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Salivary Gland Determination in Drosophila: A Salivary-Specific, *fork head* Enhancer Integrates Spatial Pattern and Allows *fork head* Autoregulation

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In the early *Drosophila* embryo, a system of coordinates is laid down by segmentation genes and dorsoventral patterning genes. Subsequently, these coordinates must be interpreted to define particular tissues and organs. To begin understanding this process for a single organ, we have studied how one of the first salivary gland genes, *fork head* (*fkh*), is turned on in the primordium of this organ, the salivary placode. A placode-specific *fkh* enhancer was identified 10 kb from the coding sequence. Dissection of this enhancer showed that the apparently homogeneous placode is actually composed of at least four overlapping domains. These domains appear to be developmentally important because they predict the order of salivary invagination, are evolutionarily conserved, and are regulated by patterning genes that are important for salivary development. Three dorsoventral domains are defined by *EGF receptor* (*EGFR*) signaling, while stripes located at the anterior and posterior edges of the placode depend on *wingless* signaling. Further analysis identified sites in the enhancer that respond either positively to the primary activator of salivary gland genes, SEX COMBS REDUCED (SCR), or negatively to EGFR signaling. These results show that *fkh* integrates spatial pattern directly, without reference to other early salivary gland genes. In addition, we identified a binding site for FKH protein that appears to act in *fkh* autoregulation, keeping the gene active after SCR has disappeared from the placode. This autoregulation may explain how the salivary gland maintains its identity after the organ is established. Although the *fkh* enhancer integrates information needed to define the salivary placode, and although *fkh* mutants have the most extreme effects on salivary gland development thus far described, we argue that *fkh* is not a selector gene for salivary gland development and that there is no master, salivary gland selector gene. Instead, several genes independently sense spatial information and cooperate to define the salivary placode.

Key Words: **organogenesis; EGFR; fork head; homeotic gene; salivary glands; *Drosophila* embryo.**

INTRODUCTION

By the time organogenesis begins in *Drosophila* embryos, relays of patterning genes have defined segments along the anteroposterior axis and at least five major subdivisions along the dorsoventral axis (St. Johnston and Nusslein-Volhard, 1992; Ingham, 1988). This crisscrossing pattern then positions organ primordia such as the tracheal placodes (reviewed in Manning and Krasnow, 1993), imaginal disc precursors (Cohen *et al.*, 1993), and salivary gland placodes (Panzer *et al.*, 1992; Andrew *et al.*, 1994). Here, we are interested in the regulation within these primordia of genes that respond to the primary patterning information. Is there a single gene that integrates all the primary pattern and determines organ identity for all downstream genes? Alternatively, are there many genes that independently interpret the primary pattern and contribute to organ identity? And do each of these genes read and integrate all the primary information, or just parts of it? To address these questions, we have chosen to examine in the salivary placode the transcriptional regulation of *fork head* (*fkh*), an important regulator of salivary gland development that is expressed early in the placode and responds to the primary patterning information (Weigel *et al.*, 1989; Panzer *et al.*, 1992; Andrew *et al.*, 1994).

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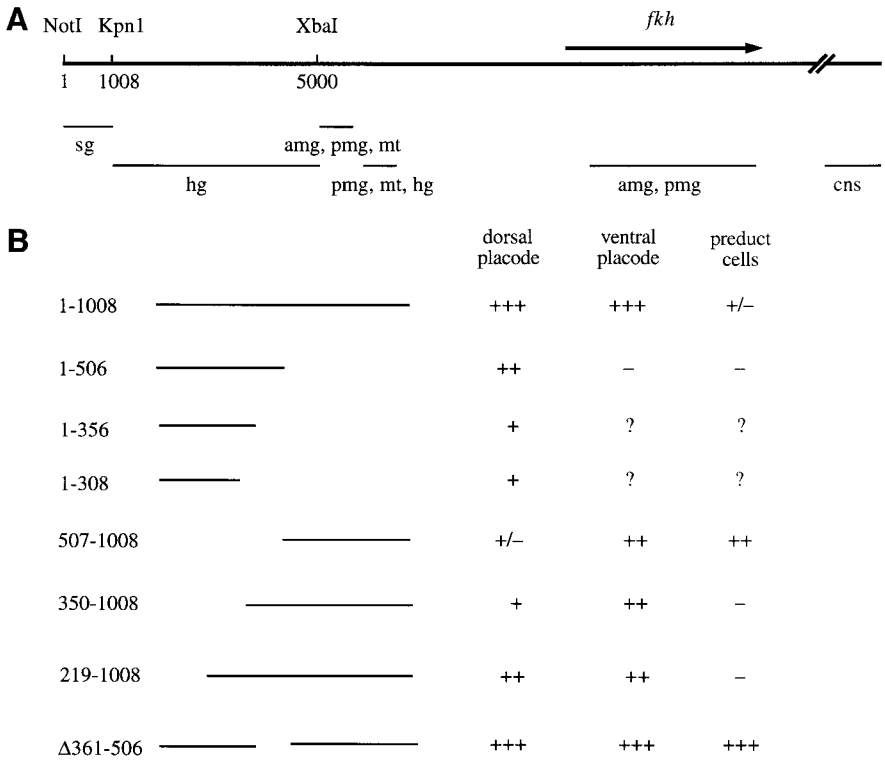

FIG. 1. Location and activity of the *fkh* salivary enhancer. (A) The salivary gland-specific *fkh* enhancer is located about 9 kb upstream of the transcriptional start. The remaining more than 20 kb of *fkh* DNA (including more than 10 kb downstream from *fkh*) contains enhancers for anterior and posterior midgut (amg and pmg), malpighian tubules (mt), hindgut (hg), and central nervous system (cns) (Weigel *et al.*, 1990). (B) Summary of the activities of parts of the 1-kb salivary enhancer. For convenience, the fragments are indicated by their end points. +++, ++, +, and − indicate strong, medium, weak, and no expression. +/− indicates variable expression. ? indicates uncertain expression (because overall expression was too weak).

There are several reasons why salivary development is useful for studying the linkage between cell patterning genes and tissue specification. First, because the salivary primordium forms shortly after the establishment of the overall body pattern, the pathway between embryonic patterning genes and salivary-specific genes appears to be relatively short. The salivary placodes, the precursors of the salivary glands proper, are morphologically distinguishable in early stage 11 at about 5.5 h of development (Panzer *et al.*, 1992). The earliest selective gene expression in the placodes, as exemplified by *fkh*, *trachealess* (*trh*), and *CrebA*, begins about 1 h earlier, in early stage 10 (Panzer *et al.*, 1992; Isaac and Andrew, 1996; Kuo *et al.*, 1996). Second, salivary tissues are morphologically simple and easily distinguishable. The placodes form in the ventral epidermis of parasegment 2 as flat discs of columnar cells that are separated from each other ventrally by about four rows of cells, two on either side of the ventral midline. The salivary ducts come from these ventral rows of PS2 cells (Isaac and Andrew, 1996; Kuo *et al.*, 1996; this paper). During the next 5 h, the glands and ducts arise by invagination from these precursors and form three morphologically distinct tissues: the simple tubular glands, the individual ducts which attach to the anterior end of each gland, and the common duct that leads anteriorly along the midline from the conjunction of the individual ducts until it opens into the pharynx. Third, all of the information for salivary determination lies within the ectoderm. In mutant embryos that lack underlying mesoderm (*twist snail* double mutants), the salivary precursors are normally determined and can invaginate (Panzer, 1994; L. Komenda and S.K.B., unpublished results). Thus, patterning information need only be sought in two dimensions, rather than three.

