Setting limits on homeotic gene function: restraint of *Sex combs reduced* activity by *teashirt* and other homeotic genes

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Each of the homeotic genes of the HOM or HOX complexes is expressed in a limited domain along the anterior–posterior axis. Each homeotic protein directs the formation of characteristic structures, such as wings or ribs. In flies, when a heat shock-inducible homeotic gene is used to produce a homeotic protein in all cells of the embryo, only some cells respond by altering their fates. We have identified genes that limit where the homeotic gene *Sex combs reduced* (*Scr*) can affect cell fates in the *Drosophila* embryo. In the abdominal cuticle *Scr* is prevented from inducing prothoracic structures by the three bithorax complex (BX-C) homeotic genes. However, two of the BX-C homeotic genes, *Ultrabithorax* (*Ubx*) and *abdominal-A* (*abd-A*), have no effect on the ability of *Scr* to direct the formation of salivary glands. Instead, salivary gland induction by *Scr* is limited in the trunk by the homeotic gene *teashirt* (*tsh*) and in the last abdominal segment by the third BX-C gene, *Abdominal-B* (*AbdB*). Therefore, spatial restrictions on homeotic gene activity differ between tissues and result both from the regulation of homeotic gene transcription and from restraints on where homeotic proteins can function.

Introduction

Homeotic mutations cause one part of an animal to develop as a copy of a different part. Such dramatic transformations identify homeotic genes as master regulators of developmental pathways. Homeotic gene organization and function are remarkably similar among both invertebrates and vertebrates (McGinnis and Krumlauf, 1992), indicating that these regulatory pathways are probably common to all animals. Many of the homeotic genes are located in clusters, including the HOX complexes in mammals and the Antennapedia Complex (ANT-C) and bithorax Complex (BX-C) in flies (Duncan, 1987; Kaufman *et al.*, 1990). Homeotic genes encode transcription factors (Levine and Hoey, 1988; Scott *et al.*, 1989), each of which is thought

to regulate specific arrays of ‘downstream’ target genes. The differential transcription of homeotic genes along the anterior–posterior axis determines differences in cell fates.

Both mutations and engineered genes have been used to observe the effects of the ‘ectopic’ expression of a homeotic gene, i.e. expression at novel locations. Such mis-expression and the resulting misplaced body parts usually have disastrous consequences for the animal. Things could be worse: although ubiquitous expression of homeotic proteins leads to homeotic transformations, not all segments are visibly affected. Limitations on homeotic gene activity are revealed in the transformations that do not occur when a homeotic gene is expressed everywhere in the developing organism. In *Drosophila*, *Caenorhabditis elegans* and mammals only a subset of the cells ectopically expressing a homeotic protein exhibit any transformations (Schneuwly *et al.*, 1987; Kuziora and McGinnis, 1988; Balling *et al.*, 1989; Gibson *et al.*, 1990; Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes *et al.*, 1990, 1992; Kessel *et al.*, 1990; Mann and Hogness, 1990; Jegalian and De Robertis, 1992; Lufkin *et al.*, 1992; Morgan *et al.*, 1992; Salser and Kenyon, 1992; Salser *et al.*, 1993).

In some cases, homeotic gene activity is known to be restricted by other homeotic genes normally expressed in more posterior segments (Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes *et al.*, 1990, 1992; Mann and Hogness, 1990). The reason for this ‘posterior prevalence’ is unknown, and the details of the homeotic gene interactions vary depending on the tissue (Heuer and Kaufman, 1992; Zeng *et al.*, 1993).

To understand the basis for spatial restrictions on homeotic gene function, we have focused on structures that require the homeotic gene *Sex combs reduced* (*Scr*) for their formation. Larval salivary gland development is dependent on *Scr*; salivary glands do not develop in loss of function *Scr* mutants (Panzer *et al.*, 1992). Although *Scr* is expressed in both the posterior head and anterior thorax, salivary glands form only in the head. *Scr* directs the two different patterns of cuticular structures, one in the posterior head and one in the anterior thorax (Kaufman *et al.*, 1980; Lewis *et al.*, 1980; Wakimoto and Kaufman, 1981; Struhl, 1982; Sato *et al.*, 1985).

The patterns of epidermal cuticular structures in the posterior head and anterior thorax depend on *Scr* expression in parasegments 2 and 3 (PS2 and PS3). Parasegments, units that encompass the posterior part of one segment and the anterior part of the adjacent more posterior segment (Martinez-Arias and Lawrence, 1985), are useful divisions because some homeotic genes function in parasegmental domains (Hayes *et al.*, 1984). *Scr* transcripts first appear in PS2, which corresponds to the posterior maxillum and anterior labium, just after cell membranes form at the post-cellular blastoderm stage (Kuroiwa *et al.*, 1985; Martinez-Arias *et al.*, 1987). *Scr* protein can be detected immediately after the onset of gastrulation, after the ventral and cephalic

furrows have formed (Mahaffey and Kaufman, 1987; Riley *et al.*, 1987; LeMotte *et al.* 1989; Mahaffey *et al.* 1989). Salivary glands form by the invagination of a ventrolateral subset of the *Scr* expressing cells of PS2. Later, during germ band extension, the *Scr* gene is also expressed in PS3, which corresponds to the posterior labium and anterior prothorax (Mahaffey and Kaufman, 1987; Riley *et al.*, 1987; LeMotte *et al.*, 1989; Mahaffey *et al.*, 1989).

The mis-expression of the *Scr* protein in regions of the body where it is not normally made, either through gain of function mutations or through engineered genes, leads to dominant transformations of body segments to a labial or prothoracic (T1) identity (Tokunaga, 1966, 1972; Struhl, 1982; Gibson *et al.*, 1990; Heuer and Kaufman, 1992; Zeng *et al.*, 1993). When *Scr* protein is ubiquitously expressed in the developing embryo and adult, not all segments are transformed. For example, the prothoracic or 'T1' beard can be ectopically induced only in the two posterior thoracic segments, the mesothorax (T2) and metathorax (T3), but not in abdominal segments (Gibson *et al.*, 1990). Similarly, the expression of *Scr* protein everywhere in the embryo leads to the expression of some salivary gland markers only in segments anterior to the normal salivary gland producing cells (Panzer *et al.*, 1992; Zeng *et al.*, 1993; and this work). Thus the influence of *Scr* is limited by its domain of transcription and, if it is mis-expressed, also by a restricted ability to affect cells where it is not normally transcribed.

Why do salivary glands form only in the head and not the thorax, despite *Scr* expression in both places? How does *Scr* direct the formation of different patterns of cuticular structures in the different segments where it is expressed? What modulates the effect of *Scr* on cell fate: are necessary cofactors available only in certain cells, or is the influence of *Scr* limited by other genes that suppress its action?

We find that restrictions on *Scr* protein activity are imposed by different genes in different tissues. We demonstrate first that BX-C homeotic genes limit *Scr* transcription and function in the cuticle, and then that a different gene, *teashirt* (*tsh*), limits *Scr* transcription and function in the formation of salivary glands.

