnt, rho,* and *spi* embryos appear to meet at the ventral midline. The placodes are completely fused in *sim* embryos.

responsible for the transition from an irregular to a regular structure.

The *fkh* expressing cells in the placode clearly overlap the anterior *en* stripe which lies just posterior to the parasegment (PS) 1/2 border. In older embryos (in which *fkh* expression is uniform) the anteriormost placode cells are also the anteriormost cells in this *en* stripe. The placodes therefore extend to the PS1/2 border. In contrast, the placodes do not overlap the *en* stripe which defines the PS2/3 border, but appear to abut it. In many of these embryos, some posterior placode cells appear darker than the other cells in the placode. At early times, this is probably due to the non-uniform intensity of *fkh* expression mentioned above. In older embryos, such as the one shown in Fig. 2B, we find that the cells appear darker because *en*-expressing neuroblasts are located just below these cells (data not shown). Thus, we believe that the placodes normally extend to, but do not overlap, the PS2/3 border. Along the dorsoventral axis the placodes extend approximately half way to the top of the labial lobes and are separated ventrally by approximately four cells.

The homeotic gene Sex combs reduced *is required for salivary gland determination*

To identify genes involved in determining placode position along the anterior-posterior axis, we looked for salivary gland defects in embryos mutant for null mutations of *labial (lab), Deformed (Dfd), Sex combs reduced (Scr), Antennapedia (Antp),* and *fushi tarazu (ftz),* segmentation or homeotic genes expressed in or near PS2. *lab, Dfd,* and *Antp* mutant embryos have salivary glands with no visible defects (Fig. 3A, B, D). We found the phenotype of *lab* embryos (which is reproducible using each of the three null alleles tested) surprising because Merrill et al. (1989) failed to detect salivary glands in tissue sections and dissections of these mutant embryos. While we have not resolved this apparent contradiction, we speculate that the salivary glands degenerate in the very late embryos that were examined by these investigators. Because of their cuticle, these embryos are not suitable for whole-mount immunohistochemistry and have not been analyzed here. We conclude that *lab, Dfd,* and *Antp* are not required for salivary gland development, at least up until the cuticle forms. In contrast, *Scr* mutant embryos lack detectable salivary glands at all stages examined (Fig. 3C). We have confirmed this result using two other markers for the placode, anti-*Tl* and N33, an enhancer trap line expressing β-galactosidase in salivary glands. While we have not examined tissue sections to rule out the possibility that a placode forms but does not express any of the markers tested, the simplest interpretation of these results, considering that *Scr* is required for the proper identity of PS2 (Wakimoto and Kaufman, 1981; Sato et al. 1985), is that in *Scr* mutant embryos determination of the salivary placode does not occur.

In embryos mutant for the pair-rule gene *ftz,* parasegments 1 and 2 are fused and *Scr* is restricted to a stripe in the posterior half of this parasegment (Riley et al., 1987). In these embryos, the placodes appear to occupy the posterior half of the fused parasegment (Fig. 2C, D). Thus, it appears that even when segmentation is disrupted, placode determination is still restricted to the *Scr* expression domain. Embryos mutant for the gap gene *hunchback (hb)* have a strongly reduced domain of *Scr* expression (Riley et al. 1987), and we observe a corresponding reduction in placode size (data not shown). These results suggest that *Scr* is the primary determinant of salivary gland placode position along the anterior-posterior axis.

This hypothesis raises a new question: why, if *Scr* is expressed in both PS2 and PS3 (Mahaffey and Kaufman, 1987; Carroll et al. 1988; Mahaffey et al. 1989; LeMotte et al. 1989), is the placode restricted to PS2? Parasegment 3 normally gives rise to the posterior compartment of the labial lobe and the anterior compartment of the prothorax, while PS2 gives rise to the corresponding parts of the maxillary and labial lobes. This difference in fate is at least partially due to the expression of *Antp* in PS3 (Carroll et al. 1986; Carroll et al. 1988; Bermingham et al. 1990). Therefore, we examined double-labelled *Antp* mutant embryos for *fkh*-expressing placode cells in PS3. These embryos are indistinguishable from similarly treated wild-type embryos (compare Fig. 2E with 2A,B). It therefore appears that the absence of cells determined to be salivary gland from PS3 is not due to repression by *Antp.* An attractive alternative is that *Scr* is required for salivary gland determination prior to stage 11, since *Scr* expression is confined to PS2 during these early stages (Mahaffey and Kaufman, 1987; LeMotte et al. 1989).