How does the salivary primordium interpret positional information in the ectoderm? We have previously shown that the formation of the salivary placodes requires the action of *Sex combs reduced* (*Scr*), *decapentaplegic* (*dpp*), and the *spitz* group genes (Panzer *et al.*, 1992). The homeotic gene *Scr*, which is expressed throughout parasegment 2 from cellular blastoderm onward and spreads to parasegment 3 at the end of germ band elongation (Riley *et al.*, 1987; LeMotte *et al.*, 1989), is the primary inducer of salivary gland formation. *dpp* and the *spitz* group genes, on the other hand, provide dorsal and ventral spatial limits on

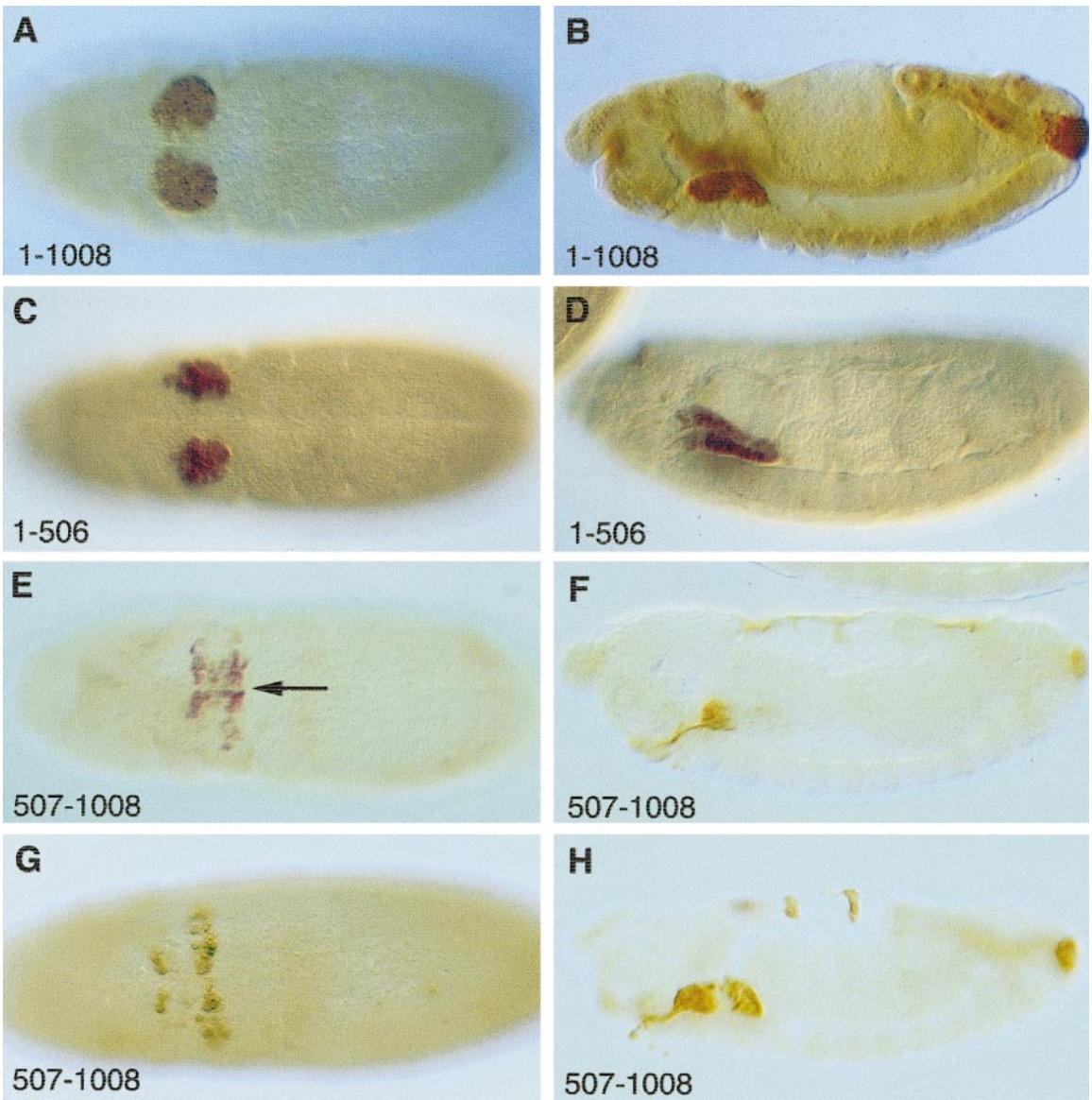

FIG. 2. The *fkh* salivary enhancer is located within a 1-kb DNA fragment. Panels on the left side are ventral views showing staining at the placode stage. Panels on the right are lateral views showing staining of invaginated glands. (A, B) The 1-kb *Not*I/*Kpn*I DNA has strong salivary enhancer activity. In some embryos, weak expression is seen in the cells ventral to the placodes (see text). (C) Expression driven by 1–506 is limited to the dorsal part of the placodes. The space between the stained regions is about eight cells instead of about four cells. (D) After invagination, expression is limited to the posterior part of the salivary gland. (E) 507-1008 drives expression in the ventral part of the placode. In addition, there is ectopic expression ventral to the placodes, all the way to the ventral midline (arrow). Expression in the dorsal part of the placode is limited to anterior and posterior stripes. The staining of these stripes is not penetrant and varies even between the two placodes of the same embryo. (G) Slightly earlier, when 507-1008:*lacZ* expression is just beginning, only the anterior and posterior stripes are seen. (F, H) After invagination, the 507-1008 expression is predominantly limited to the duct and anterior part of the salivary gland but is variably present in the posterior part of the gland.

placode formation (Panzer *et al.*, 1992; Kuo *et al.*, 1996; Henderson *et al.*, 1999). The reason that *Scr* expression in PS3 does not induce a salivary placode is that *teashirt* (*tsh*), a zinc finger gene, blocks *Scr* expression in ventral PS3 and its activity in PS3–PS13 (Andrew *et al.*, 1994).

The *fkh* gene, which encodes a founding member of the winged-helix family of transcription factors (Weigel and Jäckle, 1990; Kaufmann and Knöchel, 1996), is one of the earliest genes that responds to these spatial cues. It begins to be expressed as the salivary placode forms and continues

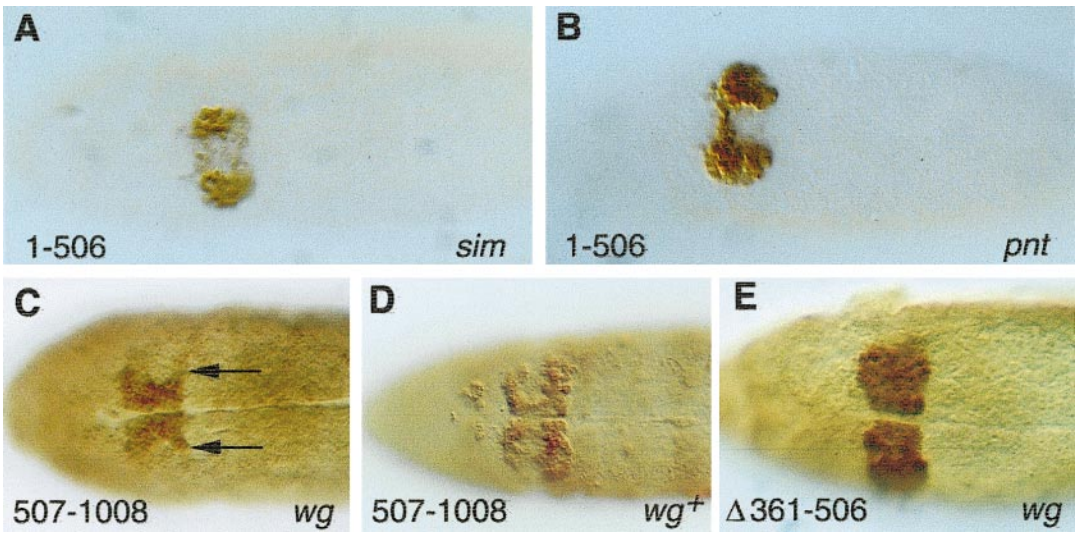

FIG. 3. EGFR and *wg* signaling define subdivisions within the placode. (A, B) Expression directed by 1–506 in *spitz* group mutant embryos is partially derepressed ventrally. (A) 1–506:*lacZ* in a *sim* embryo. (B) 1–506:*lacZ* in a *pnt* embryo. (C) In a *wg* embryo, 507-1008:*lacZ* does not give stripes (stage 11). The arrow denotes the missing posterior stripe. (D) Expression of 507-1008 in a wg^{+} embryo from the same collection. (E) Expression of Δ361–506:*lacZ*, which marks both salivary glands and ducts, in a *wg* embryo at stage 11. Note the intact, stained salivary primordium, demonstrating that all salivary cells in these embryos are capable of expressing *lacZ*.

