Development 117, 847-856 (1993)
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Distal-less is a downstream gene of *Deformed* required for ventral maxillary identity

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SUMMARY

The homeotic selector (HOM) proteins are required for the diversification of the anterior-posterior axis of the *Drosophila* body plan, assigning unique identities to regional domains of cells comprising one or a few parasegments or segments. The HOM proteins apparently accomplish this task by the transcriptional regulation of numerous downstream genes. At present few downstream genes are known, so models of how downstream genes mediate HOM functions are based more on intuition than information. Our results indicate that *Distal-less* is a downstream gene of the HOM gene *Deformed,* and *Distal-less* function is required for the elaboration of a subset of the maxillary epidermal identities specified by *Deformed.* The regulatory effect of *Deformed* on *Distal-less* is mediated by a ventral maxillary-specific enhancer located 3′ of the *Distal-less* transcription unit. We propose that *Deformed* and *Distal-less,* both of which encode homeodomain transcription factors that are persistently expressed in ventral maxillary cells, combinatorially specify a subsegmental code required for a group of cells to differentiate maxillary cirri.

Key words: *Distal-less, Deformed*, maxillary cirri, *Drosophila*

INTRODUCTION

In *Drosophila melanogaster*, the assignment of stable and different anterior-posterior axial identities in the gnathal, thoracic and abdominal regions, eventually to be realized in morphological variation in different segments, is under the control of the homeotic selector genes (Lewis, 1978; Kaufman, 1983; Lawrence, 1984). Most of the known *Drosophila* homeotic selector genes reside within the Antennapedia and Bithorax gene complexes. The Antennapedia complex (ANT-C) selector genes regulate the identities of the posterior head and thoracic segments (Kaufman et al., 1990), while the Bithorax complex (BX-C) selector genes regulate the identities of posterior thoracic and abdominal segments (Lewis, 1978; Sanchez-Herrero et al., 1985; Duncan, 1987). The ANT-C and BX-C homeotic selectors, collectively referred to as the HOM-C or HOM genes, have been cloned and extensively characterized (reviewed by Kaufman et al., 1990; Akam, 1989; McGinnis and Krumlauf, 1992; Cribbs et al., 1992). Each of the eight HOM genes plays a pivotal role in determining the identity of a few hundred embryonic cells and their descendants. We wish to address the question of how the relatively crude spatial identities specified by HOM proteins are refined to smaller spatial domains.

The limits of HOM gene activation within specific parasegmental boundaries in early embryos are initially specified by the maternal, gap, pair-rule and segment polarity genes. These four gene classes also produce the overall polarity and basic metameric pattern of the embryo (Nüsslein-Volhard and Wieschaus, 1980; Martinez-Arias and Lawrence, 1985; Ingham and Martinez-Arias, 1986; White and Lehman, 1986; Akam, 1987; Riley et al., 1987; Irish et al., 1989; Harding and Levine, 1988; Jack et al., 1988, Jack and McGinnis, 1990). After their activation in unique domains, HOM proteins presumably dictate cellular identities by regulating the spatial and temporal expression patterns of many downstream genes. They do so at least in part by acting as DNA binding transcriptional regulators, each binding DNA via a sixty amino acid homeodomain (Desplan et al., 1985, 1988; Jaynes and O'Farrell, 1988; Thali et al., 1988; Beachy et al., 1988; Hoey and Levine, 1988; Hayashi and Scott, 1990).

Some of the downstream genes directly targeted by HOM proteins are likely to have effector or 'realizator' functions (Garcia-Bellido, 1977), and would include genes that regulate cell movement, shape and mitotic orientation, and genes involved in cell-cell communication. It seems likely that another class of direct downstream targets would include genes whose products are involved in further spatial subdivisions of the large metameric primordia specified by HOM proteins. Some of these putative sub-segmental identity proteins might be required to act in combination with HOM proteins, and some might function indepen-

dently. Each HOM protein presumably controls the developmental fates of cells within its expression domain by acting in combination with many gene products, some of which are likely to be other transcription factors.

Since few HOM downstream genes have as yet been identified, it is not known how many such genes each HOM protein directly activates or represses or to what extent individual downstream genes are targeted by more than one HOM protein. The best characterized regulatory targets of the HOM proteins are other genes of the HOM complex. For example, each HOM gene expressed in the thorax and abdomen is transcriptionally repressed by more posterior HOM-C proteins (Struhl, 1983; Hafen *e*t al., 1984; Struhl and White, 1985). Also, the HOM genes *Deformed* and *labial* positively regulate their own transcription in epidermal cells, and *Ultrabithorax (Ubx)* autoregulates in visceral mesoderm cells (Bienz and Tremml, 1988; Kuziora and McGinnis, 1988; Chouinard and Kaufman, 1991).

Excepting cross-regulation and autoregulation, surprisingly few genes are known to be HOM-regulated. These include *decapentaplegic (dpp),* encoding a TGF- homolog, regulated by *Ubx* and *abdominal-A (abd-A)* within the visceral mesoderm, and *wingless (wg)*, a homolog of the mammalian proto-oncogene *int-1*, also regulated by *abd-A* within the visceral mesoderm (Reuter et al., 1990; Immergluck et al., 1990). In addition, two transcripts currently known as 35 and 48 are regulated in a *Ubx*-dependent fashion in the central nervous system (CNS) and ventral-lateral epidermis (Gould et al. 1990). Finally, the *spalt-major* gene is down-regulated by *Antp* in imaginal disk cells (Wagner-Bernholz et al., 1992).

The HOM gene *Deformed (Dfd)* is required for the identities of the maxillary and mandibular segments of the embryonic head, and is particularly important in the cells that give rise to the maxillary cirri (noninnervated triangular papillae located above the mouth), the ventral organs and the mouth hooks. *Dfd* null mutants are missing these structures, and ectopic expression of Dfd protein in embryos induces the development of ectopic cirri, ventral organs and mouth hooks in the labial and thoracic segments. During head involution *Dfd* also plays a role in orchestrating the intricate cell movements involved in the spatial positioning of head structures (Kaufman, 1983; Merrill et al., 1987; Regulski et al., 1987).