Results

Ectopic salivary gland formation is limited to cells anterior to PS2 in HS-SCR embryos

Salivary glands arise during embryogenesis from two ventrolateral 'placodes' of 80–100 cells each which span PS2 (Panzer *et al.*, 1992). All cells that contribute to salivary glands derive from *Scr* expressing cells of the embryonic ectoderm. The salivary gland placodes visibly differentiate during germ band extension, when the cells of the posterior of the embryo move dorsally and anteriorly and come to lie above the more anterior cells of the embryo. At the beginning of germ band retraction, the stage when the posterior cells

Fig. 1. Salivary glands are induced by ectopic expression of an *Scr* cDNA. Embryos were collected for 4 h, aged 1 h and heat shocked for 45 min. Expression of β -gal protein resulting from a *lacZ* insertion element in the *jalapeño* gene (A and B) or a *lacZ* insertion element in the *hückebein* gene (C and D), and of D3 antigen (E and F), was detected by antibody staining in wild-type (A, C and E) and HS-SCR (B, D and F) embryos. The heavy dark arrow indicates the normal salivary glands derived from PS2. The open arrows indicate additional salivary glands induced by HS-SCR expression. Note that the additional cells expressing each marker often fuse with the PS2 salivary gland. Staining seen posteriorly corresponds to cells of either the ventral nerve cord (C and D) or the trachea (A, B, E and F).

move back approximately to their original position in the embryo, cells near the lateral edge of each placode invaginate and move dorsally and posteriorly. Lumen-specific antigens are detected just after the salivary gland cells begin to invaginate (Figure 1E). By the end of germ band retraction, the salivary gland cells arrive at the most posterior extent of their migration, reaching the middle of the third thoracic segment and lying dorsolateral to the ventral nerve cord (Figure 1A and C).

Genes expressed in the developing salivary glands were identified through screens of enhancer trap line collections from different laboratories (D.J.Andrew and M.P.Scott, unpublished data). Enhancer trap lines contain P-element transposons bearing a largely inactive promoter fused to the *E.coli lacZ* gene inserted at one or a few sites in the genome (O'Kane and Gehring, 1987). The expression of β -galactosidase (β -gal) in these lines often mimics the expression pattern of a gene near the site of insertion due to the activation of the promoter by nearby enhancers. Several of the enhancer trap lines that produce β -gal early in salivary gland differentiation were used as markers to assay the effects of both the loss of *Scr* function and the ubiquitous expression of *Scr* protein. For all markers tested, the loss of *Scr* function results in the loss of marker expression in PS2, the region of the embryo where salivary glands normally form (data not shown).

When *Scr* protein is expressed everywhere using an *Scr* cDNA under the control of the hsp70 heat shock-inducible promoter (HS-SCR), salivary gland markers are expressed in several parasegments in addition to PS2. In each case, the ectopic expression occurs in the same dorsal–ventral position as the normal salivary glands, suggesting that normal dorsal–ventral restrictions of salivary gland formation are still operative. When *Scr* protein is ubiquitously expressed, each of the markers tested, including enhancer trap inserts in the *jalapeño* (*jal*) (Figure 1A and B) and *hückebein* (*hkb*) genes (Weigel *et al.*, 1990) (Figure 1C and D), become active only in cells anterior to the normal salivary gland expressing cells (Figure 1B and D). No such ectopic expression is observed when control animals that do not contain the HS-SCR transgene are heat shocked and stained with antibodies to β -gal (Figure 1A and C). Similar changes in expression patterns were detected using a *lac-Z* insert in the gene *dCREB-A* (line B204; Zeng *et al.* 1993), an antibody to the *dCREB-A* protein itself (Figure 5A and C) and an antibody (D3) against an unknown antigen found in the lumen of the salivary gland and other invaginating ectodermal structures (Figure 1E and F). All of the markers used in this study are ectopically induced in the two parasegments anterior to PS2 in response to HS-SCR (Figures 1 and 2). In contrast, the *fork head* (*fkh*) marker used in previous studies is induced in more posterior segments as well (Panzer *et al.*, 1992; Zhao *et al.*, 1993).

By all available criteria the cells induced by HS-SCR to express the marker proteins appear fully determined to be salivary glands. The size of the nuclei in the additional salivary gland cells is equivalent to that of the normal glands, suggesting the same level of polyteny. This is a meaningful criterion because polyteny occurs early in salivary glands relative to other tissues (Smith and Orr-Weaver, 1991). The marker expressing cells invaginate to form a lumen and express the lumen antigen, D3 (Figure 1E and F). The intensity of signal with each marker is equivalent to the signal in endogenous glands. Frequently the extra salivary gland

cells fuse with the normal salivary glands of PS2 to form an extensive array of marker expressing cells (Figure 1B and D). The behavior of the extra salivary gland cells suggests not only a full transformation of the cells to a salivary gland fate but also mutual recognition among all cells determined to be salivary glands.

The overall organization of the ectopic glands depends on the number of cells involved. If enough cells are recruited, the shape of the gland is very much like that of endogenous salivary glands. If only a few cells express the salivary gland markers, irregularly organized glands smaller than normal are seen. The variability in the number of cells forming salivary glands seems to be related to the amount and/or duration of exposure to *Scr* protein; longer heat shock treatments (45 versus 30 min) result in a larger number of cells expressing each marker (data not shown).

SCR-induced prothoracic structures are blocked in abdominal epidermis by BX-C genes

Because the expression of *Scr* protein everywhere in the embryo leads to salivary gland formation only in PS0–2, either some positively acting factors necessary for salivary gland formation are functional only in PS0–2, or factors suppressing salivary gland formation act in more posterior parasegments, or both. Based upon previous observations, the ANT-C and BX-C homeotic genes expressed posterior to PS2 are likely candidates for negatively acting factors (Duncan, 1987; Kaufman *et al.*, 1990). Ectopic expression of the homeotic genes *Antennapedia* (*Antp*) and *Ultrabithorax*

Fig. 2. Expression patterns in wild-type and HS-SCR animals of salivary gland markers are summarized. At the top is a grid of the segmented portion of the embryo. Anterior (A) is to the left, posterior (P) to the right, dorsal (D) is up and ventral (V) is down. *dpp* represses salivary gland formation dorsally and the 'spitz group' genes repress salivary gland formation along the ventral midline (Panzer *et al.* 1992). In wild-type embryos, salivary glands form exclusively in PS2 and correspondingly all salivary gland markers are shown to be expressed in PS2. In HS-SCR animals, marker proteins are ectopically expressed anteriorly, in PS0 and PS1, and sometimes posteriorly (*fkh* marker). Removing *tsh* or *AbdB* function allows expression of one marker (*dCREB-A*) to expand into posterior regions. The density and shading of pattern in each grid roughly indicates the relative number of cells within each parasegment expressing the particular marker. The pattern density does not indicate the intensity of staining within single cells; each ectopically expressing cell stains with the same intensity as cells within the endogenous salivary glands.