Expression of Scr *under control of the* hsp70 *promoter leads to ectopic salivary gland formation*

A key prediction of the hypothesis that salivary gland determination is under the control of *Scr* is that widespread expression of *Scr* at the appropriate time in development will cause cells outside of PS2 to develop as salivary tissue. To test this prediction, we examined the phenotype of embryos bearing a fusion of the *hsp70* promoter to the *Scr* gene (Gibson et al. 1990). These embryos were heat shocked at 37°C for 30 minutes at either 1-4 hours or 4-7 hours of development. In embryos heat shocked during the later period there was little ectopic *fkh* expression. In contrast, embryos heat shocked between 1 and 4 hours of development showed strong ectopic *fkh* expression in ventrolateral parts of PS1 and PS3, in A8, and in a part of the procephalon possibly corresponding to the antennal region (Fig. 4A-D). Each of these stained regions (except for A8) was observed in some embryos to invaginate and form a tubular structure, which strongly suggests that these cells form supernumerary salivary glands. To confirm that the ectopic *fkh* expression represents bona fide salivary tissue, we crossed a construct (HNK) containing *lacZ* under the control of a *fkh* enhancer fragment, which directs expression to a subset of the *fkh*-expressing tissues (Weigel et al. 1990; B. Zhou, personal communication), into flies bearing the *hsp70-Scr* fusion and then monitored the expression of

β-galactosidase. In wild-type flies, HNK is expressed most strongly in salivary glands and more weakly in the anal pads. When *Scr* expression is induced throughout the embryo, strong ectopic β-galactosidase is present in the places described above for *fkh,* as well as in ventrolateral patches in nearly every segment along the length of the embryo (Fig. 4E, F). We believe that more ectopic sites are detected in this experiment than in the previous one because β-galactosidase is a more sensitive indicator than *fkh,* perhaps because β-galactosidase is quite stable in *Drosophila* embryos (Hiromi et al. 1985). This experiment indicates that *Scr* expression can induce *fkh* expression at many sites in the embryo, and that this ectopic *fkh* expression probably corresponds to salivary glands rather than other tissues that normally express *fkh,* such as the foregut or hindgut. While we have not yet eliminated the possibility that the ectopic *fkh* and *lacZ* expression corresponds to anal pads, the fact that these ectopic patches often invaginate and that they form at an equivalent dorsoventral position as do normal placodes suggests that *Scr* expression is sufficient to cause salivary determination at these ectopic positions.

decapentaplegic-*dependent functions block placode determination dorsally*

The salivary gland placode forms in PS2 but is restricted to a subset of *Scr*-expressing cells. We wondered whether this restriction to a specific dorsoventral domain is established by inhibitors acting in the more dorsal or more ventral cells of the parasegment. If so, removal of one of these inhibitors should allow the placode to expand either dorsally or ventrally. We therefore examined the phenotype of embryos bearing mutations in genes regulating dorsoventral pattern. One such gene is *decapentaplegic (dpp),* which is expressed in the dorsal 40% of wild-type embryos (St. Johnston and Gelbart, 1987). We find that *dpp* null embryos have a nearly complete ring of *fkh*-expressing cells, corresponding to the salivary gland placodes, which extends over their lateral and dorsal surfaces. The only break in the ring is located ventrally, as indicated by the presence of *fkh*-expressing neuroblasts in the unstained gap (Fig. 5A-C). Furthermore, when these embryos are stained with anti-*Tl* antibodies, a similar broken ring is detected, and the ventral midline, as indicated by a stripe of *Tl*-expressing cells (Gerttula et al. 1988), passes through the gap (data not shown). These observations indicate that the placodes are expanded dorsally to form a continuous structure in *dpp* embryos. Thus, we believe that in wild-type embryos *dpp* acts negatively to define the dorsal boundary of the placode.