In this report we show that *Dfd* activates transcription from the homeobox gene *Distal-less (Dll)* within the maxillary segment. *Dll* is required for the development of the distal regions of all segmented adult appendages; legs, antennae, maxillary palps, labium, labrum and proboscis (Cohen, 1990; Cohen and Jürgens, 1989; Cohen et al., 1989; Sato, 1984; Sunkel and Whittle, 1987). A number of larval structures also require *Dll* for their formation (Sunkel and Whittle, 1987, Cohen and Jürgens, 1989). These include the labral, the antennal, the maxillary, and the labial sense organs of the head, the Keilin’s sense organs of the thorax and some minor elements of the head skeleton. As shown here, *Dll* function is also required for the development of a subset of the maxillary cirri, and the regulatory effect of *Dfd* on *Dll* is exerted within cirri progenitor cells. An enhancer from a 3 region of the *Dll* locus controls the expression pattern of *Dll* in cirri primordia, and contains a regulatory element that can be specifically activated in response to Dfd protein expression. The regulation of *Dll* by *Dfd* may be an example of a HOM protein activating a downstream target gene whose product will combinatorially interact with its HOM regulator at later stages to define a sub-segmental identity.

MATERIALS AND METHODS

Antibody staining and transcript localization in embryos

For single antigen detection in whole-mount embryos, embryos were collected for 1 hour and aged at 25°C to the desired stage of development. Embryos were then harvested, dechorionated, fixed with paraformaldehyde, and stained with rabbit anti-Dfd or mouse anti- -galactosidase antibodies as previously described (Jack et al., 1988; Bergson and McGinnis, 1990). To detect both Dfd and -galactosidase proteins in *hsp70-Dfd* embryos, 30 minute collections were aged 2.5-3 hours at 25°C to bring the embryos to the cellular blastoderm stage of development, heat shocked for 1 hour at 37°C, and aged for 7 hours at 25°C. The primary antibodies, a polyclonal rabbit anti- -galactosidase (1:500) (Promega) and a polyclonal mouse anti-Dfd (1:500), were added simultaneously; detection was with separate secondary antibodies, biotinylated goat anti-mouse IgG (1:500; Cappel) and alkaline-phosphatase-conjugated goat anti-rabbit IgG (1:500; Jackson Immunoresearch). Adsorption times, buffers, and wash regimens were performed as in Jack et al. (1988). Dfd antigen was detected using the Vector Labs ‘ABC’ Horseradish Peroxidase detection kit without the addition of $CoCl_2$ to the developing solution. -galactosidase antigen was detected using an alkaline phosphatase detection buffer (Vector Labs). After dehydration, singly-stained embryos were cleared with two rinses of methyl salicylate and mounted in a 1:1 mixture of methyl salicylate and Permount (Fisher) and doubly stained embryos were cleared with two rinses of xylene and mounted in Permount.

The localization of transcripts by whole-mount in situ hybridization followed the method of Tautz and Pfeifle (1989). The probe used for *Dll* was a 1.4 kb *Eco*R1 cDNA fragment containing 1.2 kb of coding sequence (Cohen et al., 1989).

Larval cuticular preparations and phenotypes

Larval cuticular preparations were done as previously described (Van der Meer, 1977). *Dll* mutant larvae with non-involuted heads were generated by crossing desired mutations (e.g. $Dll^{SA1}$and Dll^{5}) into a backgound containing the *hsp70-Dfd/UbxHD* construct (Kuziora and McGinnis, 1989) and subjecting embryos to a mild heat shock (10-20 minutes at 37°C) at 6-8 hours after egg lay, and then allowing the embryos to age for 24-36 hours at 25°C. This treatment of *hsp70-Dfd/UbxHD* embryos causes almost complete lack of head involution, but induces little or no homeotic transformation of head segments. Cirri were counted on a total of 22 Dll^{SA1}; *hsp70-Dfd/UbxHD* larvae. The maxillary lobes of these animals had an average of 5 cirri missing from the dorsal row (range 4-6), and an average of 7 cirri missing from the ventral row (range 6-8). Similar results were obtained with Dll^{5}; *hsp70-Dfd/UbxHD* larvae.

Plasmid construction and embryonic injections

A 5.8 kb *Hin*dIII fragment containing the *Dll* ventral-lateral maxillary enhancer (ETD6) was subcloned into HZ50PL, a P-element injection vector containing an *hsp70* basal promoter fused to the *lacZ* gene (Hiromi and Gehring, 1987). This construct was coinjected with the helper plasmid *p*π*25.7wc* into embryos of the ry^{506}

strain (Rubin and Spradling, 1982; Pirotta et al., 1988). Three independent transgenic strains, ETD6-57, 69, and 71 were tested for expression patterns in wild-type and mutant backgrounds.

RESULTS

Dfd regulates the *Dll* expression pattern

Dll expression evolves from a single anterior-dorsal patch at the cellular blastoderm stage to the complex pattern shown in Fig. 1B by late stage 12 of development (Cohen, 1990). Of particular importance to this study is the domain of *Dll* expression within epidermal cells of the ventral-lateral maxillary segment, which is initiated during early germ band retraction. The ventral-lateral maxillary *Dll*-expressing cells are entirely contained within the larger *Dfd* expression domain in the maxillary segment of stage 12 embryos (Fig. 1A). Fate mapping studies have indicated that the ventral-lateral maxillary region will give rise to the cirri, mouth hooks, and the ventral (sense) organs of the larval head (Turner and Mahowald, 1979; Jürgens et al., 1986).

To test whether *Dfd* function is involved in the establishment of the normal expression pattern of *Dll* in the maxillary segment, we performed in situ hybridizations to detect *Dll* transcripts both in *Dfd* mutant embryos and in *hsp70-Dfd* embryos. The *Dll* expression pattern in a *Dfd* mutant embryo is shown in Fig. 1C. *Dll* transcripts are no longer detectable within the ventral-lateral maxillary cells and are ectopically expressed in several mandibular segment cells. This regulatory relationship is non-reciprocal, as *Dfd* expression is not detectably altered in *Dll* mutant embryos (data not shown).

hsp70-Dfd embryos are homozygous for a P element insertion containing a *Dfd* cDNA fused to a heat inducible promoter. Heat-shocking these embryos for 1 hour at 37°C during the cellular blastoderm stage of development induces