to be transcribed in the salivary glands throughout embryonic and larval development, regulating many, if not all, salivary-specific genes in larvae (Mach *et al.*, 1996; Lehmann and Korge, 1996). In the embryo, *fkh* is required for salivary morphogenesis; in *fkh* mutant embryos, the placodes do not invaginate and eventually disintegrate (Weigel *et al.*, 1989). Thus, working out details of its transcriptional control should give us insights into salivary tissue determination. Here, we show that a 1-kb region of *fkh* regulatory sequence can integrate both AP and DV patterning information to direct salivary placode expression. Within this 1 kb, we identify short sequences that respond to *Scr* activation, *EGFR*-pathway repression, and *fkh* autoregulation. Thus, two important aspects of salivary gland organogenesis—initiation and maintenance—are built into the *fkh* transcriptional regulatory elements. As an unexpected bonus, dissection of the enhancer revealed patterning within the salivary placode. Subregions of the enhancer control expression in different parts of the salivary primordium. By marking these subdomains and following them into the mature salivary glands and ducts, we have been able to construct a salivary fate map.

MATERIALS AND METHODS

DNA Construction and Germ Line Transformation

For initial characterization of the salivary enhancer, DNA fragments were excised from pD1.NS which contains the *fkh* gene and about 10 kb of upstream sequence (Weigel *et al.*, 1990). These fragments (Fig. 1) were cloned into the P-element expression vector pHZ50PL for transformation (Hiromi and Gehring, 1987). For finer analysis, the 1-kb *Not*I/*Kpn*I fragment was subcloned in pBluescript and directional deletions were made by using Bal31 nuclease. The resulting subfragments were first cloned in pUC119, and then excised (*Xba*I/*Kpn*I) and cloned in pHZ50PL. The subfragment with an internal deletion, Δ361–506, was made by inverse PCR using primers flanking the region to be deleted. Subfragments were sequenced by using the dideoxy chain termination method and T7 DNA polymerase (Sequenase; US Biochemical).

For many constructs, sequences were concatemerized to give three or four tandem copies before they were inserted in either the pHZ50PL or CZIII transformation vectors. The concatemerization procedure was based on the method described by Rosenfeld and Kelly (1986). The concatemerized DNA was cloned into appropriate vectors and verified by sequencing. Detailed descriptions of the cloning procedures are available from the authors on request.

$cn;ry^{506}$ or w^{1118} flies were used for injection strains. The transformation procedures were essentially as described by Rubin and Spradling (1982). Multiple lines from each construct were recovered and examined.

Cloning the D. virilis fkh Salivary Enhancer

Two probes were used to screen a *D. virilis* genomic library (gift from M. Scott). A probe for the N-terminal fragment of the *fkh* structural gene was made by *Bam*HI/*Hin*dIII digestion of pD1.NS, and an enhancer probe was made from the *Sph*I/*Kpn*I fragment (50–1007) of the 1-kb *fkh* enhancer DNA. Screening was as described by Church and Gilbert (1984) with some modifications. Filters were prehybridized and hybridized in 0.5 M sodium phosphate, pH 7.2, 7% SDS, 100 μg/ml denatured herring sperm DNA (Sigma) at 50°C. Filters were washed with 40 mM sodium phos-

phate, pH 7.2, 1% SDS at 55°C until signal was only several times above background. Phage clones that were positive for both probes were chosen, purified, and the genomic fragments of interest subcloned into Bluescript.

Protein/DNA Binding Assays

The *fkh* DNA binding domain was amplified by PCR from the pD1NS plasmid using the following two primers: fkh-RI, 5′-GACAAACCAGAATTCATCAGAAGGAGCTACACGCAT-3′; fkh-*Xho*I, 5′-TGTGCAGCTGCTCGAGCCTCCTTCTTCTC-GTCCTTG-3′.

The amplified product was cloned in frame to pMAL-cR1 (*Eco*RI/*Bam*HI) and transformed into TB1. Saturated cultures were diluted 1/100 into LB with 100 mg/liter ampicillin and shaken 2–3 h before inducing with 0.5 mM IPTG (isopropyl thio-β-D-galactoside) for an additional 3 h. Cells were harvested and sonicated. The lysed cells were then centrifuged and the pellet discarded. MBP-FKH was purified by using amylose resin (New England Biolab) and was eluted with elution buffer (10 mM maltose, 1 mM DTT in PBS). The yield was about 0.3 mg/liter culture.

Two double-stranded oligonucleotides were used for FKH binding, NK460 (39 bp, 457–495) and fkh11. The latter contains four tandem copies of the 11-bp FKH-consensus site from NK460. Its sequence is: 5′-GATCATTGACAAAGTATTGACAAAGTATT-GACAAAGTATTGACAAAGT-3′. Binding was performed on ice in 20 μl 20 mM Hepes, pH 6.9, 40 mM KCl, 1 mM dithiothreitol, 10% (by vol) glycerol for 20 min in the presence or absence of specific (fkh11) or nonspecific competitor [poly(dG/dC)]. Electrophoresis was performed at 4°C in 50 mM Hepes, pH 6.9, in 7% acrylamide (19:1, by vol) at 10 mA.

Antibody Staining and Photography

Embryos collected on molasses/agar plates were dechorionated and fixed for 30 min with 1:1:2 mixture of PBS:10% formaldehyde (E.M. grade; Polysciences):heptane (HPLC grade; Sigma). Embryos were devitellinized by shaking in a 1:1 mixture of heptane:90% methanol, 5 mM EGTA, followed by thorough washing in methanol. Antibody staining was performed essentially according to Patel *et al.* (1989), using polyclonal rabbit anti-β-galactosidase (5′→3′ Inc.), biotinylated goat-anti-rabbit secondary antibody (Jackson ImmunoResearch), and the Vectastain Elite kit (Vector Laboratories). Color was developed by using 0.5 μg/ml diaminobenzidine and 0.06% H_2O_2. Stained embryos were washed with ethanol and cleared in methyl salicylate. Embryos were staged as described by Campos-Ortega and Hartenstein (1985) and were photographed on a Leica DMRB microscope fitted with Nomarski DIC optics.

Drosophila Stocks

wg^{IG22} and $pnt^{9\text{-}J31}$ were obtained from the Bowling Green Stock Center. sim^{H9} and dpp^{48} were obtained from K. Anderson. Mutant embryos except *dpp/dpp* were distinguished from nonmutants by the use of marked balancers, either SM6B*eve-lacZ* (Panzer, 1994) or TM3*ftz-lacZ* (a gift of Y. Hiromi).

RESULTS

A 1-kb DNA Fragment Upstream of the fkh Gene Works as a Salivary Gland Enhancer

Previous analysis showed that *fkh* regulatory sequences are spread over 25 kb of DNA that flanks the gene (Weigel *et al.*, 1990). Within this large region, a 5-kb genomic DNA fragment was found that has both salivary gland and hindgut enhancer activity (Fig. 1). The salivary expression pattern driven by this 5-kb fragment is consistent and identical to the expression of *fkh* itself (Weigel *et al.*, 1990; and data not shown). Several subfragments of the 5-kb DNA were inserted upstream of *lacZ* and tested for enhancer activity by P element-mediated transformation. If the 5-kb DNA is denoted as 1–5000 (starting from the distal *Not*I site), only the first 1-kb subfragment (1–1008) directs detectable salivary *lacZ* expression (Figs. 2A and 2B). As measured by anti-β-gal antibody staining, the enhancer activity of the 1-kb DNA is similar to that of the 5-kb fragment (Weigel *et al.*, 1990), indicating that most of the elements of the salivary enhancer are confined to this 1-kb region. The 3400–5000 fragment has at least partial hindgut enhancer activity but no salivary activity, indicating that these two enhancers are separable (data not shown).