Fig. 3. BX-C genes limit *Scr* function in the larval cuticle. Cuticle sheaths were prepared of (A) wild-type, (B) HS-SCR, (C) HS-SCR; *Ubx*⁻, (D) HS-SCR; *Df(3R)P9*, (E) and (F) HS-SCR; *Ubx*^{6.28/+} larvae that had been heat shocked for 1 h between 6 and 7 h AEL. Large arrowheads demarcate segmental borders involving thoracic segments. Small arrowheads indicate borders between abdominal segments. Arrows denote the T1 beards, which normally form only in T1.

(*Ubx*) can suppress the function of *Scr* in the epidermis (Gibson *et al.*, 1990). Phenotypic changes in the larval cuticle induced by HS-ANTP and HS-UBX are limited to the anterior segments by the more posteriorly expressed homeotic genes (Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes *et al.*, 1990; Mann and Hogness, 1990). Therefore we examined the effects of HS-SCR on both the cuticle and the salivary glands in embryos lacking homeotic gene functions.

HS-SCR affects embryonic cuticular structures only in the head and thorax; no changes occur in the abdominal cuticle (Figure 3B; Gibson *et al.*, 1990; Zeng *et al.*, 1993). The effects of HS-SCR were examined in animals mutant for *Scr*, *Antp*, *Ubx*, *abdominal-A* (*abdA*) and *Abdominal-B* (*AbdB*). All alleles used are reported to completely lack gene function (see Materials and methods). Induction of HS-SCR for 1 h at 6.5 h after egg laying (AEL) in otherwise wild-type embryos leads to a transformation of the pattern of bristles (denticles) on the T2 and T3 cuticle into a T1 pattern (Figure 3B; Gibson *et al.*, 1990; Zeng *et al.*, 1993). The transformation is most easily seen as the ectopic formation of the T1 'beard' in T2 and T3. The T1 beard is a patch of denticles normally found only in the middle of the ventral prothorax (Figure 3A). Similar transformations of T2 and T3 are seen with HS-SCR in either *Scr*⁻ or *Antp*⁻ mutant animals (data not shown). Thus, cuticular transformations caused by the HS-SCR transgene do not require the endogenous *Scr* gene, and *Antp* does not prevent *Scr* function in the T2 and T3 cuticle.

In contrast to *Antp*, all three BX-C genes limit T1 beard induction by HS-SCR. In HS-SCR; *Ubx*⁻ embryos, T1 beards are found in the first abdominal segment (A1) (Figure 3C). In animals deficient for the entire BX-C, ectopic *Scr* expression leads to T1 beard formation in every thoracic and abdominal segment from T1 to A8 (Figure 3D). Mutations in BX-C genes lead to derepression of endogenous *Antp* in posterior segments (Hafen *et al.*, 1984; Carroll *et al.*, 1986), but HS-SCR; *Antp*⁻ *Ubx*⁻ animals have prothoracic beards in segments corresponding to T1–A1 (data not shown). Therefore, the effects of HS-SCR in mutants for the BX-C are not dependent upon derepression of the endogenous *Antp* gene in abdominal segments.

Ubx is haplo-insufficient for development of the adult haltere (Lewis, 1951, 1963). We find that two copies of *Ubx* are also needed to block HS-SCR-induced T1 beards. HS-SCR expression in animals heterozygous for a *Ubx* null allele (HS-SCR; *Ubx*^{6.28/+}) causes beard formation in A1, but in each case the beard is smaller than those seen in the A1 segments of sibling HS-SCR; *Ubx*^{6.28/Ubx}^{6.28} animals (Figure 3E and F). Because in HS-SCR; *Ubx*^{6.28/+} animals the main band of denticles in the A1 segment is not visibly transformed into a thoracic segment, this result suggests that the level of *Ubx* protein relative to the level of *Scr* protein produced by HS-SCR determines whether or not a T1 beard will form.

Each of the genes in the BX-C is independently capable of suppressing T1 identity in the abdominal cuticle. In heat shocked HS-SCR; *Df(3R)P9* animals, where the entire BX-

Fig. 4. Salivary gland induction in HS-SCR embryos mutant for different homeotic genes occurs posterior to PS2 in *AbdB*⁻ embryos but not in the absence of other BX-C functions or *Antp*. HS-SCR animals with the following genotypes were tested for expression of the salivary gland marker *dCREB-A* protein: (A) *Antp*²⁵ *Ubx*^{6.28}, (B) deficient for *Ubx* and *abdA*, (C) *Ubx*^{6.28} alone and (D) *AbdB*^{D16}. Only animals missing *AbdB* function have any salivary gland expression of *dCREB-A* protein posterior to PS2. Filled arrows indicate endogenous PS2 salivary glands; open arrows indicate salivary glands induced by HS-SCR. HS-SCR; *Antp*²⁵, HS-SCR; *abdA*^{MX1} and HS-SCR; *Df(3R)P9* animals (not shown) were also examined. Ectopic salivary gland formation occurred posterior to PS2 only in animals mutant or deficient for *AbdB*. (A) HS-SCR; *Antp*²⁵ *Ubx*^{6.28}; (B) HS-SCR; *Ubx*^{6.28}; (C) HS-SCR; *Df(3R)P109*; (D) HS-SCR; *AbdB*^{D16}.

C is missing, every trunk segment in the embryo has a T1 identity. In heat shocked HS-SCR; *Df(3R)P109* embryos that lack only *Ubx* and *abdA*, T1 identity is limited to T1–A7 (not shown). Thus, the *AbdB* gene is capable of blocking T1 identity in A8. In *AbdB* mutant embryos, *abdA* expression expands posteriorly into the *AbdB* domain (Macias *et al.*, 1990). In HS-SCR; *AbdB*⁻ animals, T1 beard formation is limited to the thorax (data not shown). Therefore *abdA* is also independently capable of preventing T1 identity in A8. When both *Scr* and *Ubx* proteins are expressed everywhere in the embryo for 1 h at 6.5 h AEL (HS-SCR plus HS-UBX) not only do ectopic T1 beards fail to form, but the endogenous T1 beard is reduced or absent (data not shown). Thus, each of the abdominal BX-C homeotic genes is capable of suppressing *Scr* function irrespective of their effects on *Scr* transcription.

Abdominal-B suppresses ectopic salivary gland formation in PS14

Although the effect of *Scr* on cuticle pattern is controlled by each of the BX-C genes, the removal of *AbdB*, but not of *Ubx* or *abdA*, allows ectopic induction of salivary glands by *Scr* protein in the posterior. Using the *dCREB-A* antibody, we examined salivary gland formation in animals ectopically expressing *Scr* protein and mutant or deficient for *Antp*, *Ubx*, *abdA* or *AbdB* (Figure 4A–D). Salivary gland induction by HS-SCR was also examined in animals lacking both *Antp* and *Ubx* function (Figure 4A). All four of the homeotic genes tested here are expressed in some part of the region where salivary gland formation is suppressed (Akam, 1983; Hafen *et al.*, 1983, 1984; Levine *et al.*, 1983; Akam *et al.*, 1985; Beachy *et al.*, 1985; White and Wilcox, 1985a,b; Carroll *et al.*, 1986, 1988; Martinez-Arias *et al.*, 1987; Celniker *et al.*, 1989; Bermingham *et al.*, 1990; DeLorenzi and Bienz, 1990; Macias *et al.*, 1990). Surprisingly, only mutations in the *AbdB* gene affect where salivary glands are induced. In HS-SCR; *AbdB*^{D16} embryos (Figure 4D) and HS-SCR; *Df(3R)P9* embryos, which removes all three BX-C functions (data not shown), ectopic salivary glands can be induced to form in PS0, PS1 and PS14. In animals

