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ladybird, a tandem of homeobox genes that maintain late *wingless* expression in terminal and dorsal epidermis of the *Drosophila* embryo

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SUMMARY

***ladybird early* and *ladybird late* genes, tandemly located in the *Drosophila* 93E homeobox gene cluster, encode highly related homeodomain-containing transcription factors. Here we report the cloning of the complete cDNA sequences of both genes and a study of their expression and regulatory interactions with the segment polarity gene *wingless* in the epidermis. *ladybird* genes are co-expressed with *wingless* in epidermal cells close to the posterior parasegmental boundaries and in terminal regions of the body. In mutant embryos with altered *wingless* function, transcription of *ladybird early* and *ladybird late* is changed; it disappears completely from the epidermis in *wingless*$^{-}$ embryos, indicating *wingless*-dependence. After 6 hours of development, *wingless* expression is maintained by *gooseberry* in the ventral epidermis. However, in the dorsal epidermis and the terminal regions of the body, expression of *wingless* is independent of *gooseberry* but requires a *wingless-ladybird* regulatory feedback loop. Loss of *ladybird* function reduces the number of *wingless*-expressing cells in dorsal epidermis and leads to complete inactivation of *wingless* in the anal plate. Consequently, mutant *ladybird* embryos fail to develop anal plates and ubiquitous embryonic expression of either one or both *ladybird* genes leads to severe defects of the dorsal cuticle. Lack of late *wingless* expression and anal plate formation can be rescued with the use of a heat-shock-*ladybird* transgene.**

Key words: *ladybird* genes, anal plate, dorsal epidermis, homeobox, *wingless*, *Drosophila*

INTRODUCTION

Cell identity in the segmented epidermis of *Drosophila* embryos is specified by a network of segment polarity genes (for review see Peifer and Bejsovec, 1992; Perrimon, 1994). Most of them are highly conserved in evolution, providing a model system for cellular interactions. Initially activated by the pair rule genes, expression of the segment polarity genes becomes interdependent. One of the best examples of such a regulation is the mutual dependence between *wingless (wg)* (Baker, 1987) and *engrailed (en)* (Poole et al., 1985). These genes are expressed on either side of the parasegmental border and define two signaling centres that play a key role in epidermal patterning. *wg* itself encodes a secreted protein that acts on neighbouring cells and is required to generate naked cuticle in a restricted part of each segment (Heemskerk et al., 1991; Ingham and Hidalgo, 1993). In addition to its epidermal function, *wg* plays a role in the specification of neuroblast identity (Chu-LaGraff and Doe, 1993) in embryonic mesoderm formation (Wu et al., 1995) and imaginal development (Wilder and Perrimon, 1995). A putative Wg receptor (Bhanot et al., 1996) and the products of other genes, *armadillo (arm), dishevelled (dsh)* (Noordermeer et al., 1994) and *shaggy/GSK-3* (Bourouis et al., 1990) are thought to be required for the transduction of the Wg signal.

After 6 hours of development maintenance of *wg* activity becomes dependent on two functionally redundant *gooseberry* genes *(gsb* and *gsbn)*, which encode transcription factors containing highly related paired domains and *prd*-type homeodomains (Gutjahr et al., 1993; Li and Noll, 1993). Interactions between *wg* and *gsb* are restricted to the ventral epidermis and the loss of *gsb* function results in defects in cuticle differentiation leading to a lawn of denticles (Li and Noll, 1993). At the same time, cells of the dorsal epidermis and the terminal regions of the embryo undergo distinct patterning processes, and maintenance of *wg* activity in these cells requires other regulatory mechanisms.

We have isolated two *Drosophila* homeobox genes called *ladybird* (Jagla et al., 1993, 1994). Like *gsb* and *gsbn* (Li and Noll, 1993), they are clustered in tandem and are expressed in the epidermis, mesoderm and central nervous system (CNS) of embryos. The *ladybird* genes are located in the 93E homeobox gene cluster, just distally to *bagpipe (bap)* (Azpiazu and Frasch, 1993) and proximally to *S59* (Dohrmann et al., 1990). The most 5′ located gene, *ladybird early (lbe)*, is activated during germ band elongation slightly earlier than its relative *ladybird late (lbl)*. *lbl* follows the expression pattern of *lbe* and both *lb* genes encode transcription factors bearing a specific Ladybird-type homeodomain (Jagla et al., 1994). In addition to the *Drosophila* genes, orthologous genes have been found in mouse *(Lbx1)* and human *(LBX1)* (Jagla et al., 1995), suggesting that the *ladybird* genes could have an evolutionarily conserved role in development.

Here we report the cloning of full length cDNA sequences of both *lbe* and *lbl* genes, analyse their specific epidermal expression patterns and discuss regulatory interactions with *wg*. We show that activity of *lb* genes in the epidermis is regulated by the segment polarity gene network and depends on Wg signaling. Analysis of embryos homozygous for a deficiency uncovering the *lb* locus, as well as embryos with ubiquitous expression of both *lb* genes, indicates a requirement for *lb* in dorsal epidermis and anal plate development.