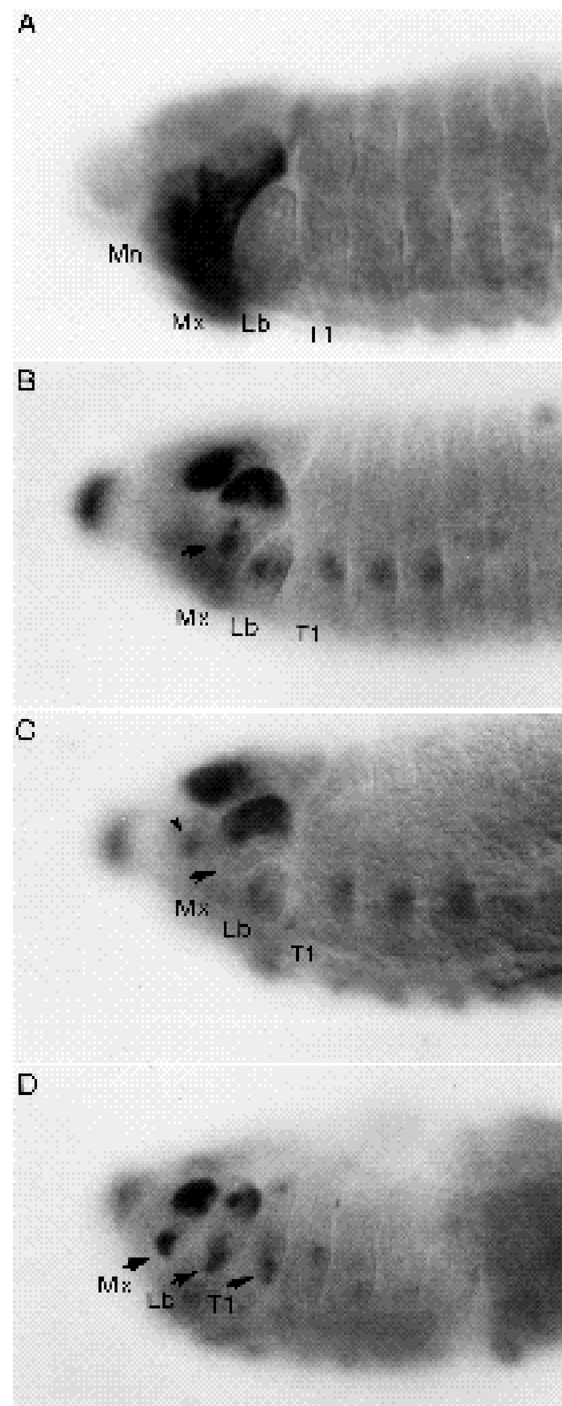

Fig. 1. *Dll* expression in wild-type, *Dfd* mutant and *hsp70-Dfd* embryos. *Dll* transcripts were visualized with digoxigenin-labeled probes, and Dfd protein was detected with mouse anti-Dfd antibodies as described in Materials and Methods. Anterior ends of the embryos are on the left and all embryos are at stage 12 of embryonic development, the end of germ band retraction (Campos-Ortega and Hartenstein, 1985). Mn, mandibular segment; Mx, maxillary segment; Lb, labial segment; T1, first thoracic segment. (A) In the epidermis of a wild-type embryo at stage 12, Dfd protein is specifically expressed in the ventral and posterior regions of the maxillary and mandibular segments. (B) *Dll* transcript expression pattern in a wild-type embryo at stage 12. Transcripts are expressed in subregions of the clypeolabrum, as well as in the antennal, maxillary, labial, and thoracic segments (Cohen, 1990). The transcript pattern in the maxillary segment consists of a dorsal domain, which includes the primordia of the maxillary sense organ, and a ventral-lateral domain (arrow). Fate maps indicate that the ventral-lateral maxillary region includes the primordia of the cirri, mouth hook, and ventral organ (Jürgens, et al., 1986). *Dll* transcripts are first detected in the ventral-lateral domain during early germ band retraction (late stage 11). All of the *Dll* expressing cells within the ventral-lateral region persistently express Dfd protein throughout embryonic development. (C) The *Dll* transcript pattern in a Dfd^{RX1} mutant embryo. *Dll* expression is not initiated in the ventral-lateral maxillary segment cells (arrow). In addition, there are a few cells within the ventral mandibular segment that ectopically activate *Dll* transcription (arrowhead). (D) *Dll* transcript pattern in a *hsp70-Dfd* embryo heat shocked for one hour at the cellular blastoderm stage of development and aged to stage 12. The *Dll* expression pattern in the labial and first thoracic segments adopts a ventral-lateral maxillary-like pattern (arrows). No ectopic expression is seen in the abdominal segments. The positive regulatory effect is most variable in the 2nd and 3rd thoracic segments (as shown). The expression of *Dll* in the antennal region is repressed in *hsp70-Dfd* embryos.

ectopic expression of Dfd protein that persists (via autoregulation) in the antennal segment and ventral regions of the labial, thoracic, and abdominal segments (Kuziora and McGinnis, 1988). *Dll* expression in *hsp70-Dfd* embryos is shown in Fig. 1D. The *Dll* pattern is strikingly altered in head and thoracic segments. *Dll* is no longer activated within the antennal segment (which correlates with a deletion of the antennal sense organ in *hsp70-Dfd* larvae; unpublished results), and the labial pattern closely resembles that of the maxillary segment, including an ectopic ventral-lateral domain of *Dll* transcription. In thoracic segments, the wild-type *Dll* expression domains in the posterior-lateral epidermis (which will give rise to Keilin's organs; Cohen, 1990) are repressed with a variable penetrance. In addition, *Dll* transcripts are ectopically expressed in novel thoracic domains that resemble the ventral-lateral maxillary *Dll* pattern in their more ventral positioning on the D/V axis.

From these experiments, we concluded that *Dfd* function is required for the ventral-lateral maxillary domain of *Dll* transcription, and *Dfd* expression is sufficient to induce ectopic 'ventral-lateral domains' of *Dll* expression in the labial and thoracic segments.

Dll is required for normal cirri development

The maxillary cuticular structures that are deleted in *Dfd* mutant larvae are the cirri, ventral organs, and mouth hooks (Merrill et al., 1987; Regulski et al., 1987). The maxillary sense organ, which develops from a dorsal-anterior region of the maxillary segment that persistently expresses *Dll* but not *Dfd* transcripts and protein (Fig. 1A,B), is less strongly affected in *Dfd* mutants. The fact that the *Dll* ventral maxillary expression domain is dependent on *Dfd* suggested the possibility that *Dll* expression in this region might mediate part of the *Dfd* morphogenetic function. Previous studies have not provided a detailed picture of ventral maxillary development in *Dll* mutants due to the difficulty of scoring structures deriving from this region on involuted or partially involuted heads. To determine if any ventral maxillary derived structures are dependent on *Dll* expression, we analyzed the cuticular phenotype of *Dll* mutant larvae in which head involution was prevented (see Materials and Methods).