homozygous for a deficiency removing *Ubx* and *abdA*, but not *AbdB*, ectopic *Scr* expression leads to extra salivary glands only in PS0 and PS1 (Figure 4C). The lack of effect of *Ubx* and *abdA* is not due to derepression of *Antp* in PS3–13 because in both HS-SCR; *Antp*⁻ (data not shown) and HS-SCR; *Antp*⁻ *Ubx*⁻ (Figure 4A) ectopic *dCREB-A* expression occurs only in cells anterior to PS2. Thus, the *AbdB* gene, which prior to germ band retraction is expressed only in PS14 (Celniker *et al.*, 1989; DeLorenzi and Bienz, 1990), prevents salivary gland induction in that parasegment. The remaining ANT-C and BX-C homeotic genes have no discernible positive or negative effect on salivary gland induction by HS-SCR (Figure 4A–D).

The endogenous *Scr* gene is not required for HS-SCR salivary gland induction

Why does HS-SCR induce extra salivary glands only in the anterior head? A potential explanation is that ectopic gland formation is due to HS-SCR activation of the endogenous *Scr* gene, which might occur only in PS0–2 due to earlier acting regulators or to BX-C repression (Pelaz *et al.*, 1993). Salivary gland formation was examined using the *dCREB-A* antibody in HS-SCR animals simultaneously mutant for *Scr*. Without ectopic *Scr* expression, this mutation in the *Scr* gene results in the complete absence of salivary glands (Panzer *et al.*, 1992 and Figure 5B). Global expression of *Scr* in these animals results in salivary gland formation in PS0–2, despite the absence of endogenous *Scr* gene function (Figure 5D). Although *Scr* protein produced by the HS-SCR transgene is sufficient to direct salivary gland formation, ectopic salivary glands are still limited to PS0–2. Spatial restrictions on salivary gland induction by HS-SCR are therefore not due to the differential activation of the endogenous *Scr* gene.

The trunk gene, *tsh*, suppresses salivary gland formation in parasegments 3–13

Because posteriorly acting ANT-C and BX-C homeotic genes have no effect on ectopic salivary gland induction in PS3–13, other candidate regulators known to be expressed

Fig. 5. Salivary glands are induced in HS-SCR animals missing endogenous *Scr* function. HS-SCR was used to induce salivary gland formation with or without a functional endogenous *Scr* gene. The salivary gland marker is the *dCREB-A* protein, detected with antibodies. Filled arrows indicate PS2 salivary glands derived from cells that express the endogenous *Scr* gene and therefore are independent of HS-SCR function (A) or derived from cells that express both the endogenous *Scr* protein and *Scr* protein from the HS-SCR construct (C). Open arrows indicate marker expressing cells dependent on HS-SCR. Heat shock treatments were for 45 min at 37°C, at 1–5 h after egg laying. (A) wild-type; (B) *Scr*^{-/-}; (C) HS-SCR; (D) HS-SCR; *Scr*^{-/-}.

in this region of the embryo at the time salivary glands are determined were tested. One candidate is the homeotic trunk gene, *tsh*, which is expressed in PS3–13 prior to and during germ band extension (Fasano *et al.*, 1991), when ectopic salivary glands can be induced. The *tsh* gene is required together with *Scr*, *Antp* and the BX-C genes to prevent development of cuticular head structures in the trunk region of the embryo (Röder *et al.*, 1992). *tsh* is thought to define the trunk segment identity upon which the ANT-C and BX-C homeotic genes act to modify segmental identity (Fasano *et al.*, 1991; Röder *et al.*, 1992). Both T1 (a trunk segment), and the labium (a posterior head segment) contain *Scr* protein (Röder *et al.*, 1992), but their morphology differs due to the expression and function of *tsh* in T1 but not the labium. Where salivary glands can be induced by HS-SCR, in PS0–2, *tsh* is not expressed. *tsh* is expressed in cells posterior to PS2, where ectopic salivary glands do not form. Thus the timing and domain of *tsh* expression, as well as its proposed function, make the *tsh* gene a possible suppressor of salivary gland induction in the trunk.

The loss of *tsh* function results in ectopic transcription of the endogenous *Scr* gene in PS3 but not in PS4–14 (Figure 6A and C; Fasano *et al.*, 1991). The ectopic PS3 *Scr* expression in *tsh*^{-/-} embryos is associated with PS3 salivary gland formation (Figure 6B and D). In animals mutant for either of two strong *tsh* alleles, *tsh*⁸ or *tsh*^{2757R8}, expression of *dCREB-A* is detected in PS3, in keeping with the gland formation observed there. Ectopic *dCREB-A* expression can be detected quite early in *tsh*^{-/-} embryos, about as early as the normal PS2 expression (Figure 6D). In later stage embryos, the PS3 *dCREB-A* positive cells, together with the salivary gland cells of PS2, form a continuous lumen. The salivary gland cells of PS3 simply invaginate following the salivary gland cells of PS2 and contribute to the mature elongated salivary glands observed in later stage *tsh*^{-/-} embryos (not shown). We conclude that

tsh prevents salivary gland formation in PS3, at least in part by regulating *Scr*.

tsh is in fact a crucial factor restricting HS-SCR induction of salivary glands in PS3–13. Salivary gland formation was examined in animals homozygous for loss of function *tsh* mutations and carrying induced HS-SCR. Under these conditions, *dCREB-A* was expressed in nearly all the segments of the body, from PS0–13 (Figure 6E and F). Thus *tsh* prevents salivary gland induction by ectopic *Scr* protein in parasegments 3–13, where the *tsh* gene is expressed prior to and during germ band extension.

In the larval epidermis, *tsh* does not block HS-SCR function. In fact, it does just the opposite. Work by Röder *et al.* (1992) demonstrates that *tsh*⁺ is required for induction of ectopic T1 beards by HS-SCR in T2 and T3. Instead of playing a repressive role in the larval epidermis, *tsh* is a necessary collaborator for HS-SCR in directing formation of the T1 cuticle pattern.

The suppression of salivary gland formation by *Antennapedia* and *Ultrabithorax* proteins is due to repression of the endogenous *Scr* gene

Production of *Antp* protein in all cells with a HS-ANTP construct suppresses salivary gland formation in PS2 (Zeng *et al.*, 1993). HS-UBX also suppresses PS2 salivary gland formation (Figure 7C). Yet in HS-SCR embryos the removal of *Antp* and *Ubx* function does not permit glands to form in PS3–6. Both *Antp* and *Ubx* repress the endogenous *Scr* gene in posterior regions of the ectoderm (Riley *et al.*, 1987; Pelaz *et al.*, 1993), and we find diminished *Scr* protein accumulation in HS-ANTP and HS-UBX embryos relative to wild-type embryos (Figure 7D–F). Thus, salivary gland formation in PS2 is suppressed by HS-ANTP and HS-UBX through their repression of endogenous *Scr* expression.