Further support for this model comes from the phenotype of embryos laid by mothers mutant for the maternal effect mutation *dorsal (dl).* In these embryos *dpp* is expressed at all positions along their dorsoventral axis (K. Arora, cited in Ip et al. 1991). In most embryos, no *fkh* expression corresponding to either salivary gland placodes, foregut, or anterior midgut is detected. Only yolk nuclei, hindgut and posterior midgut were detected (Fig. 5G). In approximately 25% of embryos (27/108 embryos aged at least 7 hours) a small cluster of stained nuclei was observed on the surface of the embryo. Although we could not determine their identity, it is possible that they represent very small patches of salivary tissue. Our results support the notion that the salivary placodes are strongly reduced, if not completely absent, in embryos laid by *dl* mothers. While *dl* may be a positive factor for expression of *fkh* in foregut and anterior midgut, we do not believe this is the case in the salivary glands, since the dorsalmost PS2 cells of *dpp* mutant embryos, which are not expected to contain nuclear *dl* protein (Roth et al., 1989; Rushlow et al. 1989; Steward, 1989), still can become determined as salivary gland. We therefore interpret the absence in *dl* embryos of cells determined to be salivary gland as a consequence of indiscriminate *dpp* expression. The prediction of this interpretation, that *dpp* mutations will be epistatic to *dl* with respect to salivary gland determination, is confirmed below.

In addition to *dpp,* we have examined mutations in other genes thought to control development of the dorsal region of the embryo. Embryos mutant for the zygotic dorsoventral gene *zerknüllt (zen),* which is required for proper development of the amnioserosa, do not appear to have any salivary gland defects (data not shown). We have also examined embryos mutant for the genes *tolloid (tld)* and *shrew (srw),* which appear to lack part of the dorsal epidermis and amnioserosa (Jürgens et al. 1984). No obvious defects in salivary gland determination were detectable in these embryos either. Our analysis of these phenotypes is limited, however, in that we would not have detected a dorsal expansion of the placodes causing a small increase in the number of stained cells without causing the placodes to appear fused or closer together.

dorsal-*dependent functions block placode determination ventrally*

To determine whether genes specifying ventral fate establish the ventral limit of the salivary gland placodes, we examined the phenotype of *dpp* embryos laid by *dl* mothers. Such embryos possess only lateral cuticular structures (Irish and Gelbart, 1987). Strikingly, these embryos are surrounded by an unbroken ring of *fkh*-expressing salivary gland cells (Fig. 5D-F). This result indicates that *dl,* or zygotic dorsoventral genes downstream of *dl,* limit the placodes ventrally. Furthermore, the absence of placodes in embryos from *dl* mothers must be due to inappropriate expression of *dpp* because the addition of a *dpp* mutation suppresses this phenotype. In contrast to the situation with salivary glands, *fkh* expression in the foregut and anterior midgut is not detected in *dpp dl* double-mutant embryos, and these tissues may be absent altogether. Thus the requirement for *dl* in the anlagen of these tissues is independent of its effects on *dpp*.

The *spitz* group genes are required for the proper development of the ventralmost cuticle and the CNS midline (Mayer and Nüsslein-Volhard, 1988), and the genes in this group that have been molecularly