When displayed on the surface of a non-involuted maxillary lobe, the structures that develop from the ventral maxillary segment are much easier to visualize. These structures; cirri, mouth hook, and ventral organ, are shown in Fig. 2 on a wild-type (A) and non-involuted larval head (B). The mouth hook develops from the most ventral region of the lobe while the ventral organ and two rows of cirri develop from the adjacent ventral-lateral region (Fig. 1A). The anterior row of cirri in the top of the frame of Fig. 2B (which will become the more dorsal row on the pseudocephalon if head involution takes place) contains 9-10 cirri, and the posterior row contains approx. 14 cirri. The ventral organ, consisting of a small dome shaped structure flanked by a smaller papilla, is located just under the two anterior row papillae farthest from the mouth hook.

Dll mutant larvae have normal mouth hooks and ventral organs, but are missing an average of five cirri from the anterior row, and seven cirri from the posterior row (Fig.

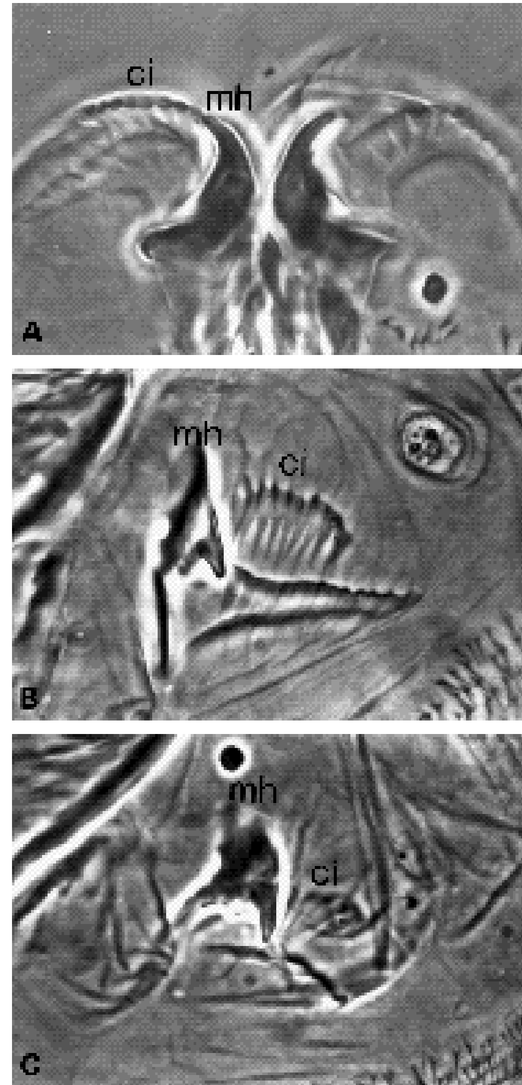

Fig. 2. Cirri phenotype of *Dll* mutant embryos. Non-involuted larval heads were obtained by brief heat shocks of Dll^{mutant}; *hsp70-Dfd/UbxHD* embryos at the cellular blastoderm stage of development. Both Dll^{SA1} and Dll^{5} null mutants were tested (Cohen and Jürgens, 1989; Cohen et al., 1989) Dll^{SA1} cuticles are shown. Larval cuticles were prepared as described in Materials and Methods. (A) Head of a wild-type first instar larva. ci, cirri; mh, mouth hooks. (B) First instar larval maxillary segment on a non-involuted head induced by *hsp70-Dfd/UbxHD* in a Dll^{+} background. The anterior of the larval body axis is at the top and ventral to the left. There are two rows of cirri (ci); the anterior row (which would be dorsal on an involuted head) consists of 9-10 cirri and the ventral organ, visible as a dome papilla flanked by a black dot, and the posterior row (which would be ventral on an involuted head) consists of approx. 14 cirri. The maxillary sense organ is in the upper right-hand corner of the frame and the mouth hook (mh) at left. (C) First instar larval maxillary segment on a non-involuted head induced by *hsp70-Dfd/UbxHD* in a Dll^{SA1} background. Approximately five cirri are deleted from the mouth hook-proximal end of the anterior (dorsal) cirri row and seven proximal cirri are missing in the posterior (ventral) row. Note that the mouth hook seems relatively unaffected by *Dll* mutations, and as previously reported (Sunkel and Whittle, 1987; Cohen and Jürgens, 1989) the maxillary sense organ is deleted.

Distal-less

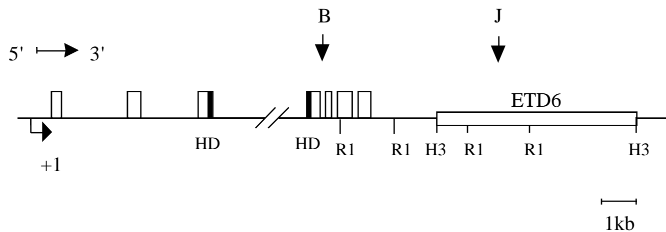

Fig. 3. Map locations of the *Dll*^{B} and *Dll*^{J} breakpoints. The horizontal line represents the *Dll* transcription unit, with the boxes marking the approximate positions of *Dll* exons. Vertical arrows point to the restriction fragments that contain the *Dll*^{B} and *Dll*^{J} rearrangement breakpoints (Cohen et al., 1989). The 3 enhancer, ETD6, contains sequences required for *Dll* expression in the ventral-lateral maxillary domain (see Figs 4, 6). HD, homeodomain coding sequences. Restriction sites, *Eco*RI (R1) and *Hin*dIII (H3) are shown.

2C). In addition, the ventral organ often includes extra papillae of unknown origin. As the ventral organ and associated cirri are still present in *Dll* mutants, the missing cirri apparently derive from the most ventral region adjacent to the mouth hook.

Two *Dll* mutant breakpoints define sequences required for ventral-lateral maxillary expression

Two chromosomal rearrangment breakpoints that interrupt the *Dll* locus, diagrammed in Fig. 3, provide a hint as to the location of sequences that control the ventral-lateral maxillary expression domain of *Dll*. Both breakpoints are located 3 of the *Dll* coding regions, and transcripts are still produced from the *Dll* loci residing on the rearranged chromosomes. The *Dll*^{J} chromosome has a breakpoint that maps within a 1.7 kb *Eco*RI fragment that spans sequences from approx. 2 to 4 kb downstream of the *Dll* transcription unit, while the *Dll*^{B} breakpoint maps within a region that includes the 3 exons of the *Dll* gene (Cohen et al., 1989). Stage 12 embryos that are homozygous for the *Dll*^{J} chromosome exhibit a normal or near normal pattern of *Dll* transcripts in the ventral-lateral maxillary domain (Fig. 4D). On the other hand, embryos that are homozygous for the *Dll*^{B} chromosome do not accumulate *Dll* transcripts within ventral-lateral maxillary cells, although the remainder of the *Dll* expression pattern appears to be normal (Fig. 4C). This suggests that a *Dll* regulatory element required for the ventral-lateral maxillary domain of *Dll* transcription maps in the 3 sequences between the *Dll*^{B} and *Dll*^{J} breakpoints.