To test whether *Antp* and *Ubx* have salivary gland suppressing activity independent of their regulatory effects

Fig. 6. *tsh* represses salivary gland formation in PS3–13. *Scr* protein expression was assayed in (A) wild-type and (C) *tsh*⁸ embryos. *D-CrebA* protein expression was assayed in (B) wild-type, (D) *tsh*⁸, (E) HS-SCR; *tsh*^{2757R8} and (F) HS-SCR; *tsh*⁸ embryos. Dark heavy arrows point to PS2 salivary glands. Open arrows point to additional salivary gland marker expressing cells.

Fig. 7. Repression of salivary glands in HS-ANTP and HS-UBX embryos is through repression of the endogenous *Scr* gene. *D-CrebA* protein expression was assayed in (A) wild-type, (B) HS-ANTP, (C) HS-UBX, (G) HS-SCR, (H) HS-SCR; HS-ANTP and (I) HS-SCR; HS-UBX embryos. *Scr* protein expression is shown in (D) wild-type, (E) HS-ANTP and (F) HS-UBX embryos. Dark heavy arrows point to PS2 salivary glands, open arrows to ectopically expressing cells. Narrow diagonal arrows indicate expression of *Scr* in primordia of salivary glands.

on *Scr* transcription, salivary gland formation was assayed in animals ectopically expressing both *Scr* and *Antp* proteins or both *Scr* and *Ubx* proteins. The use of heat shock

promoters to express two homeotic proteins circumvents the repression of *Scr* by either *Antp* or *Ubx*. Both PS2 and ectopic salivary glands form in animals expressing both HS-

Fig. 8. HS-ANTP repression of salivary gland formation does not require the *tsh* gene. Expression of *dCREBA* protein was assayed in (A) HS-ANTP and (B) HS-ANTP; *tsh*⁻ embryos. Compare number of cells expressing the salivary gland marker protein, *dCREBA*, in HS-ANTP animals with number of cells expressing the salivary gland marker protein in the wild-type embryos shown in Figures 5A and 7A. The number of cells that express the salivary gland marker protein in HS-ANTP animals varies; compare Figure 7B with Figure 8A.

SCR and HS-ANTP (Figure 7H) or both HS-SCR and HS-UBX (Figure 7I). Suppression of salivary glands by HS-ANTP and HS-UBX is therefore primarily due to the repression of the endogenous *Scr* gene.

Suppression of salivary gland formation by HS-ANTP is not through the *teashirt* gene

We have previously shown (Zeng *et al.*, 1993) that HS-ANTP blocks salivary gland formation. Because HS-ANTP represses *Scr* in PS2 (Figure 7E) and *tsh* represses *Scr* expression in PS3 (Fasano *et al.*, 1991; Figure 6C), the suppression of salivary gland formation by HS-ANTP could be due to activation of *tsh* in PS2 by HS-ANTP. If *tsh* mediates the effect of HS-ANTP on salivary gland formation, then *tsh*⁻ mutations should alleviate suppression of salivary gland formation by HS-ANTP. If suppression is not mediated by *tsh*, then *tsh*⁻ mutations should have no effect on suppression of PS2 salivary gland formation by HS-ANTP. HS-ANTP expression in *tsh*⁻ animals suppresses salivary gland formation to the same extent as does HS-ANTP in a wild-type embryo (Figure 8A and B). Correspondingly, PS2 *Scr* protein expression is the same in HS-ANTP and in HS-ANTP; *tsh*⁻ embryos (data not shown).

Persistent expression of the *Scr* protein in the *Antp* P1 domain leads to ectopic salivary gland formation only when *tsh* function is also missing

The experiments described above rely on heat shock-induced expression of the *Scr* protein, which leads to a relatively brief pulse of *Scr* protein accumulation. Some activities of the *Scr* protein may not be detectable in this assay, either because expression is limited to a brief time interval or because of some other artifact of heat treatment. To control for possible artifacts of the heat shock expression system, we adapted the GAL4 method developed by Brand and Perrimon (1993) to express the *Scr* protein persistently in ectopic locations

Fig. 9. Persistent expression of the *Scr* protein in the *Antp* P1 domain leads to ectopic salivary gland formation only in the absence of *tsh* function. *Scr* expression in (A) wild-type embryos and (B and C) P1-GAL4-SCR embryos. The initial expression of *Scr* driven by *Antp* P1-GAL4 (approximately stage 11) spans a broad domain from PS4-12 (B), but later (stage 12 and onward) is expressed at highest levels only in PS4 and PS5 (C). *dCREBA* expression in (D) GAL4-SCR and (E) GAL4-SCR; *tsh*⁻ embryos. Ectopic *Scr* expression under GAL4 control induces ectopic *dCREBA* expression only in the absence of *tsh* function. Bars with numbers indicate the approximate register of PS2 and PS4 for the purpose of comparing panels. Solid arrows in (D) and (E) indicate the normal expression of the *dCREBA* marker, while open arrows indicate ectopic expression.

during the stages when *Scr* normally affects salivary gland induction.

Antp sequences sufficient to drive expression of reporter genes in a pattern nearly identical to the *Antp* P1 transcript (M.G.Petitt and M.P.Scott, unpublished data) were fused to the yeast GAL4 protein coding region and introduced into the genome via P-element mediated transformation. *Antp* P1

transcripts, and products from the P1-driven GAL4 construct, accumulate to high levels in the ectoderm of PS4 and PS5 and to lower levels in PS6–12. An upstream activation sequence (UAS) responsive to GAL4 was also fused to a cDNA containing the entire *Scr* open reading frame and the resulting plasmid was introduced into the fly genome. When flies containing the *Antp* P1–GAL4 construct were crossed to flies containing the UAS–SCR construct, the progeny expressed the *Scr* protein both in its normal domain and in the PS4–5 *Antp* P1 domain (Figure 9A). The ectopic *Scr* protein is initially observed in a broad domain, spanning PS4 through PS12 (Figure 9B), but comes to be stably expressed at high levels only in PS4–5 (Figure 9C). The *Antp* P1–GAL4–SCR system allows continuous expression of *Scr* protein in PS4–5 from the time of germ band extension through most of embryogenesis. In contrast, protein expressed using HS-SCR persists for only about 30–60 min following cessation of heat treatment (data not shown).

Embryos containing the *Antp* P1–GAL4-driven *Scr* protein were stained with the *dCREB-A* antibody to examine salivary gland induction. As expected from the HS-SCR results, salivary glands were limited to PS2 (Figure 9D). Salivary gland formation was next examined in animals containing *Antp* P1–Gal4-driven *Scr* protein and simultaneously mutant for either *Antp* or *tsh*. In *tsh*[−] embryos (Figure 9E) but not *Antp*[−] embryos (not shown), additional marker expressing cells were detected in several other parasegments. In addition to the normal *dCREB-A* expressing cells in PS2, *dCREB-A* expressing cells were also abundant in PS3, due to derepression of the endogenous *Scr* gene in the *tsh*[−] background. Furthermore, the absence of *tsh* function allowed *dCREB-A* expression at moderate levels in PS4 and PS5, as well as at lower levels in PS6–12, where GAL4-driven *Scr* expression is more transient. Thus, both methods of ectopically expressing *Scr* protein, HS-SCR and P1–GAL4–SCR, yield the same qualitative result; *Scr* protein, if produced at sufficient levels early in development, can induce salivary gland formation in segments where *tsh* function is absent.