MATERIALS AND METHODS

Chromosomal walking and analysis of deficiency breakpoints of the 93E region

A bidirectional chromosomal walk between the *bap* and *S59* genes was carried out using PCR-generated probes corresponding to the 3′ region of *bap* and 5′ region of *S59*. Genomic clones were restricted and aligned with previously obtained *lbe* and *lbl* λ clones (Jagla et al., 1993, 1994). The distal breakpoints of Df(3R)GC14, Df(3R)eF1 and Df(3R)eBS2 were analysed by PCR amplification on genomic DNA prepared from single homozygous embryos selected by their bloated gut appearance (Bodmer, 1993). A set of primers targeting *tin, bap, lbl, lbe* and *S59* genes were designed using sequences available in the EMBL/GenBank Database Library.

cDNA cloning and sequencing

Several embryonic cDNA libraries were first tested by PCR for the presence of both *lbe* and *lbl* clones. Selected libraries were screened using homeobox-containing genomic fragments of *lbe* and *lbl* as previously reported (Jagla et al., 1993, 1994). One *lbe* and three *lbl* full length cDNA clones were obtained from the embryonic λZap libraries kindly provided by K. Zinn (Caltech, Pasadena) and C. S. Thummel (University of Utah, Salt Lake City) and sequenced using the TaqDyeDeoxy Terminator Cycle kit and an automated DNA sequencer.

Preparation of His-tagged Lb proteins and anti-Lb antibodies

lbe and *lbl* coding sequences were amplified by PCR from cDNA templates, using DeepVent DNA polymerase (Biolabs), and cloned downstream to the 6His-encoding motif of a His-pET expression vector. Chimeric proteins were produced in the *E. coli* BL21 pLysS strain and purified on Ni-agarose affinity column as previously described (Jagla et al., 1994). Polyclonal and monoclonal antibodies against these proteins were produced and tested by ELISA, western blots and by whole-mount embryo immunocytochemical staining. In addition, the monoclonal antibodies were selected using immunostaining of COS cells transfected by the pSG5-*lbe* or *lbl* expression vectors.

In situ hybridization, antibody staining and cuticle preparation

Embryos were collected from apple-juice agar plates, dechorionated, fixed and processed according to the method of Tautz and Pfeifle (1989). The digoxigenin (Dig)-labeled DNA probes targeting *lb* genes were prepared by PCR (Jagla et al., 1994). The *wg* 3.0 kb cDNA probe (Baker, 1987) was labeled by random priming. After overnight hybridization with the Dig probes (final concentration of 2-4 ng/ml), embryos were incubated (1 hour) with preadsorbed anti-Dig antibody coupled with alkaline phosphatase (Boehringer) (1:2000). Colour reaction was performed using NBT and X-phosphate as substrate. For antibody staining whole-mount embryo preparations were blocked (1 hour) in 5% normal goat serum and incubated overnight at 4°C with first antibody. The secondary antibodies were biotinylated horse anti-rabbit or anti-mouse IgG, detected using an ABC-AP or Elite-ABC-horseradish peroxidase (HRP) kit (Vector Laboratories). To identify cell positions or to distinguish heterozygous embryos, some preparations were double stained with anti-β-gal, anti-En or anti-Eve antibodies. Stained embryos were dehydrated, mounted in Canada balsam and photographed using a Nomarski optics.

Cuticles were prepared essentially as described by Li and Noll (1993), mounted in Hoyer's medium and photographed under phase contrast optics.

Heat-shock *ladybird* flies

The heat-shock *lb* constructs (*hs-lbe* and *hs-lbl*) were made by inserting the full-length *lbe* and *lbl* cDNAs into the *Eco*RI site of the P-element vector pCaSpeR-hs and injected into w^{1118} embryos according to standard procedure (Rubin and Spradling, 1982). From several transformants, the *hs-lbe4A* and *hs-lbl5/1* insertions on chromosome X were used in most experiments. Double hs-*lbe/lbl* transgenic flies were generated by recombination of *hs-lbl* transgenes on hs-*lbe* containing X chromosome. Single *hs-lb* transgenes were combined with Df(3R)eF1 for the rescue experiments. Embryos homozygous for the Df(3R)eF1 deletion were identified by a lack of pericardial cells when immunostained with anti-Eve antibody.

Heat-shock treatment and temperature shift

hs-gsb, hs-lbe, hs-lbl, hs-lbe;hs-lbl and w^{1118} (wild-type control) embryos were collected, aged on agar plates at 25°C and heat-shocked (15 minutes 37°C) in water. After incubation in a humidified chamber at 25°C the embryos for in situ hybridization and immunostaining were removed at 7 to 8 hours AEL, those for cuticle preparation were left up to 24 hours AEL. wg^{IL114} embryos were aged at 18°C and shifted to the non-permissive temperature (29°C) at 6 hours AEL.

Fly strains

Flies were raised on standard *Drosophila* medium at 25°C. The null alleles ftz^{W20}, en^{IIB86} and hypomorphic eve^{ID19} were kindly provided by W. Gehring (Biozentrum, Basel). hh^{IJ35}, ptc^{IN108}, wg^{CX4}, wg^{IL114}, nkd^{7H16}, Df(2R)gsb^{IIX62}, and $y\ arm^{XK22}$ mutants were obtained from the Tübingen stock center. Df(2R)gsb^{IIX62} (Bopp et al.,1986) carries a deletion encompassing both *gsb* and *gsbn* genes. The *hs-gsb* transgenic strain was from M. Noll (Institute of Molecular Biology, Zurich) and deficiencies of the 93 region (Df(3R)eGp4, Df(3R)GC14, Df(3R)eF1, Df(3R)eBS2) were from the Bloomingthon stock center. bap^{208} and tin^{EC40} EMS alleles were provided by M. Frasch. The *Oregon R* strain was used as a wild-type control.