Closer examination revealed that the salivary expression directed by the 1-kb fragment is not quite the same as that directed by the 5-kb fragment. With the 1-kb DNA, some transformed embryos show ectopic expression of variable strength in the cells located ventrally between the two placodes. This ventral expression, which is weaker than expression in the placode itself, is rarely observed for either the 1–5000 fragment or for FKH protein itself (Weigel *et al.*, 1990; Panzer *et al.*, 1992). Within any particular transformed line, the amount of ventral expression is variable and is not dependent on the chromosomal location of the enhancer:*lacZ* transposon. These results suggest that there is at least one ventral repression element located outside the 1-kb enhancer. Without that element, the cells ventral to the placode can weakly express *lacZ*. In the normal chromosomal context, repression elements within and outside the 1-kb enhancer would interact for effective ventral repression. Consistent with these suggestions, in experiments described later, a region within the 1-kb enhancer DNA was identified to be necessary for ventral repression.

The Salivary Placode Is Assembled from Multiple, Overlapping Domains

Further dissection of the 1-kb enhancer (1–1008) showed that salivary expression of *fkh* is regulated in a spatially complex pattern. Figure 1B lists the subfragments that were used for making transformation constructs. The first half of the 1-kb enhancer, 1–506, drives expression in most of the placode, but not in its most ventral two rows of cells (Fig. 2C). This lack of expression in the ventral cells of the placode can be clearly seen by the greater separation between the stained regions of the placodes in these embryos

than in embryos expressing the 1-kb or 5-kb constructs or FKH protein itself (six to eight cells apart across the ventral midline instead of about four cells apart). The overall expression level directed by 1–506 is easily detectable but significantly lower than that by the 1-kb enhancer.

The other half of the enhancer does not direct the simple, complementary pattern. 507-1008 drives expression not only in the ventral part of the placode, but also in the cells ventral to the placodes (Fig. 2E). In addition, in more than half of the 507-1008:*lacZ* embryos, expression at the anterior and posterior edges of the placode extends dorsally, forming anterior and posterior stripes that overlap cells in which 1–506:*lacZ* would be expressed. The remaining embryos do not have these dorsal extensions. Again, the variability is not transformant line-dependent. When 507-1008:*lacZ* expression begins at late stage 10, only the two stripes are visible (Fig. 2G). They extend across the ventral midline, one at the front edge and one at the back edge of parasegment 2. We imagine that the pattern detected at later times (stage 11) arises by adding to the stripes a ventral domain that includes the ventral cells of the placode and the cells ventral to the placode.

In summary, both the 1–506 and 507-1008 halves of the enhancer direct reporter gene expression to salivary placode cells, but the patterns that they direct are very different. Within the placode, which had previously been seen as a homogeneous field of cells, *fkh* expression is regulated in at least four partially overlapping domains: the dorsal and ventral parts of the placode, and the anterior and posterior stripe domains.

Subdivisions within the Placode Are Defined by EGFR and wg Signaling

We have shown previously that expression of *fkh* is limited at the dorsal edge of the placode by *dpp* and at the ventral edge of the placode by *EGFR* and the *spitz* group genes (Panzer *et al.*, 1992). Expression driven by the two halves of the enhancer has identified a new dorsoventral limit, the boundary between the dorsal and ventral parts of the placode. What genes establish this boundary? Expression of 1–506:*lacZ* in *sim* or *pnt* embryos shows that the *EGFR* pathway is involved (Figs. 3A and 3B). In these embryos, staining is as strong in the dorsal placode as it is in wild-type embryos, but now there is also weak staining that extends all the way to the ventral midline. Thus, expression of 1–506:*lacZ* must normally be limited by *EGFR* signaling. In contrast to this result, *dpp* mutations had no effect on this boundary (data not shown).

In the anteroposterior dimension, establishment of parasegmental borders depends on interaction between *wingless* (*wg*) expressing cells at the posterior edge of one parasegment and *hedgehog* or *engrailed* expressing cells at the anterior edge of the next, more posterior parasegment (reviewed in van den Heuvel *et al.*, 1993). Since 507-1008:*lacZ* is expressed in anterior and posterior stripes adjacent to the borders of parasegment 2, we tested its reliance on *wg*. At the placode stage in *wg*-mutant embryos, the posterior stripe is not seen, and the anterior stripe may also be missing (Fig. 3C, compare with 3D). At slightly earlier times, when only the stripes are seen in wild-type embryos, no expression of 507-1008:*lacZ* is seen in *wg*-mutant embryos. Because *wg* embryos show extensive cell death starting shortly after placode formation, we wanted to be sure that the lack of 507-1008:*lacZ* expression was not due to death of placode cells at the front and back of the parasegment. For this reason we checked, in *wg*-mutant embryos, the expression of a construct (Δ361–506:*lacZ*) that is normally expressed in all salivary cells, both the placode and the more ventral preduct cells. The expression of this construct in all salivary cells of *wg*-mutant embryos confirms that the stripe cells are still present and are capable of expressing *lacZ* (Fig. 3E). These results suggest that *fkh* responds to *wg* signaling by expression in both posterior and anterior stripes.

The fkh Salivary Enhancer and Salivary Patterning Are Conserved during Evolution

Drosophila virilis and *D. melanogaster* diverged about 60 million years ago (Beverley and Wilson, 1984). During the 120 million years of separation, we expect that unimportant sequences will diverge more rapidly than functionally important ones. To clone the *virilis fkh* homologue, a *virilis* lambda genomic library was screened and clones that hybridized to both the *melanogaster fkh* structural gene and the 1-kb enhancer were isolated. Sequence comparisons between *melanogaster* and *virilis* reveal that the *fkh* salivary enhancer is a highly conserved regulatory region, both in its overall organization and in its sequence (Fig. 4A). The fact that multiple regions are conserved and that several of these are 80–100 bp long suggests that many elements are involved in *fkh* transcriptional control.

To test whether the *virilis* and *melanogaster* DNAs are functionally equivalent, pieces of the *virilis* DNA were used to drive β-gal expression in *melanogaster* embryos. The results for two different constructs showed that the *virilis* DNA does work as a salivary enhancer in *melanogaster*. For *virilis* DNA corresponding to 550-1008 of the *melanogaster* enhancer, the β-gal expression was found mostly in the anterior part of the glands and in the ducts (Fig. 4B), just like the expression pattern driven by the 507-1008 fragment of the *melanogaster* enhancer. In addition, when we used a piece of *virilis* DNA corresponding to 300-1008 of the *melanogaster* sequence, the expression was very similar to that of the complete 1-kb *melanogaster* enhancer or its 350-1008 fragment (Fig. 4C). The glands were well stained and there was little expression in the ducts. These results suggest that *fkh* transcriptional control and salivary patterning have been conserved during evolution.