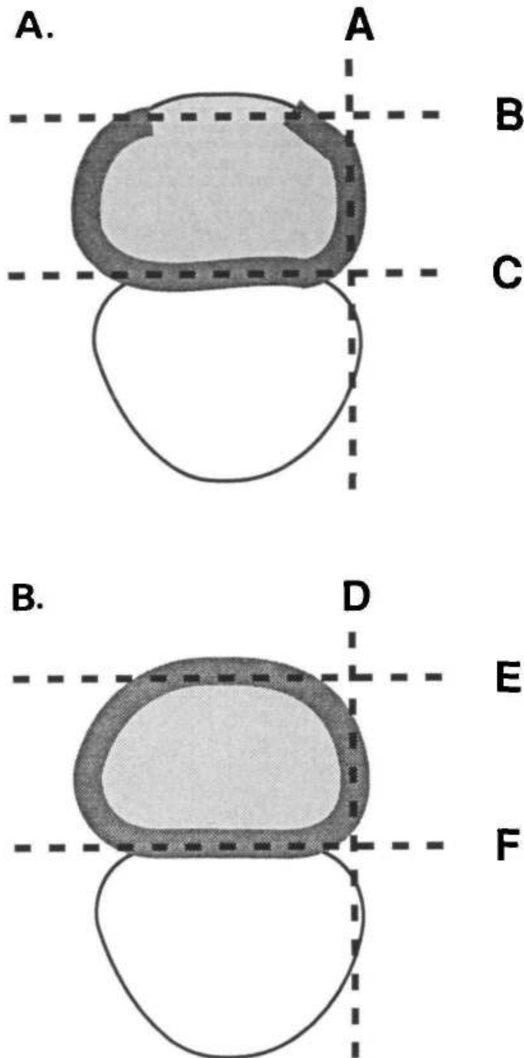

Fig. 6. Schematic representation of *dpp* and *dpp dl* embryos. Shown are transverse sections through the parasegment 2 germ band (light shading) and head region (unshaded) of (A) *dpp* and (B) *dpp dl* mutant embryos. The dotted lines indicate the plane of the optical section shown in the corresponding panel of Fig. 5. Dark shading indicates the expanded salivary placodes, stained with anti-*fkh*.

characterized are expressed in this region (Crews et al. 1988; Thomas et al. 1988; Bier et al. 1990). Thus, they are candidates for genes that might mediate the effect of *dl* on salivary gland determination. We examined the phenotypes of four zygotic members of this group: *pointed (pnt), rhomboid (rho), single-minded (sim),* and *spitz (spi).* The most profound phenotype is observed in *sim* embryos, in which the placodes are completely fused at the ventral midline, to yield one large placode (Fig. 7D). Interestingly, *fkh* expression in the placode of these embryos is initiated laterally and spreads before invagination to the ventral midline (data not shown). In the other mutants, the placodes appear expanded towards the ventral midline but not as completely as in *sim.* In *pnt, rho, spi,* the placodes appear to touch (Fig. 7A-C). In preliminary work, we have evidence that embryos mutant for *Star (S),* another *spitz* group gene, have an even weaker phenotype: the placodes appear separated by approximately two cells rather than four (S. P., unpublished data). Since the alleles of *pnt* and *spi* used are not necessarily null mutations, the less extreme phenotype of embryos bearing these mutations (compared with *sim* embryos) may be due to residual activity of these genes. The *rho* allele used, however, is likely to be a null mutation (Bier et al. 1990). Therefore, different *spitz* group mutations appear to affect the placodes to different extents.

We interpret these phenotypes as an expansion of the placodes towards the ventral midline, or in other words, a fate shift of the ventral cells towards a more lateral (placode) fate. Although we have not rigorously ruled out the possibility that these phenotypes are due to the death of the most ventral cells, this seems unlikely in the case of *sim,* in which the *fkh* expressing region seems strikingly larger than one might expect if the placodes simply moved together after the death of the intervening cells. Furthermore, Nambu et al. (1990) have found that in *sim* mutants, the mesectodermal cells which require *sim* for their proper development are still alive at stage 11. If the phenotypes we have observed are indeed the result of a transformation of ventral to lateral fate, it seems likely that repression of salivary gland determination in the ventral ectoderm by *dl* is mediated by the *spitz* group genes. Further experiments, however, will be necessary to rule out the possibility that *dl* can act more directly.