RESULTS

The homeobox genes *lbe* and *lbl* map distally to *bap* and are inactivated by the Df(3R)eF1 deletion

Our previous data (Jagla et al., 1994) showed that *lbe* and *lbl* are tandemly located in the 93E homeobox gene cluster. In order to define the position of *lb* inside the cluster, the distal breakpoints of four deficiencies were analysed (Fig. 1A-C) and a genomic walk was carried out (Fig. 1B). Initially, by PCR (see Materials and methods), we determined that *lbl* maps between the distal breakpoints of Df(3R)GC14 and Df(3R)eF1 while the *S59* gene is located outside from Df(3R)eF1 (Fig. 1B,C). Since, *bap* alleles complement Df(3R)GC14 whereas alleles of its immediate neighbour *tin* do not (Azpiazu and Frasch, 1993), the PCR results indicate that *lb* genes are located distally to *bap* and proximally to *S59*. This gene order was confirmed by our genomic walk (Fig. 1B) which also revealed that *lbe* maps distally to *lbl* and that both genes are transcribed from the opposite DNA strand compared to *tin, bap* and *S59*. In addition, as determined by Southern blot analysis (not shown), the λ clones encompassing the genomic region between *lbe* and *S59*

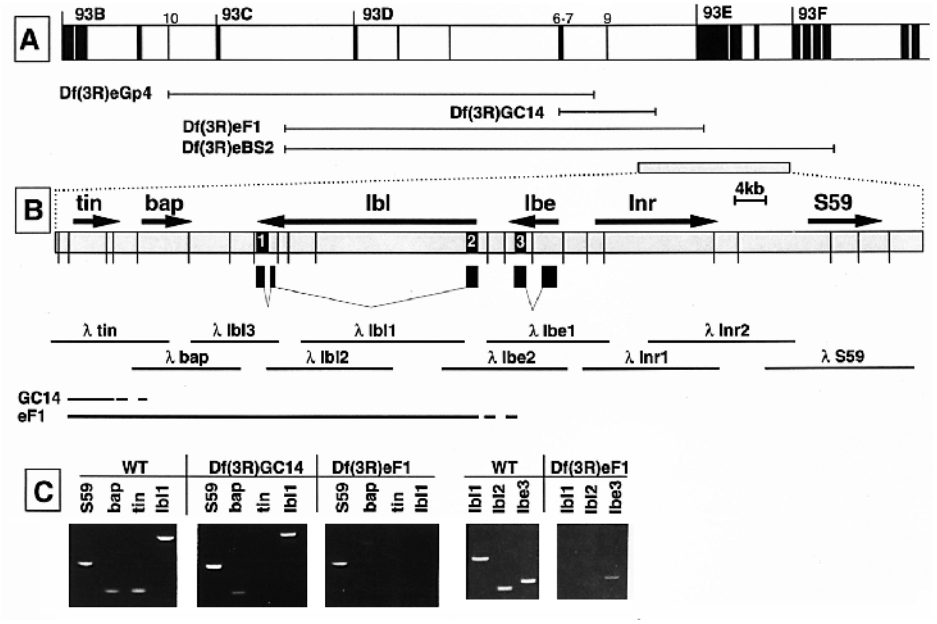

Fig. 1. Molecular organization of the *lb* locus. (A) Chromosomal extents of four deficiencies from the 93 region (Mohler and Pardue, 1984). (B) Chromosomal walk along the *lb* locus and *Eco*RI restriction map of a 115 kb genomic region encompassing *tin, bap* (Azpiazu and Frasch, 1993), *lbe, lbl* (Jagla et al., 1994), *inr* (Fernandez et al., 1995) and *S59* (Dohrmann et al., 1990). Arrows indicate the directions and the extents of 93E gene transcripts. Black boxes, within *lbl* and *lbe* coding sequences (numbered from 1 to 3) correspond to the regions amplified by PCR. Exon/intron organization of the *lb* genes is depicted below the restriction map. The λ genomic clones isolated during this walk are indicated below, as well as the positions of distal breakpoints of Df(3R)GC14 and Df(3R)eF1 deficiencies (dashed lines). (C) Mapping of the distal breakpoints of Df(3R)GC14 and Df(3R)eF1 deficiencies. Each panel corresponds to a PCR amplification of DNA from a single homozygous embryo.

(Fig. 1B) contained a *Drosophila* homologue of the *insulin receptor (inr)* gene (Fernandez et al., 1995). The position of the distal breakpoint of Df(3R)eF1 was analysed by PCR using the *lbe* and two different *lbl* primers targeting 3′ *(lbl1)* and 5′ *(lbl2)* coding sequences (Fig. 2B,C). The *lbl* gene was found to be inside and *lbe* outside the region deleted by Df(3R)eF1. However, the lack of *lbe* activity in the Df(3R)eF1 homozygous embryos (Fig. 8H) suggests that Df(3R)eF1 deficiency carries an additional mutation within the *lbe* gene, or deletes some *lbe* regulatory sequences located downstream of the gene.

lbe and *lbl* cDNA sequences predict structurally related nuclear proteins

As found previously, Lbe and Lbl share highly homologous regions extending downstream from the homeodomain (Jagla et al., 1994). The sequence of full length *lb* cDNA clones revealed conservation of additional regions located immediately upstream from the homeodomain (Fig. 2A,B). The 2045 bp *lbe* cDNA clone (not shown, see GenBank accession number) contains a leader sequence of 227 bp, an ORF of 1440 bp, and a trailer of 378 bp. Analysis of the coding sequence,