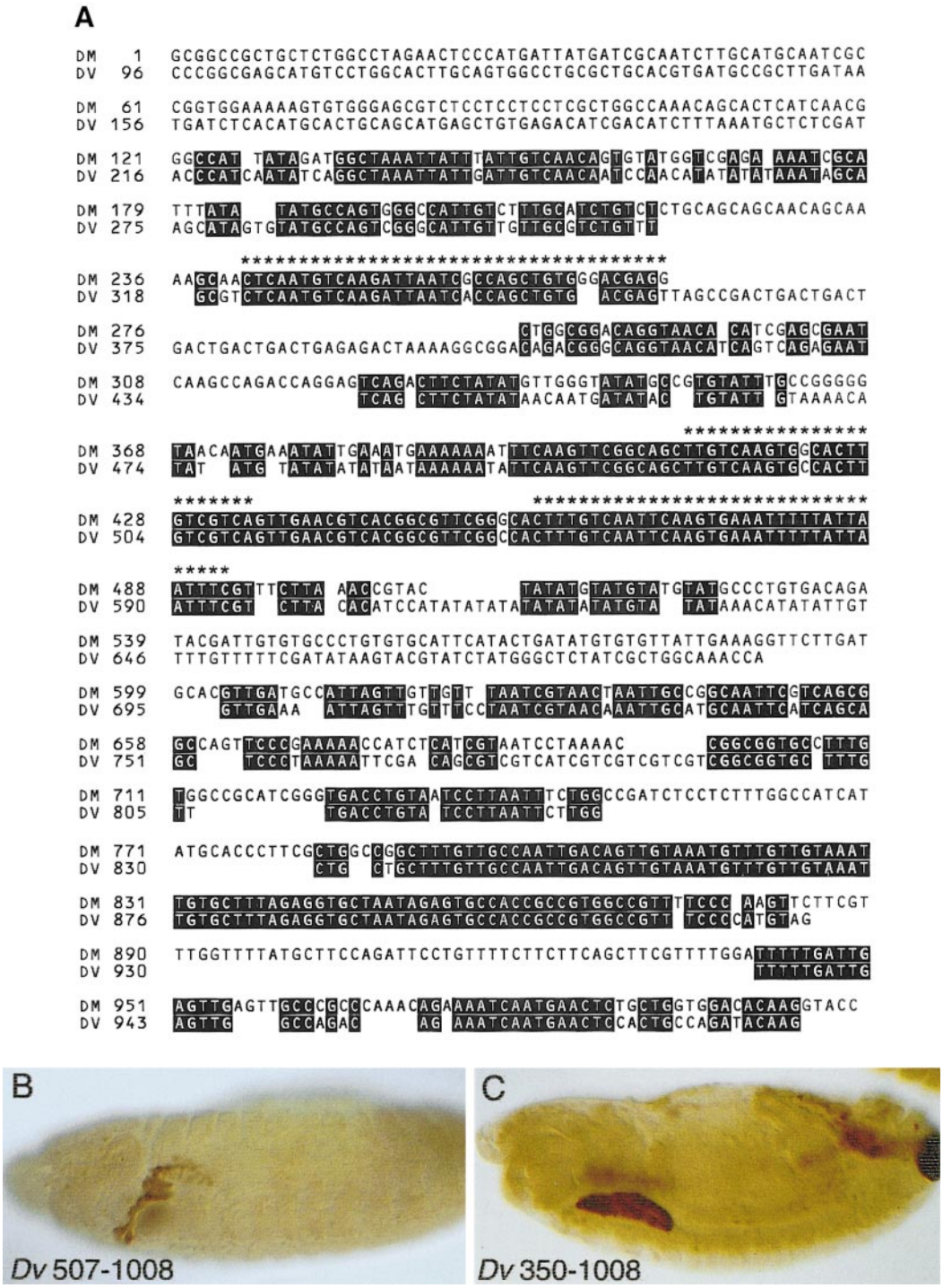

FIG. 4. (A) Sequence comparison between the *D. melanogaster* and *D. virilis fkh* salivary gland enhancers reveals multiple conserved domains. Conserved regions are highlighted. Asterisks indicate the 250, 420, and 460 elements. (B, C) The *fkh* salivary enhancer is functionally conserved between *D. melanogaster* and *D. virilis*. (B) The *virilis* 507-1008 homolog in *melanogaster*, showing *lacZ* expression in the anterior part of the salivary glands and in the ducts. (C) The *virilis* 350-1008 homolog in *melanogaster*: expression in the salivary glands but not the ducts.

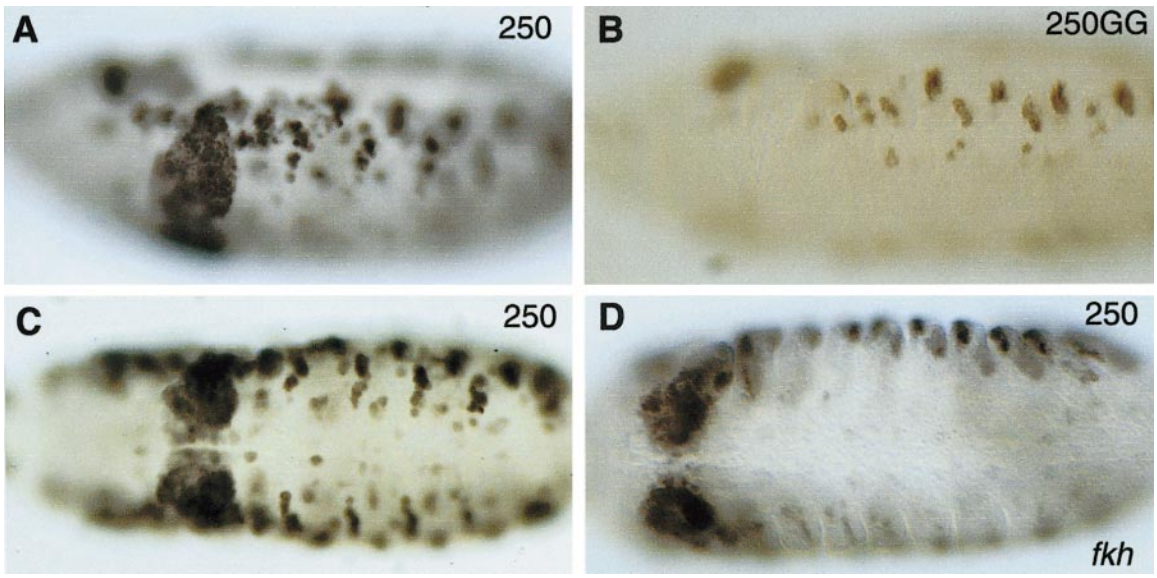

FIG. 5. The 250 element is a parasegment 2 enhancer and a probable SCR target. (A, C) $(250)_3$:*lacZ* directs expression in PS2. These ventrolateral and ventral views of a stage-11 embryo show expression throughout PS2 and in a repeating pattern in other ectodermal segments. (B) A 2-nt mutation within the potential SCR binding site of the 250 element eliminates the PS2-specific expression. Expression in other parasegements is not much affected. (D) $(250)_3$:*lacZ* expression is not dependent on *fkh*. At stage 13, the uninvaginated salivary gland cells of a *fkh*-mutant embryo continue to express $(250)_3$:*lacZ*.

Deletion Analysis of the fkh Salivary Enhancer Reveals Several Spatially Distinct Transcriptional Elements

This conserved organization along with additional dissection of the enhancer has allowed the identification of several important regulatory regions that have distinct functions (Fig. 1B). First, because 219-1008 activity is stronger than 350-1008, there is likely to be an activating element between 219 and 350. In addition, there is little difference between the enhancer activities of 1–308 and 1–356, suggesting that this activator does not lie between 308 and 356. From this analysis, we concluded that there is probably an activating element within the 219–308 region. By similar reasoning, there is likely to be an activating element within the 357–506 region and multiple activating elements within the 507-1008 region.

We also identified a negative element in the 350–506 region. In addition to its placode expression, 507-1008 gives strong ectopic expression in the cells ventral to the placodes, expression that is not seen for *fkh* itself and is only occasionally and weakly seen with the 1-kb enhancer. The strong ventral derepression indicates that 507-1008 lacks a ventral repression element that is present in the 1-kb enhancer. Further dissection located this element between 350 and 506, because 350-1008 is ventrally repressed (Fig. 1B). Further analysis has concentrated on the positive 219–308 region and on the 357–506 region that contains both positive and negative elements.

The 250 Region Acts as a Parasegment 2 Enhancer

In the 219–308 interval, 242–277 is strongly conserved in the *D. virilis* enhancer (Fig. 4A), and we have designated it as the 250 region for further analysis. To test the activity of this 36-bp region, three copies of 250 were placed upstream of the minimal heat shock promoter and *lacZ*. Multiple transformed lines containing this construct were obtained, and all showed β-galactosidase expression throughout the ectoderm of PS2, as well as in a few cells in each trunk segment and patches in the head (Fig. 5).

These results suggest that, in its normal context, 250 activates *fkh* expression in the salivary placode, perhaps by binding the homeotic regulator SCR. Within the 250 element, there is one region with two overlapping sequences of ATTAAT that are similar to homeodomain core binding sites (Fig. 4A). To test whether this consensus site is important for PS2 expression, the two contiguous As were changed to Gs. These changes should destroy homeodomain protein binding, but have minimal effect on the surrounding regions. Staining of transformants carrying this 250-GG construct is consistent with the direct involvement of *Scr* in the enhancer activity of the 250 element. The few cells in PS2 that continue to stain are not specific to PS2 but are part of a segmentally iterated pattern seen in other segments of the embryo in addition to PS2 (Fig. 5B). The specific disappearance of staining from PS2 indicates that the substituted nucleotides are normally bound by a PS2-specific transcription factor, probably SCR (see Discussion).