Discussion

Scr and the initiation of the salivary gland developmental program

Our study has revealed restrictive influences, summarized in Figure 10, which limit where along the anterior–posterior axis *Scr* has the potential to induce salivary glands. *Scr* is the sole known positive signal for initiating the program of salivary gland morphogenesis in *Drosophila*. The *Scr* expression profile, and its requirement and sufficiency in otherwise wild-type cells to induce salivary gland formation, support a model in which *Scr* controls the expression of early-acting genes in the salivary gland pathway. Because *Scr* is expressed in all of the primordial cells of the salivary gland, *Scr* and the genes that set the dorsal–ventral salivary gland boundaries (Panzer *et al.*, 1992) probably directly determine the number and placement of cells that will participate in forming salivary glands. Genes downstream of *Scr* that act early in salivary gland development may control events such as cell shape changes, nuclear movement, and the initiation of polytenization, all early events in salivary gland morphogenesis.

Fig. 10. Summary of regulation of salivary gland formation. (A) Salivary glands are normally formed from PS2 primordia, where *Scr* but not TSH accumulates. The bars are a simplified summary of locations of proteins in the ectoderm, at the time and dorsal–ventral position of salivary gland formation. McGinnis and Krumlauf (1992) provide additional references on details of the expression patterns. (B) HS-SCR, by producing *Scr* protein in all cells as shown, causes formation of ectopic glands in PS0 and PS1 in an otherwise wild-type embryo. The same effect is seen when either *Scr* or *Antp* or both *Ubx* and *abdA* function is removed. Thus none of these genes is necessary to facilitate or block salivary gland formation. The elimination of *AbdB* function allows HS-SCR to activate salivary gland markers in PS14 as well as PS0 and PS1. PS14 is where ABDB protein, but not TSH protein, is produced. If *tsh* function is removed HS-SCR will then activate salivary gland markers in a region extending from PS0 to PS13. The markers are expressed at a lower level in PS4–13, perhaps due to effects of *Antp*, *Ubx* and *abdA*. (C) HS-ANTP and HS-UBX both repress the endogenous *Scr* gene, thus preventing activation of salivary gland markers. However if *Scr* protein is provided with a heat-inducible construct immune to repression by ANTP or UBX, salivary gland markers can be induced where neither TSH nor ABDB proteins are found.

How are the various regulators of salivary gland development related? Panzer *et al.* (1992) and Zhao *et al.* (1993) have shown that *fork head* (*fkh*) can be activated by HS-SCR in PS0, 1, 3 and 14 at the proper dorsal–ventral position. Yet HS-SCR does not induce four other salivary gland markers in PS3–13 unless *tsh* function is absent. Thus, PS3 *fkh* activation is not absolutely dependent on expression of any of the four marker genes. Also, the induction of *fkh* in PS3 does not activate transcription of the four marker genes in PS3. Whether *fkh* alone, in the absence of *tsh* function, can activate transcription of any of the four marker genes is unknown. Expression of at least two of the genes, *dCREB-A* and *hkb*, however, are not dependent on *fkh* function; their expression is unchanged in *fkh* loss of function mutants (S.Beckendorf, personal communication). Whatever the regulatory relationships between *fkh* and the other salivary gland markers, it is clear that different components

of the salivary gland genetic hierarchy are differentially regulated.

fkh and at least two of the marker genes used to assay for salivary glands, *dCREB-A* and *jal*, are good candidates for direct control by *Scr*. All three genes are expressed early in embryogenesis in patterns coincident with the domain of *Scr* protein expression in the salivary gland placode. All the genes that mark salivary glands are also expressed and presumably required in other tissues and at other developmental stages. In fact, no single gene dedicated exclusively to salivary gland development has been identified from the expression patterns of all known cloned genes and screens of hundreds of enhancer trap lines from various laboratories. Thus, activation of the salivary gland program by *Scr* appears to require the regulation of multiple subservient genes, each of which also acts in other pathways; it is the unique combination of these activities that specifies and carries out salivary gland development.

Blocking *Scr* activation of the salivary gland program

Both *tsh* and the BX-C genes control trunk differentiation at two levels: (i) the regulation of transcription of more anteriorly expressed homeotic genes (Hafen *et al.*, 1984; Carroll *et al.*, 1986; Fasano *et al.*, 1991; Röder *et al.*, 1992; Pelaz *et al.*, 1993) and (ii) the suppression of function of the same anterior homeotic genes when transcriptional control has been overridden, as in heat shock promoter and GAL4 experiments (Kuziora and McGinnis, 1988; Gibson *et al.*, 1990; Gonzalez-Reyes and Morata, 1990; Gonzalez-Reyes *et al.*, 1990, 1992; Mann and Hogness, 1990).

The multi-level control exemplified by limits on *Scr* activity provides both stability and flexibility of gene function on which evolutionary forces can work. Mutations resulting in mis-expression of a regulatory gene may be tolerated if initially other molecules inhibit its activities. In its new expression domain, the regulatory protein might eventually acquire new activities not limited by the same inhibitory molecules. The different regulators in salivary gland control and epidermis also provide flexibility. In the present case, for example, a mutation resulting in *Scr* transcription becoming resistant to repression by homeotic genes in the trunk would change the epidermis but not induce salivary glands.

Expression of *Scr* in PS2 spans the dorsal–ventral axis, yet salivary glands form only at a specific location along this axis. Panzer *et al.* (1992) demonstrated that the *decapentaplegic* (*dpp*) and ‘*spitz* group’ genes impose dorsal–ventral constraints on where salivary glands form. Presumably the same genes limit effects of ectopic *Scr*. A parallel to the *dpp* block of salivary gland induction is seen in the suppression of PIT-1/GHF-1 transcriptional activation of the growth hormone gene, GH, in mammals by activin (Struthers *et al.*, 1992). Activin, like *dpp*, is a member of the TGF- β gene family (Ling *et al.*, 1986; Vale *et al.*, 1986; Massagué, 1987) and PIT-1/GHF-1 is a POU class homeodomain-containing transcription factor (Bodner *et al.*, 1988; Ingraham *et al.*, 1988). The mechanism used to block *Scr* induction of salivary glands by *dpp* may be analogous to the (as yet unknown) mechanism used to suppress PIT-1/GHF-1 DNA binding and transcriptional activation by activin. The *dpp* restriction, however it works, must be superimposed on the *tsh* block to *Scr* function but the dorsal–ventral limitation and anterior–posterior limitation systems can work independently.