Dll 3′ sequences contain an enhancer that is specifically activated in ventral-lateral maxillary cells

One possible function for the interval between the *Dll*^{B} and *Dll*^{J} breakpoints is that of a ventral maxillary-specific transcriptional enhancer. To test this, a 5.8 kb *Hin*dIII fragment, designated ETD6 (Fig. 3), was cloned upstream of the basal promoter in the *lacZ* reporter vector HZ50PL (Hiromi and Gehring, 1987). Transgenic embryos carrying this construct (ETD6 strains) express -galactosidase in ventral-lateral maxillary cells in a pattern that exhibits a spatial and temporal overlap with the normal *Dll* ventral maxillary expression domain (Figs 4A, 5).

Dll transcription within the ventral-lateral maxillary segment is initiated during early germ band retraction and includes 20-25 cells by the end of germ band retraction (Fig. 5A). Interestingly, this number is approximately the same as the number of cirri (approx. 23-24) that typically develop from ventral-lateral maxillary epidermis. In comparably staged embryos, the ETD6 enhancer directs -galactosidase expression in approximately 16 cells that overlap the most ventral cells in the *Dll* ventral-lateral maxillary domain (Fig. 5B). During head involution (stage 14), the maxillary segment undergoes a slight rotation, and ventral maxillary cells come to occupy anterior positions relative to the formerly dorsal (now posterior) cells. It is during this stage that the first morphological signs of cirri appear, as some of the ventral-lateral maxillary cells gradually become organized into two orderly rows. The ventral row of cirri cells, derived from a relatively posterior position in the ventral-lateral maxillary lobe, and the dorsal row of cirri cells (anteriorly derived) can be first visualized between stages 14 and 15 (Turner and Mahowald, 1979). During this period, *Dll* transcripts are expressed in these apparent cirri precursors (Fig. 5C; Turner and Mahowald, 1979). The ETD6 enhancer expression pattern is limited to the same group of cells (Fig. 5D).

During stages 16 and 17 the cirri-producing cells become clearly visible, now localized on the anterior-ventral aspect of the pseudocephalon. Expression of -galactosidase from the ETD6 enhancer, at stage 16, is detected in approximately 16 cirri-producing cells, 8-10 cells in the dorsal row and approx. 6 cells in the ventral row. In both rows, the ETD6-expressing cells include those that are closest to the developing mouth hooks and thus correspond to positions that do not produce cirri in *Dll* mutant larvae. In stage 17 larvae, ETD6 expression persists in the proximal cirri (nearest the mouth hooks) of the dorsal row, but appears to be gradually extinguished in the ventral row (Fig. 5E). *Dll* expression, as measured by whole-mount in situ hybridization, appears to be extinguished in cirri at some time during stage 16-17. The *Dll* transcript patterns in cirri during these very late stages resembles the ETD6 expression pattern, but is difficult to accurately define due to the low resolution of in situ hybridization, coupled with the spatial clustering of other *Dll*-expressing structures (antennal sense organ, maxillary sense organ) in the pseudocephalon of late stage embryos (data not shown).

Ectopic *Dfd* is sufficient to activate ectopic expression of the *Dll* 3′ ventral-lateral maxillary enhancer in other segments

The spatial overlap of the ETD6 enhancer expression with the ventral maxillary domain of the *Dll* pattern suggested that this 3 regulatory element supplies most of the ventral-lateral maxillary pattern of *Dll.* To test whether the ETD6 enhancer is regulated in a *Dfd*-dependent manner, the ETD6 expression pattern was analyzed in *Dfd* mutant and *hsp70-*

Dfd embryos. As mentioned earlier, the ETD6 expression pattern is localized to Dfd-protein-expressing cells in the ventral-lateral maxillary epidermis, as is shown in the doubly stained embryo in Fig. 6B. In *Dfd* mutant embryos, the ETD6 enhancer is not active at any stage of embryonic development (data not shown).

In *hsp70-Dfd* embryos, heat shocked for 1 hour at the cellular blastoderm stage and aged to retracting germ band stage, the ETD6 enhancer (blue) is ectopically activated in the antennal, labial and thoracic segments within cells ectopically expressing *Dfd* (brown) (Fig. 6C). The pattern of ectopic activation of ETD6 is very similar to the pattern of ectopic activation of ventral-lateral patches of *Dll* transcription in *hsp70-Dfd* embryos after heat shock (Fig. 1D). Note that even in the labial and thoracic segments only a subset of the cells that ectopically express the Dfd protein also express the 3 enhancer. In addition, although Dfd protein is also ectopically expressed in abdominal segments, the ETD6 enhancer is very rarely activated in the abdomen, and if so, only in one or two cells.

Dll mutations do not revert the homeotic transformations induced by ectopic *Dfd* expression in embryos

The foregoing results suggest that *Dll* may be an important downstream mediator of the *Dfd* morphogenetic function in the maxillary segment. To test whether *Dll* is equally important when *Dfd* is expressed ectopically, we placed the