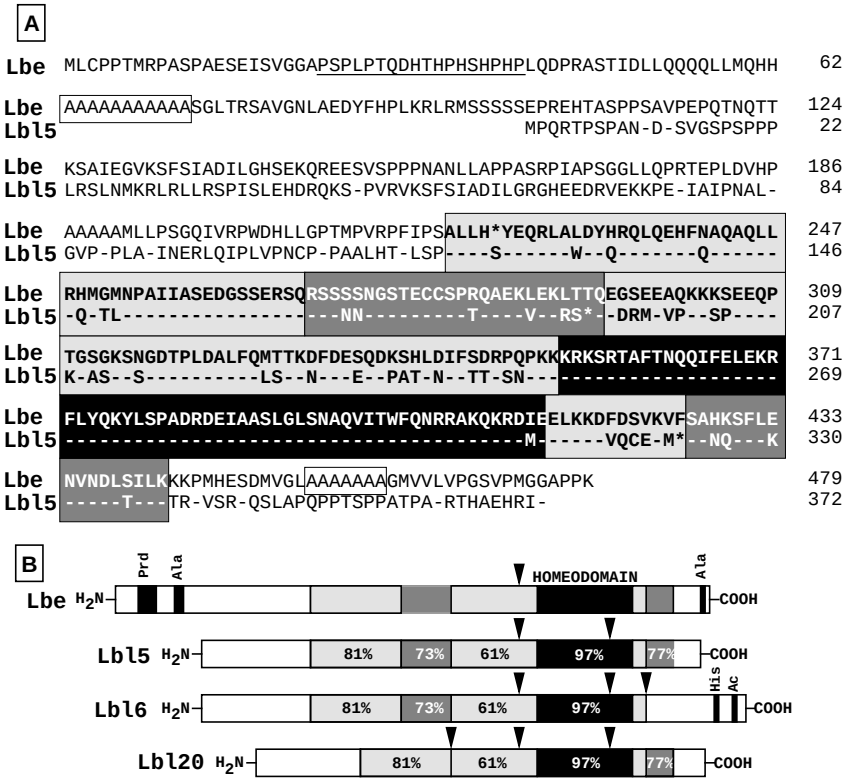

Fig. 2. Sequence and similarities between the Lbe and Lbl proteins. (A) Alignment of the predicted amino acid sequence of Lbe and one of the Lbl isoforms. The Prd-like, His/Pro rich domain in Lbe is underlined and two poly-Ala streches are boxed. The homeodomains are in black, the conserved flanking regions are in grey and the regions corresponding to the small introns, retained in *lbl* transcripts are in dark grey. The highly conserved regions between both Lb proteins are in bold type. (B) Comparative scheme of Lbe and three different Lbl protein isoforms deduced from the sequence of cDNA clones. Black, grey and dark grey boxes are as in A. Positions of Prd-like, poly-Ala, poly-His and acidic domains are indicated. Amino acid identity between both proteins are percentages. Introns are depicted by arrowheads. Note that short introns retained as coding sequences in *lbl* cDNAs (dark grey boxes) share high similarity with corresponding regions in the Lbe protein. The longest ORF encoded by *lbl6* cDNA results from the splicing of a 52 bp intron (dark grey box downstream from the homeodomain) alternatively retained in *lbl5* and *lbl20* cDNAs. The shortest *lbl20* ORF is devoided of the 75 bp intron (dark grey box upstream from the homeodomain).

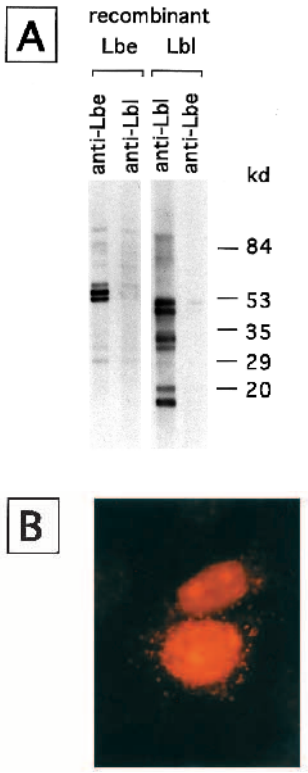

Fig. 3. Specificity of anti-Lbe and anti-Lbl antibodies and nuclear localization of Lb proteins. (A) Western blot of His-tagged recombinant Lbe and Lbl proteins probed with rabbit anti-Lbe and rabbit anti-Lbl serum. Multiple bands given by Lbl recombinant protein result from the amino-terminal localization of the 'tag', allowing purification of the non-terminated proteins. (B) Nuclear localization of the transiently expressed Lbe protein in transfected COS cells, detected by monoclonal antibody.