The inability of HS-*Scr* to induce salivary glands in segments containing the *tsh* or *Abd-B* proteins might depend on the absolute level of *Scr* protein and the persistence of *Scr* protein expression over time. Heat shock-induced gene expression leads to a pulse of protein accumulation which does not necessarily persist for a sufficient length of time to be fully functional, and heat treatment itself could affect target gene expression at many levels, including transcription, translation and RNA processing (Lindquist, 1986). By using the GAL4-mediated expression system, we have circumvented artifacts intrinsic to heat treatment and have also provided a kinetic profile of *Scr* expression more consistent with the normal profile of *Scr* expression. Under these circumstances, *tsh* still acts to prevent the induction of salivary glands by *Scr*, indicating that the results of heat shock experiments are not artifacts of the heat shock expression system.

HS-*Scr* induces salivary gland formation in nearly every segment of the embryo in *tsh*[−] embryos, but the size of the salivary glands induced in posterior body segments is sometimes small relative to the salivary glands formed in anterior segments. Double labelling experiments with the *en* and *dCREB-A* antibodies indicate that the smaller ectopic glands are usually found in PS6–13 in HS-*Scr*; *tsh*[−] embryos (data not shown). The salivary gland size differential may indicate that in the absence of *tsh* function more posteriorly acting homeotic genes may partially suppress salivary gland induction. Alternatively, other as yet unidentified inhibitors may be responsible for the smaller size of salivary glands in posterior segments. We have examined salivary gland formation in the zygotic gap mutants and see no effects on salivary gland formation other than those that can be explained by the changes in the expression of *Scr*, *tsh* or both genes (D.J. Andrew and M.P. Scott, unpublished results).

Four models of how *tsh* and *AbdB* block HS-*Scr* function

How do *tsh* and *AbdB* impose limits on where salivary glands can form? *AbdB*, like all homeotic genes in the ANT-C and BX-C, encodes a homeodomain-containing DNA-binding protein (DeLorenzi *et al.*, 1988; Celniker *et al.*, 1989). The *tsh* protein is not closely related to any protein currently in the data base, but it contains three putative zinc fingers (Fasano *et al.*, 1991). *tsh* protein may therefore be a transcriptional regulator that interacts either positively or negatively with homeotic proteins as they act upon target enhancers. The combinatorial action of *tsh* with homeotic genes in other cases (Röder *et al.* 1992) provides strong support for this hypothesis. A key experiment will be to examine the interactions of *Scr* and *tsh* proteins with an enhancer that responds to both proteins, but no such enhancer has yet been identified.

We present four models of mechanisms for the repressive effects of *tsh* and *AbdB*. The first is a simple competition model: *Scr* and either *tsh* or BX-C proteins compete for the binding of the same or overlapping sites within target genes. In this model, *tsh* and *AbdB* would compete better than *Scr* for key target genes relevant to salivary gland formation. In the cuticle, all three BX-C proteins would compete better than *Scr* for target site binding. Competition between homeodomain proteins, and between homeodomain and other types of proteins, has been described in the nematode and in mammalian systems. The *mec3* gene, which encodes

a homeodomain-containing protein required for specifying a particular neuronal cell fate, is activated by another homeodomain protein, *unc-86*, and is positively autoregulated. Both proteins bind *mec-3* regulatory DNA *in vitro* but binding of *unc-86* protein precludes binding of *mec-3* protein at some sites (Xue *et al.*, 1992). Some type of post-transcriptional competition occurs when the *mab-5* and *lin-39* proteins, both nematode HOM complex homeodomain proteins, neutralize each other during neuron development (Salser *et al.*, 1993). Another example of competition comes from aldolase B regulation in human hepatoma cells. Binding and transcriptional activation of the aldolase B promoter region by HNF-1 (a POU homeodomain protein) is inhibited by HNF-3, another DNA binding protein normally expressed in liver and derivative liver cell lines (Gregori *et al.*, 1993). Both HNF-1 and HNF-3 bind to the same site within the aldolase B enhancer, so the mechanism of inhibition by HNF-3 may be through direct competition with HNF-1 for the binding site. In flies, competition between *Ubx* and *AbdB* proteins has been proposed as a mechanism for posterior prevalence (Lamka *et al.*, 1992). The competition model predicts that binding sites for all three proteins, *Scr*, *tsh* and *AbdB*, are present within the enhancers of *Scr* salivary gland target genes and are very tightly linked.

In the second model, *Scr*, *tsh* and BX-C proteins compete for activating cofactors. Again, both *tsh* and *AbdB* would be better at competing than *Scr* in the salivary gland primordia, and all three BX-C proteins would be better competitors than *Scr* in the larval cuticle forming cells. An apparent precedent for this type of regulation is known. One of the mechanisms by which the chick ovalbumin upstream promoter transcription factor (COUP-TF) might indirectly inhibit transcriptional activities of the vitamin D3 receptor (VDR), the thyroid hormone receptor (TR) and the retinoic acid receptor (RAR) is by competitive binding to a necessary coregulator, the retinoid-X receptor (RXR). The formation of a COUP-TF/RXR heterodimer titrates out the RXR protein, which is necessary for target gene activation by VDR, TR and RAR (Kliwer *et al.*, 1992; Cooney *et al.*, 1993). In applying this model to salivary gland regulation, it is expected that the regulatory regions of the salivary gland target genes will contain binding sites for the *Scr* protein but may or may not also contain binding sites for the *tsh* and/or *AbdB* proteins.

In the third model, both *tsh* and *AbdB* directly affect the ability of the *Scr* protein to bind and/or regulate downstream target genes. The inactivation of *Scr* by *tsh* and *AbdB* could be mediated by the formation of inactive heterodimers with the *Scr* protein, like those observed with the helix-loop-helix (HLH) class of transcriptional regulators (e.g. Benezra *et al.*, 1990; Ellis *et al.*, 1990; Parkhurst *et al.*, 1990; Peterson *et al.*, 1990). Negatively acting HLH proteins cannot bind DNA but can form stable heterodimers with positively acting HLH proteins that can bind DNA. Similarly inactive *Scr*-*tsh* or *Scr*-*AbdB* complexes might form in posterior segments of the *Drosophila* embryo. This model predicts *Scr* binding sites, but neither *tsh* nor *AbdB* binding sites, within regulatory regions of salivary gland target genes, and only *Scr* binding sites in regulatory regions of T1 epidermal target genes.

A fourth mechanism for the suppression of salivary gland induction in posterior regions of the embryo is the alteration of chromatin by *tsh* to make downstream target genes inaccessible to *Scr*. The similarity of the zinc fingers in the

tsh protein to those found in *Suvar(3)7* (Fasano *et al.*, 1991), a gene affecting position-effect variegation in *Drosophila* (Reuter *et al.*, 1990), suggests a role for the *tsh* gene in affecting chromatin configuration. The most common kind of position-effect variegation is due to juxtaposition of a regulated gene to heterochromatin and concomitant loss of expression. Since *Suvar(3)7* affects position-effect variegation in a dose-dependent manner, it is possible the protein encoded by this gene, and also *tsh*, directly associates with chromatin. This model applied to salivary gland regulation predicts that binding sites for all three proteins, *Scr*, *tsh* and *AbdB*, exist in the neighborhood of direct *Scr*-regulated target genes, but do not overlap.