Identification of the 420 Element as a Negative Regulator That Responds to EGFR Signaling

As mentioned above, comparison of the 507-1008 and 350-1008 constructs identified an element between 350 and 506 that mediates ventral repression. If this location is correct, deletion of this region from the otherwise intact 1-kb enhancer should lead to ventral derepression. As shown in Figs. 6A and 6B, Δ361–506, an internally deleted construct that removes most of this region, does indeed direct strong expression in both the placode and the ventral cells. The ectopic expression in the ventral cells is as strong as in the placodes, indicating that few if any, functional ventral repression elements exist in the remaining 0.85 kb of the enhancer. This is the same pattern of expression that we saw for *fkh* itself or for the 1-kb *fkh* enhancer in embryos that lack *EGFR* signaling (Panzer *et al.*, 1992; and data not shown). Therefore, we propose that an *EGFR*-dependent repressor normally acts on the 361–506 region to prevent *fkh* expression in the cells ventral to the placode.

Comparison to the *virilis* enhancer sequence does not help to localize the negative element more precisely because almost the entire 350–506 region is conserved. However, preliminary footprinting experiments with crude embryonic extracts showed protection of the 415–424 region which overlaps two adjacent e-box sequences separated by a single nucleotide. To test whether this region mediates the ventral repression signal, we constructed three internally deleted versions of the 1-kb enhancer that lack either 361–424, 413–424, or 413–506. All three lack 413–424. When tested in embryos after transformation, all three constructs gave stronger duct staining than the 1-kb enhancer (data not shown). The 413–506 deletion which extends furthest to the right has the most dramatic effect, suggesting that there is more than one repression element within the 361–506 region. The smallest deletion, 413–424, must disrupt at least one of these elements.

These experiments showed that this region (hereafter called the 420 region) is necessary for ventral repression. To test whether it is sufficient, three copies of a 21-bp fragment (411–431) were added to the Δ361–506 construct, which by itself is expressed throughout the salivary gland and duct primordia. As a result, ventral repression was restored (Figs. 6C and 6D). Similarly, when this 21-bp fragment was inserted between 507 and 1008 and the basal promoter, *lacZ* expression was limited to parts of the placode, or at later times, to the salivary glands (data not shown).

We conclude that the 420 region contains an element that mediates the ventral repression signal. This repression normally overrides activation signals for the reporter or *fkh* expression in the preduct region and limits *fkh* expression to the salivary placode.

The 460 Region Mediates fkh Autoregulation

As described above, there is not only a ventral repression element, but also an activating element in 361–506. To locate this element, we divided this 146-bp region into small elements and put a concatemer of each region in front of 507-1008 to test for enhancement of its salivary gland activity. This strategy should allow detection of even minor enhancements, because 507-1008 by itself is not expressed in all cells of the placode or salivary gland. The 460 element, which includes nucleotides from 457 to 495, gave a strong response in this assay, indicating that 460 contains an activating element (Fig. 7A). To determine whether this element itself is sufficient to be a salivary enhancer, a concatemer including four copies of just the 460 element was tested. Staining showed that this element can by itself activate *lacZ* expression that is limited to the salivary glands (Fig. 7B). However, expression of $(460)_4$:*lacZ* begins during salivary invagination, later than either *fkh* or the Scr-dependent construct 250:*lacZ*. This temporal difference suggests that the enhancing activities of 250 and 460 are different.

Sequence comparison showed that the 460 element includes a consensus FKH/HNF3 binding site sequence that is very similar to an established FKH binding site from the *Kruppel* promoter (Fig. 8A) (Kaufman *et al.*, 1994). This similarity suggested that endogenous FKH might be activating the 460 construct. To test this idea, $(460)_4$:*lacZ* was transferred to *fkh*-mutant embryos. Its activity is absolutely dependent on FKH (Fig. 7C), suggesting that *fkh* expression in salivary glands is autoregulatory.

Because of the sequence similarity between the 460 element and the FKH/HNF3 binding sequence, this interaction may be direct. To test this idea, a FKH fusion protein was expressed in *Escherichia coli* and used in gel shift experiments with two different oligonucleotides, the 460 element itself (39 bp, 457–495) and $fkh(11)_4$, a four-fold concatemer of the 11-bp, potential FKH binding site. As shown in Fig. 8B, there is a single shifted band with 460 and multiple, shifted bands with $fkh(11)_4$, consistent with its multiple repeats. In both cases, specific competitors reduce the shifted bands. Thus, binding appears to be specific for the 11 nucleotides of the consensus and does not require interaction with other sequences in the 460 element. Taken together, these experiments indicate that FKH can bind to the 460 region through the 11 nucleotides of the consensus FKH binding site and suggest that this binding accounts for $(460)_4$:*lacZ* expression *in vivo*.

Fate Mapping Salivary Tissues with fkh-lacZ Constructs

If embryos expressing one of the partial *fkh* enhancer constructs are allowed to finish invagination, only a subset of the salivary cells will be marked by *lacZ* expression. Since the salivary cells do not divide during this time, a fate map can be constructed by correlating the position of marked cells at the placode and gland stages (Fig. 9).

The 1–506 construct gives rise to expression in the dorsal part of placodes. Older embryos show staining in the distal 3/4 of the glands (Fig. 2D). This result suggests not only that the dorsal part of the placode becomes the distal part of the

salivary gland, but also that the ventral part of the placode gives rise to the proximal salivary gland. The 507-1008 enhancer construct confirms this suggestion by showing its strongest placode expression in the ventral cells and later showing strong expression in the proximal cells of the invaginated glands (Figs. 2E, 2F, and 2H). With this construct, we also see variable expression in the posterior part of invaginated glands, and we propose that this staining comes from the variable anterior and posterior stripes seen at the placode stage. The fact that some embryos have no stripes is consistent with our observation that some invaginated glands have little distal staining (Fig. 2F). This construct consistently shows little staining in mid-dorsal placode cells and in cells on the medial side of the invaginated gland about halfway along its length (Figs. 2E and 2H). We think these are the same cells at different stages. These results, along with the fact that invagination in uniformly stained placodes can be seen to initiate at the dorsal, posterior edge of the placode, can be combined to produce a consistent hypothesis for salivary morphogenesis. The first cells to invaginate are those at the dorsal end of the posterior stripe. They are immediately followed by the remaining cells of the posterior stripe and by the cells of the mid-dorsal placode. All of the cells of the dorsal placode invaginate before any cells of the ventral placode do. The ventral cells are the last placode cells to invaginate, and they form the proximal end of the gland. Finally, the duct forms after the gland has invaginated.

At stage 11, both 507-1008 and Δ361–506 give ectopic expression in cells ventral to the placodes. Examination of these embryos at later stages shows that the salivary ducts are stained in addition to part or all of the glands (Figs. 2F, 2H, and 8B). By examining embryos at intermediate stages during the invagination of the glands and ducts, we can see a continuous progression from the cells ventral to the placode to the invaginated ducts. Therefore, we conclude that the cells ventral to the placodes are the duct precursors. A similar conclusion has been reached by Isaac and Andrew (1996) and by Kuo *et al.* (1996).

DISCUSSION

Multiple Salivary Enhancer Elements Cooperate to Turn on fkh in a Precise Spatial Pattern

Based on current and previous studies of *fkh* transcriptional control (Weigel *et al.*, 1990), it is clear that of the 25 kb of regulatory DNA surrounding the *fkh* gene, only the 1-kb DNA identified in this study works as a salivary enhancer. Within this enhancer, there are several regulatory elements that direct *fkh* transcription in different parts of the salivary primordium.

Activation elements. SCR, the principal salivary activator, must act upon more than one site in the *fkh* enhancer, because the left and right halves of the enhancer can independently activate expression in salivary glands. We have identified one SCR-responsive element in the left half, the 250 element. This 39-bp element is likely to bind SCR *in vivo*, since we find that mutations in its consensus homeodomain binding site prevent parasegment 2 expression. These results have recently been extended with the demonstration that both SCR and its cofactor, the EXTRADENTICLE protein, bind to 250 *in vitro* and are necessary for 250 activity *in vivo* (Ryoo and Mann, 1999).