predicts a protein of 479 amino acids with a Prd-like repeat (Bopp et al., 1986) in the amino-terminal region, a homeodomain in the carboxy-terminal part and two poly-Ala stretches on either side of the protein (Fig. 2A,B). The *lbl* cDNA clones *lbl20* (1477 bp), *lbl5* (1867 bp) and *lbl6* (1982 bp) (not shown) correspond to transcripts differentially spliced by retention of the short introns (dark grey boxes in Fig. 2A,B), a phenomenon previously reported in human (Cooke et al., 1988). These cDNAs predict three proteins of 346, 372 and 411 amino acids (Fig. 2B). The longest (Lbl6) differs from the others at the carboxy-terminal region (Fig. 2B). The common features of *lbl* clones are (i) a short 5′ leader sequence (about 150 bp), (ii) the same initiating methionine codon, and (iii) several polyadenylation signals (AATAAA) preceding the poly(A) stretches. In addition to the short alternative introns, comparison between *lbl* genomic and cDNA sequences, revealed a large 20 kb intron upstream of the homeobox, and a 571 bp intron located inside the homeobox (Figs.1B, 2B) (Jagla et al., 1993). The predicted Lbe and Lbl proteins contain highly conserved homeodomains (97%) and regions of conservation upstream (61-81%) and downstream (77%) of the homeodomain (Fig. 2C). To further characterize these proteins, we raised antisera to His-tagged-Ladybird fusion proteins in both mice and rabbits. The anti-Lbe and anti-Lbl antibodies specifically recognize the corresponding recombinant protein (Fig. 3A) and detect nuclear proteins in COS cells transfected by the corresponding expression vector (Fig. 3B and data not shown) and in whole-mount embryos (Fig. 8C).

lbe and *lbl* display similar expression patterns

Activation of *lbe* slightly precedes that of *lbl* and appears during germ band elongation (3 hours 30 minutes AEL) in the primordium of the anal plate (Fig. 4A) and subsequently during neuroblast segregation (about 4 hours AEL) in the epiderm of gnathal segments and in some neuroblasts (Fig. 4B). Later, after completion of germ band elongation (4 hours 20 minutes AEL), epidermal domains of the terminal regions of the body contain only low levels of *lbl* transcripts (not shown) and Lbl protein (Fig. 4G,H). This is consistent with a spatial distribution of *lbl* gene products that evolves rapidly during embryogenesis into a pattern corresponding to that of *lbe*. The only difference concerns the trunk epidermis, where *lbe* transcripts are much more abundant (compare Fig. 4D and J).

Dynamic appearance of new epidermal and mesodermal domains of *lbe* and *lbl* gene expression takes place between 5 and 7 hours AEL, although the most prominent region of *lb* activity remains the anal plate (Fig. 4C,E,I,K). At about 5 hours AEL, both *lbe* and *lbl* start to be expressed in a cluster of mesodermal cells corresponding to the heart precursors (Jagla, K. and Frasch, M., unpublished data; see Fig. 4C,J). At the onset of segmental groove formation just posterior to these mesodermal cells in the thoracic and abdominal segments (A1-A7) a one-cell-wide epidermal *lb* stripe appears (Fig. 4C,J). This dorsal stripe broadens anteriorly up to 4-5 cells at 7 hours AEL (Fig. 4D) and then up to 6-7 cells after germ band retraction (Fig. 4F,L). Surprisingly, no expression of either *lb* genes is detected in the dorsal epidermal cells of the most posterior abdominal (A8 and A9) segments (Fig. 4C,F,I,L). At the late extended germ band stage *lb* transcripts also appear in the ventral, but not the lateral epidermis (Fig. 4D,E). These ventral *lb* stripes are weaker than dorsal patches (Fig. 4D) and in the case of *lbl* become visible during germ band retraction (not shown). After germ band retraction, the anti-Lbe and anti-Lbl antibodies clearly label broad epidermal stripes of dorsal cells (Fig. 4F,L) which migrate towards dorsal closure. Subsequently, during head involution, *lbe* and *lbl* expression decreases throughout the ventral but not the dorsal epidermis (Fig. 4L) and still persists in the terminal regions of the body corresponding to the head segments and the anal plate (not shown). At later stages of embryogenesis, *lb* gene expression progressively disappears from the epidermis and becomes restricted to the segmental border muscles and clusters of cells in the central and peripheral nervous system (not shown).

The *lb* and *wg* genes are co-expressed in the trunk epidermis and in the terminal regions of the body

The distribution of *lb* genes products in the epidermis of the trunk is particularly reminiscent of the late *wg* expression pattern. To localise more precisely the *lb* expressing epidermal cells along the anteroposterior axis of individual segments, we used, as a positional marker the En-positive cells of the posterior compartment (Poole et al., 1985) specifically stained with anti-En antibody. Double immunostaining for Wg/En (Fig 5A), Gsb/En (Fig. 5B) and En-immunostaining of embryos previously hybridized to the *lbe* probe (Fig. 5C,D) revealed a major domain of *lb* expression that overlaps the cells expressing *wg* and *gsb*, located just anteriorly to the cells expressing *en*. The posterior-most row of *lb*-expressing cells crosses the parasegmental groove and overlaps the *en* domain, as is observed for *gsb* (Li and Noll, 1993). In the dorsal epidermis, however, *gsb* stripes disappear at 6 hours AEL (Fig. 5B) whereas *lbe* (Fig. 5D) and *lbl* (not shown) remain co-expressed with *wg*. In addition to the expression in the trunk, we also observed common epidermal domains of *lb* and *wg* activity in the