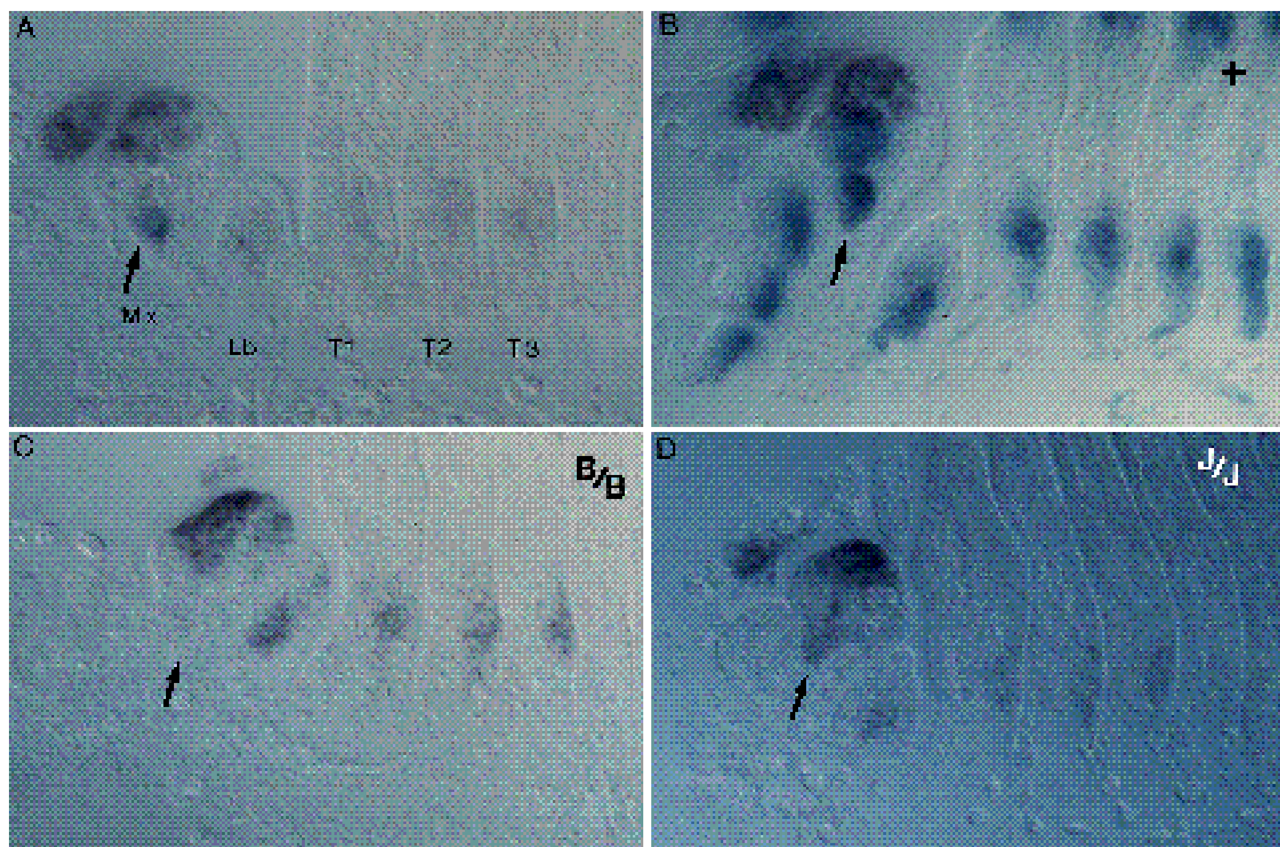

Fig. 4. Sequences required for *Dll* expression in the ventral-lateral maxillary segment are located in 3 regions of the *Dll* locus. The *Dll* expression pattern was tested in embryos homozygous for two different chromosomal rearrangement breakpoints, (Dll^B and Dll^J; Cohen et al., 1989) which both map in the 3 region of the *Dll* locus. To aid in the identification of homozygous breakpoint mutant embryos, the rearranged chromosomes were placed over a second chromosome balancer containing a *lacZ* enhancer detector inserted into the *wingless* (*wg*) locus (Perrimon et al., 1991). Double labeling to detect both *Dll* transcripts and -galactosidase was performed as described by Cohen et al. (1991). Mx, Maxillary segment; Lb, labial segment; T1-T3, thoracic segments. (A) The expression pattern of the *lacZ* gene driven by the *Dll* 3 (ETD6) enhancer (blue) and *Dll* (purple) in a stage 12 embryo. -galactosidase and *Dll* are both expressed in ventral-lateral maxillary segment cells (arrow). Note that -galactosidase is not expressed in all of the *Dll*-expressing cells of the ventral-lateral maxillary domain, only the ventralmost 16 cells (approximately). (B) A control embryo (one of the sibling progeny from a *Dll* breakpoint stock), double-stained for *Dll* (purple) and -galactosidase (blue). The insert on the balancer chromosome provides -galactosidase expression in the *wg* expression pattern. (C) The *Dll* transcript expression pattern in an embryos homozygous for the Dll^B breakpoint. This embryo was double stained for both *Dll* and -galactosidase, and is identified as Dll^B/Dll^B by the lack of -galactosidase staining in the *wg* pattern. Note that the ventral-lateral maxillary expression of *Dll* is missing (arrow). (D) The *Dll* transcript expression pattern in an embryo homozygous for the Dll^J breakpoint. The *J* breakpoint is between 2 and 4 kb 3 of the *B* breakpoint. This embryo was stained for both *Dll* and -galactosidase and is identified as Dll^J/Dll^J by the lack of -galactosidase staining in the *wg* pattern. Note that the ventral-lateral maxillary expression of *Dll* is present (arrow).

hsp70-Dfd construct in *Dll* mutant genetic backgrounds. As previously mentioned, ectopic expression from the heat inducible *Dfd* cDNA induces maxillary cirri in labial and thoracic segments. *Dll* mutations did not completely revert this homeotic transformation, as some ectopic cirri are still induced in *hsp70-Dfd* larval cuticle (data not shown).

A detailed analysis of the cirri produced in *hs70-Dfd* larvae may provide an explanation for this lack of requirement for *Dll* in ectopic cirri development. We note that among the four to six cirri typically induced by *hsp70-Dfd* in the labial segment and T1, a ventral organ is also often present. In the strongest transformations two rows of ectopic cirri are present, each with four to six cirri, with one row containing a ventral organ (data not shown). This indicates that some of the ectopic cirri formed in *hs70-Dfd* larvae correspond to the cirri most distant from the mouth hooks. As shown in Fig. 2C, these are the cirri in the maxillary segment that are not dependent on *Dll* function. These results indicate that although both *Dfd* and *Dll* are required for the development of the ventral-most cirri, *Dfd* can act either independently or in combination with other factors to induce the formation of ectopic cirri.

DISCUSSION

These experiments indicate that the homeobox containing gene *Dll* is one of the downstream transcription units regulated by the HOM gene *Dfd*. This regulation takes place in embryonic ventral-lateral maxillary epidermal cells, which represent a small subset of the cells expressing *Dfd*. *Dll* function is required in these cells for the development of the specialized maxillary structures known as cirri, thus mediating an important part of the segmental identity function of *Dfd*. The regulatory influence of *Dfd* on *Dll* is exerted principally through an enhancer element that resides in a 3 region of the *Dll* locus. At present, the limits of this *Dfd*-dependent ventral-lateral maxillary enhancer are defined by a *Hin*dIII site at the left end of the 5.8 kb ETD6 test construct, and the Dll^{J} breakpoint that maps 1-3 kilobases away (Fig. 7).