Discussion

Pattern forming genes act both positively and negatively to regulate salivary gland determination

In this study we have examined the effects of various patterning mutations on salivary gland determination. The phenotypes we observed have allowed us to identify, in molecular terms, the coordinates of the salivary gland anlage on the embryonic fate map. Several kinds of results indicate that *Scr* defines the position of the salivary placode along the anterior-posterior axis. First, in wild-type embryos the placode is restricted to parasegment 2, the domain of early *Scr* expression. Similarly, in embryos mutant for the pair-rule gene *ftz,* the placode is limited to the posterior half of the fused PS1-PS2 parasegment, the same region to which *Scr* expression is restricted (Riley et al. 1987). Second, markers normally expressed in the salivary gland placode are not expressed in *Scr* mutants, and the placode is presumably absent. Conversely, indiscriminate expression of *Scr* under the control of an inducible promoter leads to ectopic expression of these salivary gland markers in a ventrolateral stripe along most of the embryo. In many cases, the patches of cells which ectopically express these markers invaginate, supporting the conclusion that these cells are determined to become salivary gland tissue.

Appropriately timed expression of *Scr* appears to be crucial for salivary gland determination. Although *Scr* is expressed from stage 12 onward in the PS3 epidermis of wild-type embryos, this region does not give rise to salivary glands. Furthermore, we do not believe that repressors are important in preventing salivary gland determination in this region. First, mutations in *Antp,* the most likely antagonist of *Scr* function in PS3, do not alter salivary gland determination. Second, when *Scr* is expressed ectopically in PS3, salivary gland determination can ensue. However, ectopic expression of *Scr* was only effective early, during a period when *Scr* expression is normally confined to PS2. This time dependence of salivary gland determination might be mediated by a ubiquitous positively acting factor which is expressed only early. Alternatively, it might be mediated, as discussed below, by a positively acting dorsoventral patterning gene which is expressed early.

We have found that the dorsoventral position of the placode is under the negative control of at least two genetic pathways. Dorsally, salivary gland determination is repressed by *dpp* and possibly by genes whose function is dependent on *dpp. dpp* is initially expressed during syncytial blastoderm stage in the dorsal 40% of blastoderm embryos and by the end of gastrulation has begun to narrow to two dorsolateral stripes (St. Johnston and Gelbart, 1987). In *dpp* null embryos, the placodes are expanded through the *dpp* expression domain to form a single fused band which is broken only ventrally, while in embryos laid by *dl* mothers, indiscriminate *dpp* expression prevents salivary gland determination.

Other zygotic genes important for development of the dorsal side of the embryo do not appear to have a major role in restricting the salivary placode. For two reasons, however, we cannot rule out a role for *zen* in repressing salivary gland determination. First, in *zen* embryos, the amnioserosa (the dorsalmost tissue) is replaced by more ventral tissues, possibly dorsal epidermis (C. Rushlow, cited in Rushlow and Levine, 1990), suggesting that after the blastoderm stage, *dpp* expression might persist in the *zen* domain. Thus, salivary gland determination might still be under the negative control of *dpp* in the most dorsal cells of *zen* embryos. Second, *zen* expression is not properly maintained in *dpp* embryos (Rushlow and Levine, 1990). Therefore, the phenotype of *dpp* embryos might be similar to that of *dpp zen* double mutants. Negative regulation of placode determination by *zen,* visible as a dorsal gap between the dorsally extended placodes, would not be detected in *dpp* embryos.

By examining embryos doubly mutant for *dl* and *dpp,* we have found that *dl* acts to restrict the placode ventrally in the same way that *dpp* acts dorsally. The phenotypes of embryos bearing mutations in *spitz* group genes suggest that these genes also have a role in the ventral restriction of salivary gland determination. Because these genes act zygotically in the determination of the ventralmost ectodermally derived tissues (Mayer and Nüsslein-Volhard, 1988; Thomas et al. 1988), while *dl* acts maternally to control the overall dorsoventral pattern (Anderson and Nüsslein-Volhard, 1986; Anderson, 1987), it is likely that repression of placode determination by high concentrations of nuclear *dl* protein in the ventral epidermis and mesectoderm is mediated through the *spitz* group.