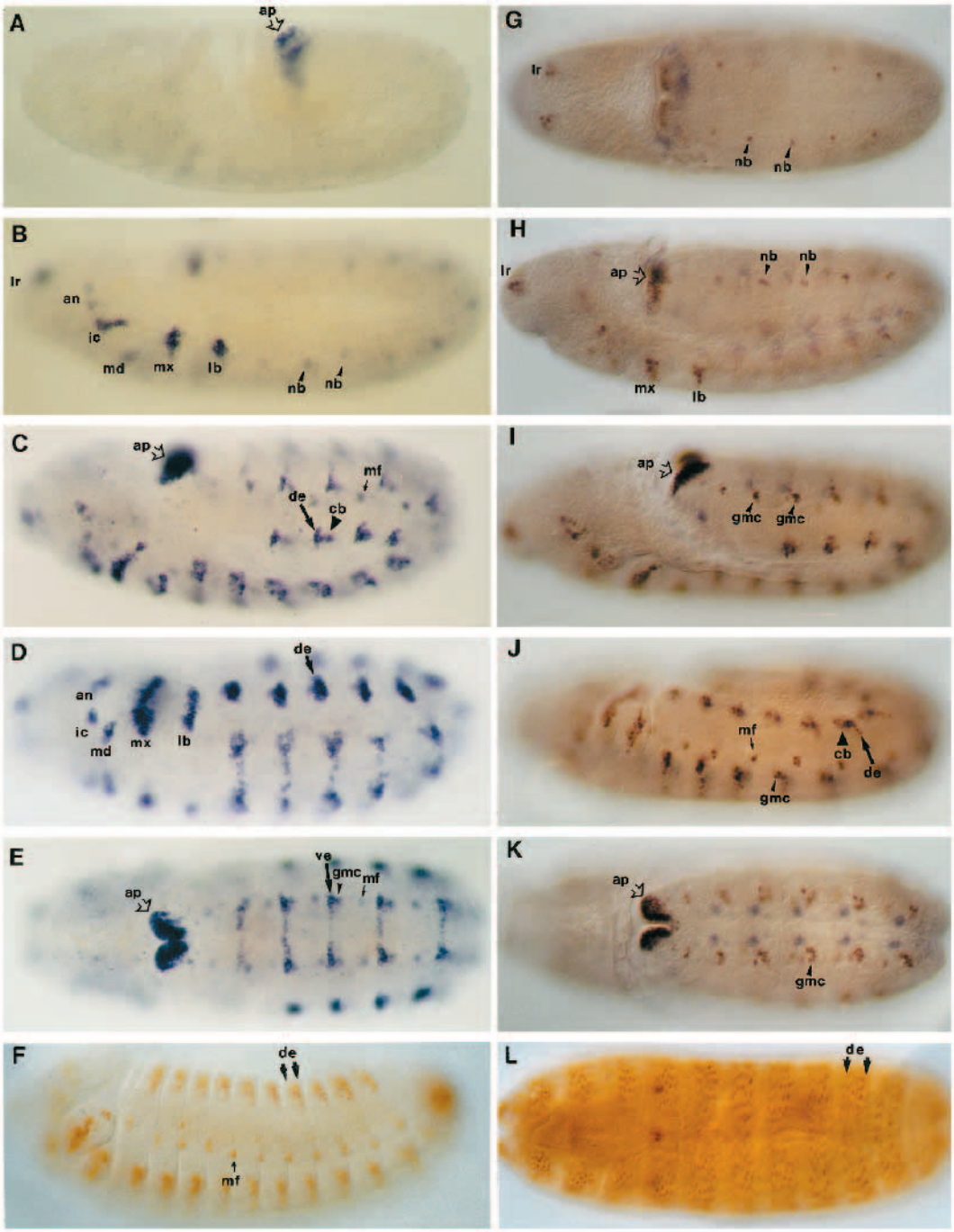

Fig. 4. Epidermal expression patterns of *lb* genes. Distribution of *lbe* (A-F) and *lbl* (G-L) gene products in wild-type embryos, visualized by whole-mount in situ hybridization (A-E) or by immunostaining with anti-Lbe (F,) or anti-Lbl (G-L) antibodies. Although *lbe* and *lbl* have a similar expression patterns, *lbe* activity precedes that of *lbl*; initially in terminal regions (compare A,B and G,H), then in trunk epidermis (compare C-E and I-K) and persists in the laterally interrupted stripes up to the dorsal closure (F). Developmental stages of the embryos: (A) germ band extension (stage 8), (G) early extended germ band (stage 9), (B-E, H-K) extended germ band (stages 10 and 11), (F) early dorsal closure (stage 13), (L) late dorsal closure (early stage 15). (D,E and J,K) are different views of the same embryos. Two thick arrows point to expanded dorsal *lbe* (F) and *lbl* (L) epidermal domains. an, antennal segment; ap, anal plate; cb, cardioblast precursors; de, dorsal epidermis; gmc, ganglion mother cells; ic, intercalary; lb, labial segments; lr, labrum; md, mandibular segment; mf, segmental border muscle founder cells; mx, maxillary segment; ve, ventral epidermis. All whole mounts are oriented with anterior to the left and photographed under Nomarski optics.

terminal regions of the body, corresponding to the labrum (Fig. 5E,F) and to the anal plate (Fig. 5E,G). These regions represent the earliest domains of activity for both *lb* genes.