None of the above models is exclusive; each could apply to phenotypic suppression both in the salivary gland and larval epidermis, and the mechanisms of suppression of *Scr* function by *tsh* and BX-C genes need not be the same. The insufficiency of one *Ubx* copy in preventing HS-SCR beard induction suggests that relative levels of *Ubx* and *Scr* are critical in determining the phenotypic consequences of *Scr* mis-expression, supporting a competition model for BX-C suppression of *Scr* protein activities. Rarely, with longer heat shock induction of *Scr* protein, a few cells in PS14 express the *dCREB-A* marker protein. This result again suggests that relative levels of *Scr* and, in this case, *AbdB*, are important. Competition, as a mechanism for phenotypic suppression, has been demonstrated with other homeotic proteins (Lamka *et al.*, 1992; Salser *et al.*, 1993).

Homeotic genes such as *Scr* control segmental identity by directly controlling organogenesis. The presence of other homeotic genes, such as the BX-C genes, and other regulatory molecules, such as *tsh* and *dpp*, set limits on where those organs can be formed by defining permissive versus non-permissive regions of the embryo. Thus, every cell expressing a particular homeotic protein does not adopt the same fate; other master regulators impinge on the phenotypic consequences of expressing a particular homeotic gene.

Materials and methods

Drosophila strains

All homeotic mutant strains in this study are described in Lindsley and Zimm (1992) and are either null mutations based on genetic criteria or are deficiencies that completely remove each gene being tested. *Df(2R)tsh⁸* is described in Fasano *et al.* (1991) and is at least a 40 kb deficiency that removes *tsh* and surrounding DNA. *tsh^{2757R8}* is a lethal revertant of an enhancer trap insert in the *tsh* gene isolated from the Scott/Fuller Laboratories' enhancer trap collection (L.Mathies and M.P.Scott, unpublished). By all criteria, including larval cuticle phenotypes (D.J.Andrew and M.P.Scott, unpublished), derepression of *Scr* in PS3 (D.J.Andrew and M.P.Scott, unpublished) and gut defects (L.Mathies and M.P.Scott, personal communication), *tsh^{2757R8}* is a weaker allele of *tsh* than *Df(2R)tsh⁸*. The genotype of each stock built and used in this work was independently verified by antibody staining and larval cuticle preparations.

Enhancer trap lines with non-lethal inserts in *hkb* (line 5953) and *jal* (line 5929) are from the Scott/Fuller enhancer screen and will be described in detail elsewhere.

Antibodies

The antibodies to *Scr* (Scr6H4) and *Antp* (Antp8C11) are both mouse monoclonal antibodies supplied by Glicksman and Brower (1988a,b). The mouse monoclonal antibody to the *Ubx* protein (FP3.38) was provided by J.Lopez and D.Hogness (Lopez and Hogness, 1991). The *en* monoclonal antibody (4D9) was supplied by the Kornberg laboratory (Patel *et al.*, 1989). The monoclonal antibody to β -gal was purchased from Promega. Rat polyclonal antibody to the *tsh* protein (*tsh*3) was provided by L.Mathies (Zeng *et al.*, 1993). Preparation and isolation of the rat polyclonal antisera to *dCREB-A* (S.M.Smolik, unpublished data) will be described in detail

elsewhere. D3 is a monoclonal antibody originally isolated in the Jan laboratory (UCSF) that stains the lumen of all invaginating ectoderm.

Embryo staining

Embryo fixation and staining were performed as described by Reuter *et al.* (1990). Homozygous mutant embryos were identified by double labelling with an antibody that recognizes the salivary gland marker and an antibody that recognizes each mutant protein in question. For example, homozygous *Scr*^d embryos were identified by staining with the *Scr* monoclonal antibody 6H4. *Scr*^d homozygotes show only very faint staining in the labial region of the ventral nerve cord with the Scr6H4 antibody. (The limited staining in the ventral nerve cord is often undetectable.) Homozygous *Antp*²⁵ embryos were similarly identified by limited, nearly undetectable, *Antp*8C11 staining in the ventral nerve cord. *Ubx*^{6.28} and BX-C deficiency homozygotes were identified by the absence of staining with the *Ubx* monoclonal antibody FP3.38. Neither *Df(2R)tsh*⁸ nor *tsh*^{2757R8} show detectable staining with *tsh* antisera (L.Mathies, personal communication).

Antibody stained embryos were visualized using Nomarski optics and photographed on a Zeiss Axiophot with either Ektachrome 64 Tungsten slide film or Ektar 25 print film.

Ectopic protein expression systems

The HS-SCR and HS-ANTP constructs are described in Zeng *et al.* (1993). The HS-SCR line used for the entire study contains an X chromosome insertion that gives >90% transformation of larval cuticle with 1 h heat shocks at 6.5 h AEL and ~80% ectopic salivary glands when a heat shock of 45 min at 37°C is applied between 1 and 5 h of development. The HS-UBX stock was provided by R.Mann and D.Hogness (Mann and Hogness, 1990).

To examine salivary gland formation, embryos were collected for 4 h, aged 1 h at 25°C, and heat shocked at 37°C for either 30 or 45 min. Embryos were then aged for ~12 h at 25°C, fixed and stained with antibodies.

The *Antp* P1–GAL4 expression construct is a fusion of ~16 kb of *Antp* regulatory sequence spanning the *Antp* P1 start site. Details of its construction are available upon request and will be presented elsewhere (M.G.Petitt and M.P.Scott, in preparation). The UAS–SCR construct was built from an *Scr* cDNA containing the entire open reading frame (D.J.A., in preparation). A *Dra*I site immediately upstream of the *Scr* initiation codon and an *Eco*RV site immediately downstream of the termination codon were used to generate a fragment that was fused downstream of the UAS–hsp70 promoter in the transformation vector pUAST (Brand and Perrimon, 1993). Germline transformation was carried out by standard methods.

To examine cuticle transformations, embryos were collected for 2 h and aged for 6.5 h after the midpoint of the collection at 25°C. After a 1 h heat shock at 37°C, embryos were allowed to develop for ~36 h at 25°C prior to fixation and cuticle preparations.

To distinguish the effects of heat shock alone from the effects due to expression of HS-SCR, mutant animals not containing the HS-SCR construct were heat shocked and prepared for study in parallel with the HS-SCR mutant animals. To demonstrate that production of ectopic *Scr* protein was necessary to give the observed transformations, animals bearing the HS-SCR construct without heat shock treatment were prepared in parallel with experimental animals.

Cuticle preparations

Embryos were dechorionated in 50% bleach for 2 min, rinsed and fixed in 50% heptane, 4% formaldehyde in 1 × PBS for 20 min. Vitelline membranes were removed by replacing the fix solution with methanol, vortexing, allowing the embryos to settle, removing the methanol and repeating the process until methanol was clear of membranes. Embryos were then incubated in 1:4 glycerol:acetic acid for 24 h at 65°C. Embryos were then placed on slides in Hoyer's mountant and allowed to clear for at least 24 h at 65°C.

Cuticle preparations were examined using either phase or dark field optics and photographed with Ilford PanF 50 black and white print film.

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