Repression by the EGFR pathway. Negative regulatory elements that respond to *EGFR* signaling appear to be concentrated in the 350–506 region of the enhancer. Although *Scr* is initially expressed throughout PS2, activation of *fkh* in the preduct cells is overridden by these negative elements.

Our previous results showed that signaling through the Spitz-EGFR pathway defines the ventral boundary of *fkh* expression and the ventral boundary of the placode (Panzer *et al.*, 1992; Kuo *et al.*, 1996). The current results add two new features to the role of this pathway. First, we have shown that an EGFR-dependent repressor acts to regulate *fkh* transcription rather than the transcription of an upstream activator like *Scr* or an *Scr* cofactor. This is shown by the identification of a ventral repression element in the *fkh* enhancer that is both necessary and sufficient to exclude expression from the most ventral cells of PS2. The identity of the protein binding to this element has not yet been determined. The only known EGFR-regulated, transcriptional repressor, YAN, is inactivated rather than activated by EGFR signaling (Gabay *et al.*, 1996).

Second, we find that EGFR signaling defines at least two different ventral boundaries in PS2: the ventral edge of the dorsal placode and the ventral edge of the ventral placode. EGFR signaling in the ventral epidermis originates from cells at the ventral midline, probably by production of a secreted form of the Spitz ligand that can activate the EGFR several cells away from the midline (Golembo *et al.*, 1996). The resulting signal is graded with its highest level ventrally (Raz and Shilo, 1993; Schweitzer *et al.*, 1995). These results suggest that the ventral edge of the dorsal placode responds to a lower threshold of EGFR signaling than does the edge of the ventral placode. In addition, there may be a second negative regulator for the edge of the dorsal placode. Even though *sim*-mutant embryos do not produce secreted Spitz at the midline, ventral expression of the 1–506:*lacZ* construct is only partially derepressed. This second negative regulator might be novel, or it might result from early activity of the EGFR before the ventral midline cells are formed (Skeath, 1998). The possibility that residual, low level activity of EGFR might partially block 1–506:*lacZ* expression would be consistent with a low threshold for 1–506:*lacZ* repression.

FKH autoregulation. The FKH-responsive site at 460 suggests that *fkh* expression is autoregulatory. This result may account for the continued expression of *fkh* after its initial activator is gone. SCR protein begins to disappear from the ventral part of parasegment 2 at about the time the salivary placode first forms (LeMotte, 1989) and is absent from invaginated salivary glands. In contrast, *fkh* continues

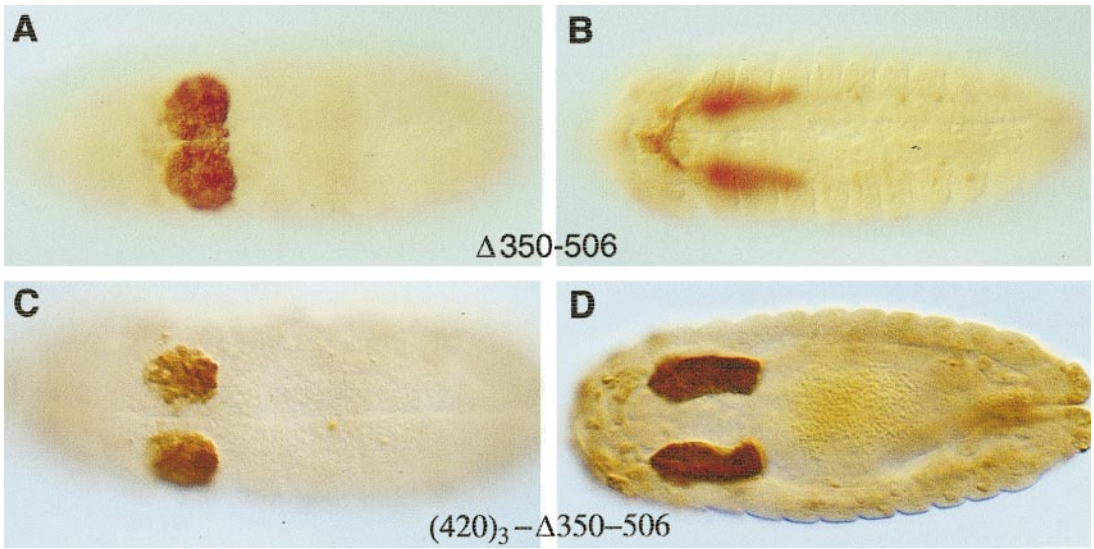

FIG. 6. The 420 element mediates ventral repression. Panels on the left are ventral views of the placode stage. Panels on the right are ventral views of invaginated glands. (A, B) Expression of Δ350–506:*lacZ*. (B) The salivary duct instead of the glands is in focus. (C, D) Expression of $(420)_3$-Δ350–506:*lacZ*. Staining in the preduct cells (C) or duct cells (D) is strongly repressed.

to be expressed in the salivary glands throughout embryonic and larval development (Panzer, 1994; Lehmann and Korge, 1996; Mach *et al.*, 1996). The expression of several salivary gland-specific genes is dependent on this continued expression of *fkh*, both in later embryos (Weigel, 1989; our unpublished results) and in third instar larvae (Lehmann and Korge, 1996; Mach *et al.*, 1996). In contrast, several genes whose expression begins in the early placode as *fkh* expression does, are transiently expressed. These include *Toll*, *huckebein*, *trachealess*, and *eye gone* (Panzer, 1994; Isaac and Andrew, 1996; Kuo *et al.*, 1996; Jones *et al.*, 1998). Expression of *trachealess* and *eye gone* has been shown to depend on SCR, and timing of the expression of all these genes suggests that they are, like *fkh*, directly activated by SCR. Their transcripts disappear from the glands at or shortly after invagination, about the time that SCR protein disappears.

Response to wg signaling. The final regulatory element we have identified is located in the right half of the 1-kb enhancer and responds to a combination of *Scr* and *wg* signaling to produce anterior and posterior stripes of expression. This pattern, at least the posterior stripe, may be important for *fkh* function in the placode, function that is required for placode invagination (Weigel *et al.*, 1989). In wild-type embryos, expression of FKH protein is initially strongest at the posterior side of the placode where invagination starts. In *wg* embryos, no obvious invagination is observed at stage 11 and later invagination often starts generally across the whole placode rather than being localized at the posterior dorsal edge (Panzer, 1994). Thus, it is possible that *wg* signaling is involved in the stronger *fkh* expression at the dorsal posterior side and that this stronger expression triggers local invagination of the salivary placode.

Subdivisions of the Salivary Primordium Are Biologically Relevant

There are additional reasons to think that the four domains of expression that we have characterized are biologically relevant rather than just artifacts of arbitrary enhancer dissection. First, several other salivary-specific genes or enhancer traps are expressed in these domains. The terminal gap gene *huckebein* is expressed in the placode and is required for normal invagination (Bronner *et al.*, 1994; Panzer, 1994). It initially appears as anterior and posterior stripes just like the stripes we see with 506-1007. *Toll* is also expressed specifically in the placode (Gerttula *et al.*, 1988), initially as a posterior stripe, then throughout the dorsal part of the placode, before it finally appears in the whole placode just before invagination (Panzer, 1994). Several other genes and enhancer trap lines are first expressed at the posterior edge before spreading throughout the placode. These observations indicate that the patterns directed by partial *fkh* enhancers are shared with other genes and also suggest that these genes share common genetic control mechanisms with *fkh*.