Wg is required for epidermal expression of *lb*

The similarity between *lb* and *wg* gene expression patterns (Fig. 6A) prompted us to analyse *lbe* transcript distribution in some of the segmentation mutants. *lbe* expression is altered by mutations of the pair rule genes *even skipped (eve)* and *fushi tarazu (ftz)* (data not shown) which are both required for proper expression of *en* and *wg* (Frasch et al., 1987, Ingham et al., 1988). However, since *lbe* gene activity appears late, during the extended germ band stage, it is unlikely to be regulated directly by the early-acting pair rule genes, but may be a target or even a component of the Wg signaling pathway. To investigate this possibility we have analysed *lbe* activity in the absence of the major elements of the *en-wg* regulatory loop (Fig. 6B-F). Using the double staining procedure with anti-En antibodies and a *lbe* specific probe we found that in *en* (Fig. 6B) and *wg* (data not shown) null mutants *lbe* transcription is down-regulated. Since *en* is not affected and *lbe* activity decays in wg^{IL114} embryos shifted to the non-permissive temperature at 6 hours AEL (Fig. 6C) we postulate that *wg* rather than *en* is directly required for *lbe* transcription. This observation is consistent with the *wg*-like distribution of *lbe* transcripts in nkd^{-} (Fig. 6D) and ptc^{-} embryos (Fig. 6E). In particular, in *ptc* mutants, the expanded

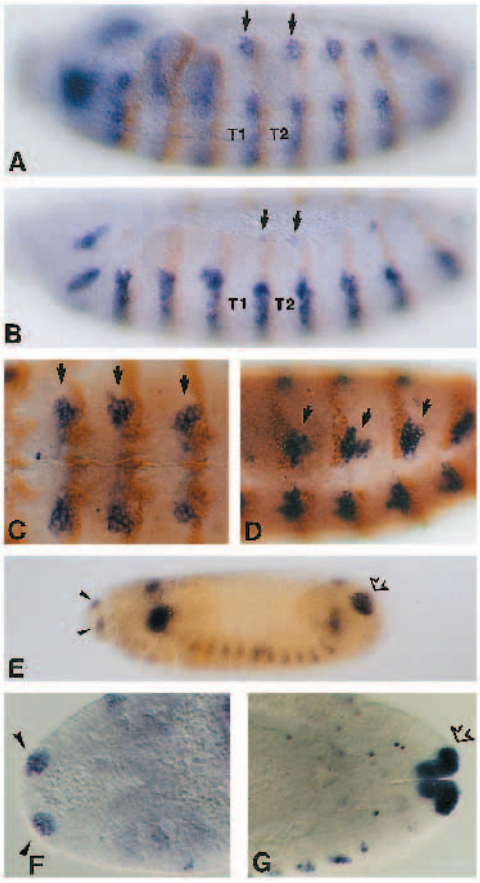

Fig. 5. Co-expression of *lb*, *wg* and *gsb* genes in the embryonic epidermis. Both *lb* genes are co-expressed with *wg* in dorsal and ventral epiderm and in terminal regions of the body. *lb-gsb* co-expression is limited to ventral stripes. The positions of *lb*-expressing cells were revealed in relation to those expressing *en*, stained with anti-En antibody (yellow/brown). (A,B) Ventrolateral, (C) ventral and (D) lateral views of stage 11 embryos, showing Wg- (A), Gsb- (B) or *lbe*- (C,D) expressing cells in the dorsal and ventral epidermis (arrows). Note that at early stage 11 Gsb protein (B) disappears from the dorsal region. (E) Dorso-lateral view of a late stage 12 embryo immunostained for Wg. (F) View of the head of a stage 10 embryo and (G) view of the tail of a stage 13 embryo hybridized with a Dig-labeled *lbe* probe. Arrowheads indicate anlage of the labrum sensory organ, and open arrows the anal plate. (T1-T3) thoracic segments. All embryos were photographed under Nomarski optics.

wg domain is limited anteriorly by a narrow ectopic *en* stripe (Ingham et al., 1991) and the *lbe* expression territory broadens to the same limit (Fig. 6E). A similar but unlimited expansion of the *lbe* domain was observed in *nkd* mutants (Fig. 6D). *nkd* is known to repress *en* autoactivation (Heemskerk et al., 1991). Since *lbe* expression is lost in *wg* mutants, we tested embryos mutant for *arm* that is thought to abolish transduction of Wg signalling in target cells. *lbe* expression decays in the majority of epidermal cells of *arm*$^{-}$ embryos (Fig. 6F) suggesting that the Wg signalling pathway is necessary for *lb* activity.

To further confirm the dependence of *lb* genes upon *wg* activity, we analysed *hs-gsb* and *hs-gsb;wg*$^{-}$ embryos (Fig. 6G-I). Previous studies of Li and Noll (1993) have shown that ubiquitous expression of *gsb* ectopically activates the endogenous *gsb* gene in cells located anteriorly to the wild-type stripe (Fig. 6G). This ectopic induction, however, was not observed in a *wg*$^{-}$ background (Li and Noll, 1993). As shown in Fig. 6I, *hs-gsb* is also able to activate ectopic *lbe* stripe formation and as for *gsb*, this phenomenon is *wg*-dependent and cannot be detected in *hs-gsb/wg*$^{-}$ embryos (Fig. 6J). Therefore, it is likely that *wg* function is required for both activation and maintenance of *lb* expression.

gsb activity maintains both *wg* and *lb* expression in the ventral, but not the dorsal epidermis