Obviously, *Dfd* expression alone is not sufficient for the activation of the *Dfd*-dependent *Dll* 3 enhancer since the enhancer is initially activated in retracting germ band embryos, hours after the establishment of Dfd protein expression in blastoderm stage embryos. In addition, the 3 enhancer is activated in only a fraction of the epidermal cells that express Dfd protein (Jack et al., 1988; Mahaffey et al., 1989). Thus other unknown factors must limit the expression of the *Dll* 3 enhancer to ventral-lateral epidermal cells and either prevent its expression prior to germ band retraction, or promote it thereafter. These factors are likely to be present in other segments of the animal as *Dll*

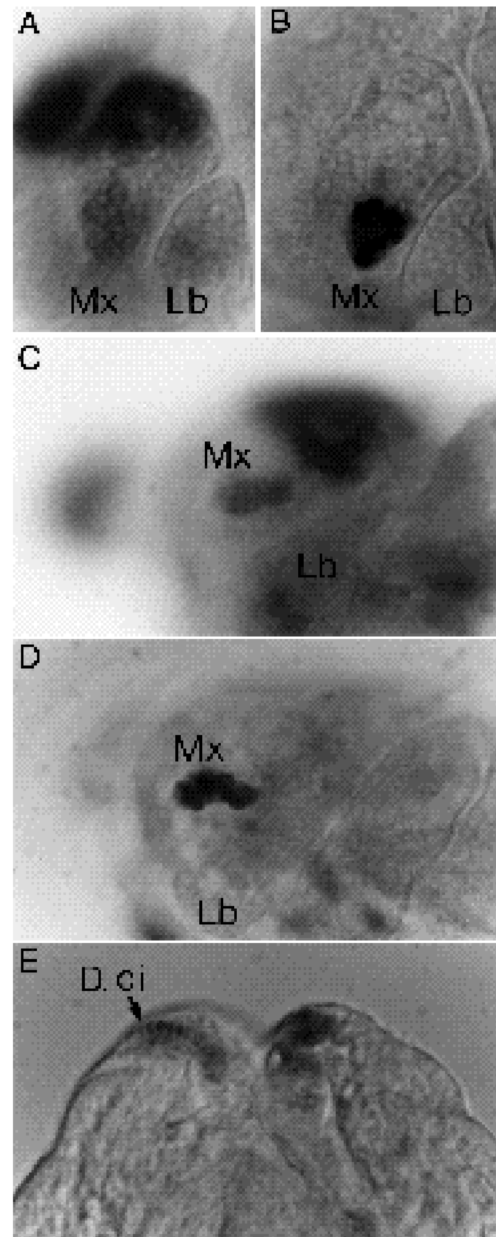

Fig. 5. A comparison of the *Dll* and *Dll* ETD6 enhancer expression patterns during late stages of embryonic development. *Dll* transcripts were detected using digoxigenin-labeled probes and -galactosidase expression from the ETD6 enhancer (Fig. 3) was detected with mouse anti- -gal antiserum (Promega) as described in Materials and Methods. Mx, maxillary segment; Lb, labial segment; D. ci, dorsal cirri. (A) *Dll* transcript expression and (B) ETD6 enhancer expression in 10 hour embryos. Within the ventral-lateral portion of the maxillary segment, 20-25 cells accumulate *Dll* transcripts, while the ETD6 enhancer is activated in an overlapping domain of approximately 16 cells. (C) *Dll* transcript expression and (D) ETD6 enhancer expression in 11 hour embryos. The maxillary segment has undergone a slight rotation by this stage, bringing ventral maxillary cells to a more anterior position. Both *Dll* and the ETD6 enhancer are expressed in a distinctive group of cells that are beginning to be arranged in an organized, double row pattern. Turner and Mahowald (1979) identified these cells as the cirri primordia. (E) ETD6 enhancer expression in a 13-14 hour embryo (stage 16). The cells in the ventral row of cirri no longer stain for -galactosidase. During subsequent stages of development dorsal row expression also declines; the last cells with detectable staining are those closest to the midline.