The complete fusion of the placodes in *sim* embryos is interesting in light of the localization of the *sim* protein to the nuclei of the ventral midline cells (Crews et al. 1988; Thomas et al. 1988). Because *sim* is expressed in only a subset of cells in which salivary determination is repressed by the *dl*-dependent ventral pathway, *sim* might act in two ways to prevent the placodes from extending into the ventralmost epidermis and mesectoderm. First, we imagine that *sim* acts autonomously to repress salivary determination in the mesectoderm. Second, *sim* could act through a cell signalling mechanism to prevent the neighboring cells in the ventralmost epidermis from becoming salivary placode. This signalling mechanism could involve other members of the *spitz* group. Sequence analysis of *rho* suggests that it encodes a transmembrane protein (Bier et al. 1990), while *spi* encodes a protein related to TGF-α (N. Perrimon, personal communication). These are types of proteins which could mediate the proposed signal. Furthermore, mutations in *sim,* which would block both repression pathways, would be expected to cause a more severe phenotype than mutations in *spitz* group genes involved in only the signalling event. This prediction is borne out by our result that in *sim* mutant embryos the placodes are completely fused, while in *spi, pnt,* and *rho* the placodes meet but are not fused.

These results establish negative regulation from both the dorsal and ventral sides of the placode. We envision two alternative mechanisms by which this negative regulation could determine the site of salivary placode formation. First, the *dpp* and *dl* pathways might directly inhibit salivary development. With this model, salivary placodes would form by default only in that part of PS2 where these pathways are inactive. Alternatively, the apparent negative regulation of salivary gland determination by these pathways might be a consequence of their capacity to negatively regulate a gene required in all segments for development of the ventrolateral ectoderm. *dpp* and *dl* would restrict the expression of this gene to a ventrolateral stripe along the anterior-posterior axis of the embryo. Because its product would be required in addition to *Scr* for salivary gland determination, the salivary placode would only form at a ventrolateral position in parasegment 2, where the expression domains of this gene and *Scr* intersect. This hypothesis could also provide an explanation for the early requirement for *Scr* in salivary gland determination. If this positively acting dorsoventral gene were only expressed early, then late expression of *Scr* under control of the heat shock promoter would fail to induce the formation of ectopic salivary tissue because the additional positive requirement would be absent. On the other hand, early expression of *Scr* throughout the embryo would be expected to result in the formation of salivary tissue along a ventrolateral stripe of the embryo. Our results with the *hsp70-Scr* fusion fulfill this

prediction, but do not distinguish this model from the possibility that salivary gland determination is directly repressed at all positions along the anterior-posterior axis by *dpp, dl,* and the *spitz* group. These two models could be distinguished by identification of a dorsoventral gene required for salivary determination. Many of the zygotic dorsoventral genes appear to be required for the proper development of the dorsalmost regions of the embryo (Jürgens et al. 1984; Wakimoto et al. 1984; Tearle and Nüsslein-Volhard, 1987; Zusman and Wieschaus, 1985; Zusman et al. 1988; reviewed in Ferguson and Anderson, 1991). Although all of these genes are important for dorsal development, mosaic analysis has shown that one of them, *short gastrulation (sog),* must be expressed in ventrolateral ectoderm of the embryo (Zusman et al. 1988), the region from which salivary glands are derived. Thus, *sog* appeared to be the best candidate for a dorsoventral gene which, in conjunction with *Scr,* would act positively to determine salivary glands. However, we have recently found that *sog* embryos possess salivary glands (S.P., unpublished data). Therefore, if a positively acting factor controls salivary placode position along the dorsoventral axis of the embryo, this factor may correspond to an unidentified gene.

We have not yet discussed why placode determination does not occur in the presumptive mesoderm of parasegment 2. One possible explantion is that *Scr* is not expressed in mesodermally derived tissues until after stage 11 (LeMotte et al. 1989). However, we have shown that expression of *Scr* throughout the embryo does not result in ectopic *fkh* expression in mesodermally derived tissues. Since in this case improper timing cannot explain the absence of salivary tissue from the mesoderm, we are left with two other hypotheses that are consistent with the fact that *dl* places a ventral limit on the placodes. As described above, there might be a positive regulator in the ventrolateral region of the embryo that is required in addition to *Scr* to determine salivary glands. Because this proposed regulator would be absent from mesoderm, no placodes could form there. Alternatively, the mesoderm might contain a negative regulator, probably also under the control of *dl,* that prevents salivary determination.