The second reason for thinking that the expression domains are important is that they align with the patterns of cell movement during invagination. Cells of different domains invaginate in a prescribed order and show little intermixing except at their borders. Invagination begins in cells that lie in the overlap between the dorsal domain of the placode and the posterior stripe. Then, the cells of the

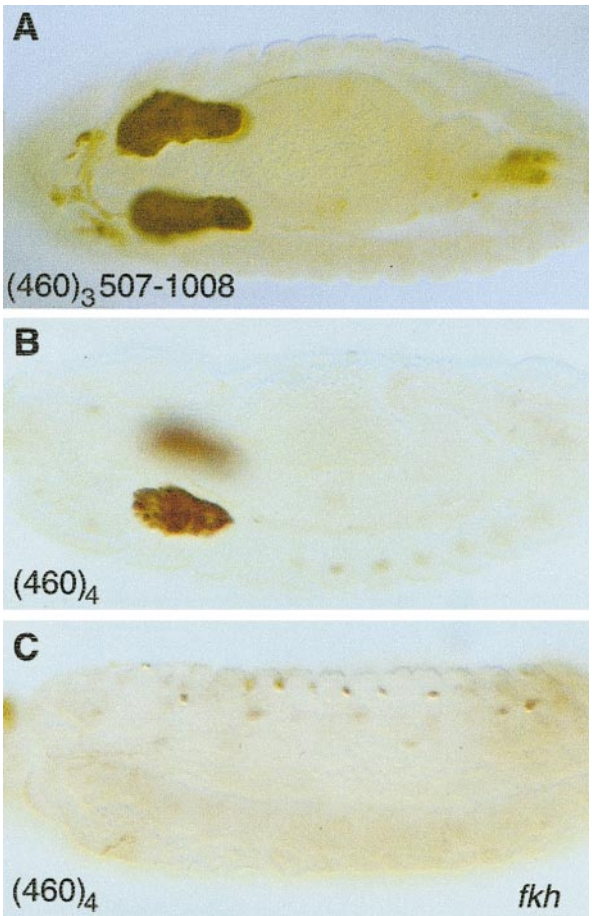

FIG. 7. The 460 element has *fkh*-dependent salivary gland enhancer activity. (A) When three copies of 460 were inserted next to the 507-1008 fragment, the gland had much stronger and more uniform expression than 507-1008 alone (compare with Figs. 2F and 2H). Interestingly, expression in the ducts is reduced, suggesting that 460 also has ventral repression activity. (B) Four copies of 460 alone are sufficient to drive expression in the salivary gland. (C) In fkh-mutant embryos, the $(460)_4$:*lacZ* expression disappears. Shown here is a stage-13 *fkh* embryo. In wild-type embryos, $(460)_4$:*lacZ* expression begins in early stage 12.

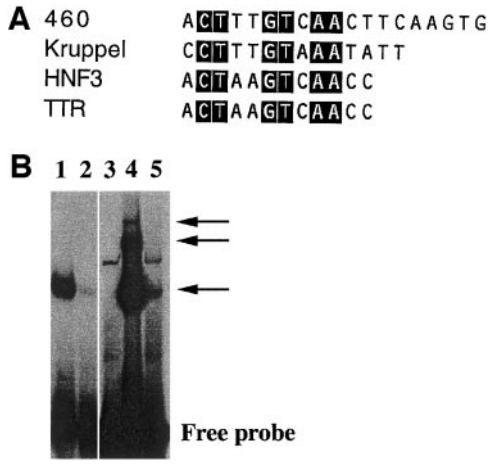

FIG. 8. (A) Shown below the 460 sequence are three strong FKH/HNF3 binding sites (Kaufmann *et al.*, 1994). (B) Mobility shift assay showing that FKH binds to the 460 element *in vitro*. In lanes 1–3, the double-stranded oligonucleotide is the 45-bp 460 element. In lanes 4–6, the oligonucleotide is the 44-bp $fkh(11)_4$, which contains four tandemly repeated copies of the potential FKH binding site from the 460 element. The reactions for lanes 1, 2, 4, and 5 included 60 ng of MBP-FKH fusion protein. Lane 1, 460 element with 60 ng of the MBP-FKH fusion protein. In addition, lane 2 had 200 ng of 460 element cold competitor (200× excess), and lane 5 had 200 ng $fkh(11)_4$ cold competitor (200× excess). Lane 3 had just the free $fkh(11)_4$ probe. Total volume for the binding reaction was 20 μl; 1 μg poly(dG/dC) was added to each reaction as a nonspecific competitor.

dorsal domain invaginate before cells of the ventral placode do. Next, the ventral placode cells invaginate before the still more ventral duct cells. Thus the cells in these domains must be functionally distinct before invagination has occurred.

Finally, after invagination the cells deriving from these domains, at least the dorsal and ventral domains, continue to be functionally distinct and these differences may be evolutionarily conserved. In *Bombyx*, the p25 gene is expressed in the posterior silk glands (silk glands and *Drosophila* salivary glands are probably homologous tissues and both are examples of labial glands). When introduced into *Drosophila*, p25 regulatory sequences drive reporter expression only in the anterior part of the larval salivary glands (Bello and Couble, 1990). This stained anterior region corresponds to about 20 cells, similar to the anterior domain we have described for embryonic glands, a domain

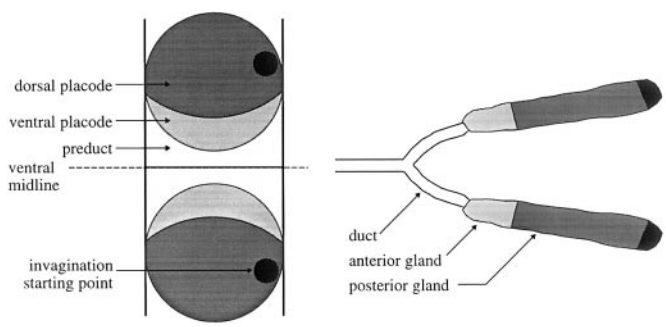

FIG. 9. Fate map of salivary tissues. On the left is a salivary precursor at the placode stage. The right shows the fates of several regions after invagination. The ventral and dorsal parts of the placode give rise to the anterior and posterior parts of the glands respectively. The region ventral to the placode gives rise to the duct.

that arises from the ventral cells of the placode. These results show that the anterior salivary gland cells are a distinct cell type, and probably one that is already distinct when the placode forms. Thus, though *Drosophila* salivary glands appear to be a homogeneous epithelium, they can be divided into multiple cell types, much like the labial glands of many other insects. In addition, at least one set of labial gland-specific transcriptional regulators has been conserved during evolution and is restricted, in both Bombyx and *Drosophila* to a subset of labial gland cells (Bello and Couble, 1990).

Is There a Salivary Selector Gene?

Since we are interested in how salivary glands are specified, we wanted to know whether there is a salivary selector gene, a gene that is activated in the salivary primordium but not surrounding tissues and then is required for the activation of all subsequent salivary-specific genes. Our finding that the salivary-specific *fkh* enhancer integrates several spatial cues suggests that a salivary selector gene does not exist.

Among the salivary-specific genes identified so far, *fkh* is the most important for salivary gland development. In *fkh* mutant embryos, invagination fails and the placodes remain on the surface of the embryo for several hours until they disaggregate (Weigel *et al.*, 1989). Despite this importance, *fkh* is not a master regulator or selector gene for salivary development. Several other genes, among them *CrebA*, *Toll*, *huckebein*, *trachealess*, and *eye gone*, are expressed in the salivary placode at about the same time as is *fkh* (Andrew *et al.*, 1994; Gertulla *et al.*, 1998; Panzer, 1994; Isaac and Andrew, 1996; Kuo *et al.*, 1996; Jones *et al.*, 1998). All that have been tested are dependent on *Scr* for salivary expression (Panzer *et al.*, 1992; Andrew *et al.*, 1994; Isaac and Andrew, 1996; Kuo *et al.*, 1996; Jones *et al.*, 1998). We have tested whether these genes or several similarly expressed enhancer trap lines are dependent on *fkh*. None are. Several of the other early genes are derepressed ventrally in *spitz*-group mutant embryos, and at least two, *hkb* and *Toll*, are expressed in anterior and/or posterior stripes in PS2 (Panzer, 1994). Thus it appears that regulation of these genes may be similar to *fkh* regulation; each may independently integrate spatially limited inducers and repressors. These data suggest that there is probably not a simple linear pathway from *Scr* through a single salivary specifier. Instead, it appears more likely that the combinatorial effects of the early, salivary-specific genes, *fkh* among them, determine salivary identity. Each of these early genes would affect the expression of a different or partially overlapping set of downstream genes. Both positive and negative regulators of placode positioning and identity would act on the expression of multiple genes to define the placode. Placode determination would arise by consensus rather than fiat.

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