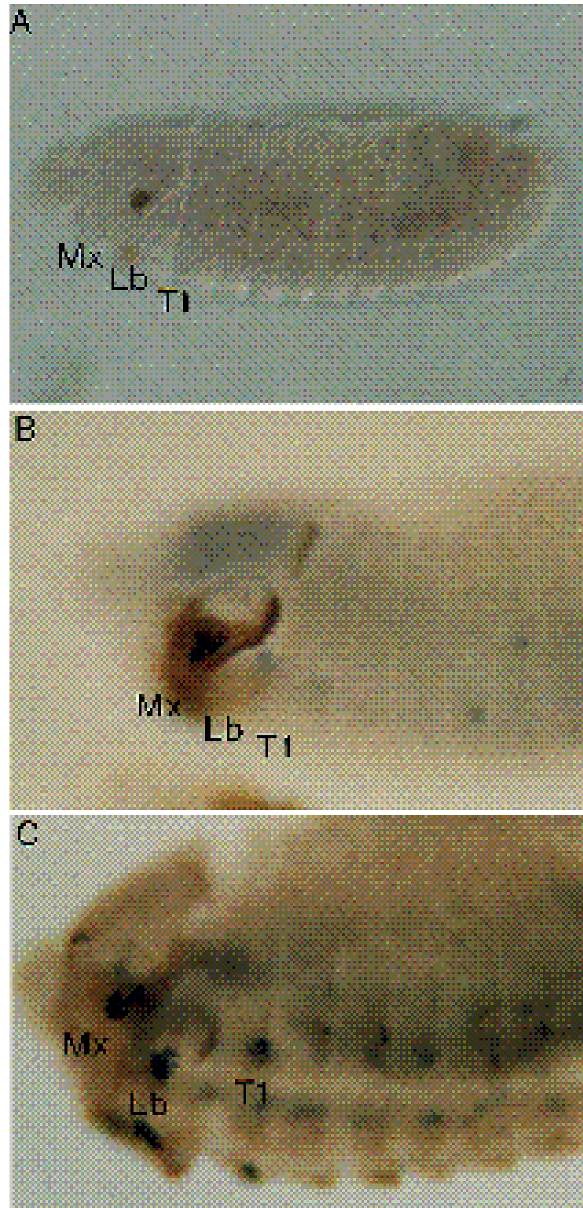

Fig. 6. Expression of the *Dll* 3 (ETD6) enhancer in *hsp70-Dfd* embryos. *hsp70-Dfd;* ETD6 enhancer/*lacZ* embryos were heat shocked for one hour at 37°C at the cellular blastoderm stage and aged for 7.5 hours at 25°C. Embryos were stained with mouse anti-Dfd and rabbit anti- -galactosidase antibodies as described in Materials and Methods. (A) -galactosidase (brown) protein pattern in a *hsp70-Dfd*; ETD6 embryo which was not heat shocked. Maxillary (Mx) expression is limited to the ventral-lateral epidermis. (B) Dfd (brown) and -galactosidase (blue) protein patterns in a *hsp70-Dfd*; ETD6-71 embryo which was not heat shocked. *Dfd* and *lacZ* are co-expressed within cells of the ventral-lateral maxillary epidermis. (C) Dfd (light brown) and -galactosidase (blue) protein patterns in a *hsp70-Dfd*; ETD6-71 embryo after heat shock. Dfd protein is ectopically expressed in the posterior ventral and ventral-lateral portions of each embryonic segment as described previously (Kuziora and McGinnis, 1988). The ETD6 enhancer is ectopically activated within a subdomain of the ectopic *Dfd* expressing cells in the labial (Lb) and first thoracic (T1) segments, with a few cells exhibiting activation of the enhancer in other segments.

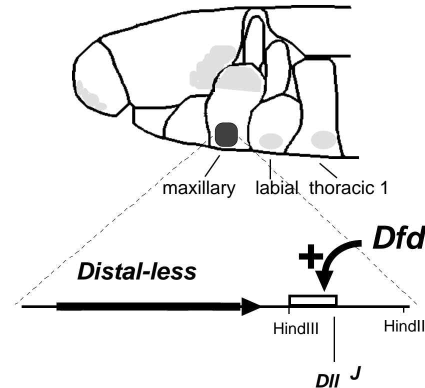

Fig. 7. *Dfd* activates *Dll* transcription in cells of the ventral-lateral maxillary segment through a 3 enhancer. Sequences sufficient to mediate the *Dfd* regulatory effect map within the region designated by the open box. Whether the *Dfd* activation effect is exerted directly or indirectly is unknown at present. Once *Dll* transcription is activated, *Dfd* and *Dll* are persistently expressed in the cirri progenitor cells and may act in combination (both proteins are homeodomain-containing transcription factors) to assign an identity to ventral-lateral maxillary epidermis that eventually results in the development of cirri.

transcription can be induced in ectopic ventral-lateral patches in *hsp70-Dfd* embryos. Whether the *Dll* 3 enhancer is directly activated by the Dfd protein is as yet unknown, but this regulatory element is an excellent candidate for a direct downstream target of the Dfd protein due to its lack of activation in *Dfd* mutant embryos and its ectopic activation in *hsp70-Dfd* embryos. Immunoprecipitation assays using Dfd protein overexpressed and purified from *E. coli* (Regulski et al., 1991; Dessain et al., 1992) indicates that the ETD6 enhancer has significant in vitro binding affinity for Dfd protein (unpublished results). However, much more information about the location and function of important sequences within the ETD6 enhancer will be required before it is known whether the 3 enhancer is directly regulated by Dfd protein.

Dll function is necessary only for the development of the cirri that develop adjacent to the mouth hook. Although we can say that these cirri most likely derive from the more ventral regions of the *Dfd* dependent *Dll* expression domain in the maxillary segment, there are no obvious morphological (or compartmental) subdivisions that separate the affected from unaffected cirri. The only difference we have detected between the affected and unaffected cirri is in their differential expression of the ETD6 enhancer construct. After stage 14 of development the *Dll* 3 enhancer is progressively inactivated in a distal to proximal direction (proximal cirri cells being those closest to the animal's ventral midline), first in the ventral row of cirri and then in the dorsal row. Thus, relative to the unaffected cirri of each row, the affected cirri-producing cells exhibit the most persistent expression of the *Dll* 3 enhancer. Perhaps *Dll* expression is only required during the late stages of cirri development, so that those cells that express both *Dll* and *Dfd* at late stages (*Dfd* is expressed in all of the cirri cells well into stage 17, Malicki et al., 1992) show a mutant phentype when lacking *Dll* function.

As *Dll* is persistently expressed in combination with *Dfd* in the ventral-lateral maxillary epidermis, and both are required for the morphological development of this region, the two homeodomain transcription factors produced from these loci are likely to represent an important part of a combinatorial morphogenetic code that assigns a specific subsegmental identity within the maxillary segment. Other factors must certainly be required for the complete combinatorial code that is sufficient to generate ventral-lateral maxillary identity, and this code is likely composed of both *Dfd*-dependent and *Dfd*-independent factors. Our results, along with those of Wagner-Bernholz et al. (1992), and Vachon et al. (1992), suggest that there is at least one intermediate level of transcription factor genes involved in spatial and/or cell-type identity in the genetic pathway between homeotic genes and the 'effector' gene products which must eventually be activated to achieve the final differentiated state. The recent work of Vachon et al. (1992) has shown that *Dll* transcription is repressed by the *Drosophila* HOM proteins Ubx and abd-A in the abdominal region of the embryo. Interestingly, the DNA element that mediates the Ubx/abd-A regulatory effect maps a considerable distance upstream of the first *Dll* exon, whereas the ETD6 element that mediates the Dfd regulatory effect maps in 3 sequences (Fig. 7). Thus, some of the intermediate regulators like *Dll* may be common targets of several homeotic genes through different regulatory elements.

The mouse genome contains genes that are structural homologs of *Dfd* and *Dll* (Price et al., 1991; Porteus et al., 1991; Robinson et al., 1991; McGinnis and Krumlauf, 1992). Dollé et al. (1992) and Bulfone et al. (1992) report that transcriptional expression from two of the mouse *Dll* homologs (*Dlx-1* and *Dlx-2*) is initiated in midstage embryos in spatially restricted domains within the mesenchyme of the four branchial arches. In the fourth branchial arch, *Dlx* expression overlaps with the expression patterns of mouse *Dfd*-like genes (Hunt and Krumlauf, 1991), which were initiated in this region at earlier stages of embryogenesis. Thus it is possible that during the process of facial development, a regulatory relationship between mammalian *Dll*-like and *Dfd*-like genes may exist that resembles the *Dfd* regulation of *Dll* in the *Drosophila* head.

We thank Nadine McGinnis for much experimental assistance with the early stages of this work, and many members of the laboratory for their commments, help and criticism. These experiments were supported by a grant to W. M. from the NIH (HD28315) and a grant to S. M. C. from HHMI.

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(Accepted 12 December 1992)