Our observations have been summarized in Fig. 8. We imagine that *Scr* and the dorsoventral genes act on one or a small number of "salivary gland determining genes" which establish and maintain the identity of this tissue. These genes would then activate other genes such as *fkh* which allow morphogenesis to occur. For several reasons we do not consider *fkh* itself to be a salivary gland determining gene. First, the defect observed in *fkh* embryos occurs after invagination has begun. Placode formation itself is normal (Weigel et al. 1989a; S. P., unpublished data). Second, we have found that the expression of several salivary gland markers, such as *Tl, dsrc29A* (Gerttula et al. 1988; Hashimoto et al., 1991; Wadsworth et al. 1990; Katzen et al. 1990), and several enhancer traps, are independent of *fkh* (M. Belvin and S. P., unpublished data). Finally, *fkh* expression in the salivary gland placode is first detected

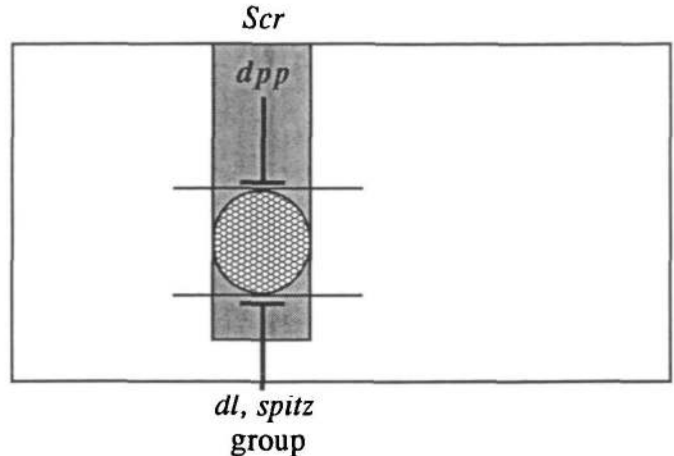

Fig. 8. Summary model. The salivary placodes and *Scr*-expressing region (shaded area) are projected onto the blastoderm embryo (large rectangle). The top of the rectangle indicates the dorsal midline of the embryo, while the bottom indicates the ventral midline of the embryo prior to mesoderm invagination. The placodes are determined only within the *Scr*-expressing region of the embryo, and are limited dorsally by *dpp* and ventrally by the *spitz* group genes and a high nuclear concentration of *dl*.

in stage 11, which occurs after approximately 6.5 hours of development. As discussed above, the strongest induction of ectopic *fkh* expression in *hsp70-Scr* is observed when *Scr* expression is globally induced much earlier, between 1 and 4 hours of development. Because of the gap between the time *Scr* appears to act and the time *fkh* expression appears, it seems likely that there is at least one intervening gene which is activated by *Scr* and, in turn, activates *fkh.*

A hierarchy of gene expression directing salivary gland development

The results we have presented here provide the background for a molecular analysis of a genetic cascade regulating salivary gland development. We believe that there are at least four levels to this cascade. At the top of the hierarchy, *Scr* and the dorsoventral patterning genes establish the location of the salivary gland placode. In the second level, the proposed salivary gland determining genes interpret the positional information provided by the patterning genes, and go on to regulate third tier genes such as *fkh,* which direct subprograms of salivary gland development, such as invagination. The *fkh* protein shows extensive sequence similarity to the mammalian transcription factor HNF-3A, and is therefore expected to be a transcriptional regulator (Weigel and Jäckle, 1990). We have identified two enhancer trap insertions in which β-galactosidase expression is dependent on *fkh* in the salivary glands (Weigel et al. 1989a; S.P. and M. Belvin, unpublished observations). The genes tagged in these lines are likely targets of *fkh,* and probably represent a fourth level of the salivary gland development cascade. It will be interesting to learn whether they control yet

another level of gene expression, or whether they act more directly to mediate morphogenesis, for instance, by interacting with cytoskeletal components or cell surface molecules. We expect that analysis of genes at various levels of this hierarchy will allow us to address the long standing question of how homeotic and other pattern forming genes lead to the formation of differentiated tissues in the mature larva.

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