The maintenance of *wg* expression becomes *gsb*-dependent during segmental groove formation (about 6 hours AEL), (Li and Noll, 1993). However since at this time the dorsal *gsb* stripes decay, the *wg-gsb* autoregulatory loop may be restricted to the ventral region. Indeed, our analysis of *wg* (Fig. 7A,B) and *lbe* (Fig. 7D,E) expression patterns in *gsb*$^{-}$ embryos clearly shows that in the dorsal epidermis (Fig. 7A,D), both *wg* and *lbe* are *gsb*-independent. Furthermore, *wg* and *lbe* expression in *gsb* mutants, also persists in the epidermal domains of the head and tail, in particular, the labrum and anal plate (Fig. 7D,E). In contrast, in *wg*$^{-}$ embryos, *lbe* (Fig. 7C) and *lbl* (Fig. 7F) activity completely disappears from the epidermis, including the head segments and the anal plate. The central nervous system seems to be the only domain of *lb* expression which is only partially or not affected by *wg* loss of function (Fig. 7C,F). This observation suggests that, from about 6 hours AEL, maintenance of *wg* expression in the dorsal epidermis, does not involve *gsb* but may require other transcriptional regulators such as the *lb* gene products.

lb is required for late *wg* expression in the dorsal and terminal epidermis and for anal plate formation

In order to test the influence of *lb* activity on *wg* expression we used embryos homozygous for Df(3R)eF1 (Fig. 8F-J) that lacks both *lbe* and *lbl* gene products (Fig. 8H). These embryos were found to lack Wg protein in the labrum and anal plate (Fig. 8F) and to have reduced levels in the dorsal epidermis from 8 hours AEL (Fig. 8G). These domains correspond to the region of *gsb*-independent expression of *wg* and *lb* (Figs 5, 7). Consequently, Df(3R)eF1 embryos do not develop the anal plate (Fig. 8J) and display defects in the dorsal cuticle (Fig. 8I). The most affected region of the dorsal cuticle corresponds to the *wg*-dependent (Bokor and DiNardo, 1996) type 4° denticles (reduced number and abnormal pigmentation) but modifications appear also in type 3° cells. This cuticular phenotype is not detected in embryos carrying Df(3R)eGp4 (Fig. 8A-E) or Df(3R)GC14 (Mohler and Pardue, 1984, see Fig. 1A) which delete together the same genomic region but retain *bap* and *lb* loci. This suggests that the phenotype observed in Df(3R)eF1 embryos is due to a loss of either *bap* or *lb* function. A loss of *bap* function does not seem to be responsible for the cuticle phenotype of Df(3R)eF1, since a wild-type cuticle pattern was observed in *bap*^{208} embryos (not shown). That it is due to *lb* was shown by rescue experiments in which continuous expression between 4 and 9 hours AEL of *lbl* gene is sufficient to restore terminal *wg* expression (not shown) and anal plate formation (Fig. 8K). In the dorsal epidermis *wg* expression is restored partially (not shown), suggesting that in this region *wg* activity requires two *lb* gene products and cannot be fully rescued by one of them. Together this data indicates that *lb* genes maintain late *wg* expression in dorsal and terminal epidermis and that a *wg-lb* regulatory feedback loop is required for anal plate formation. The function of *wg*, late, in the dorsal epidermis is not fully understood, but whatever its role, *lb* genes

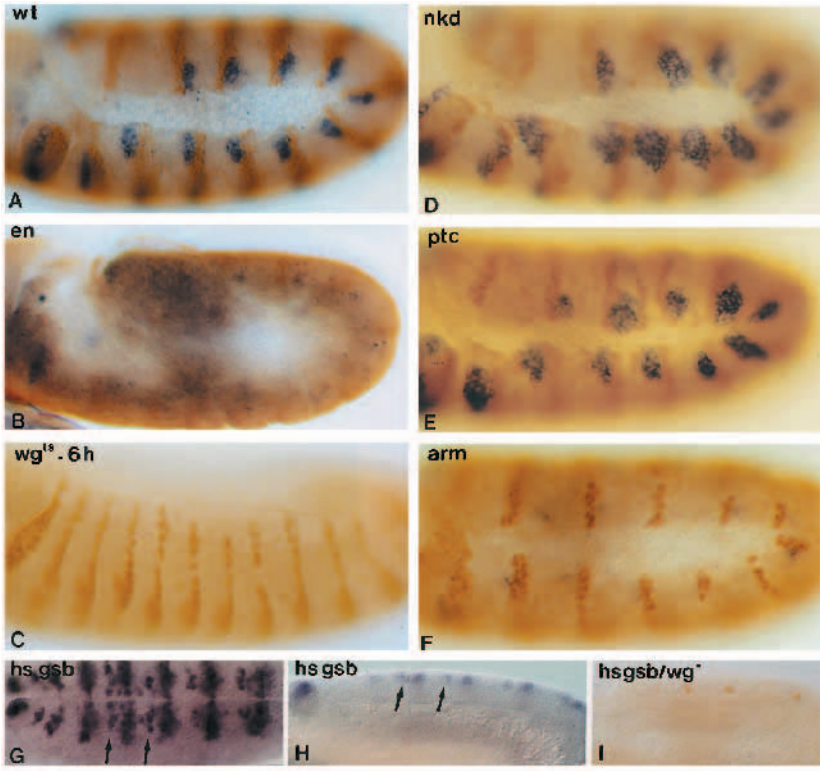

Fig. 6. Wg signal is required for *lbe* expression. (A-F) Lateral views of wild-type (A) and homozygous mutant embryos (B-F) hybridized with a Dig *lbe* probe (blue) and immunostained with an En antibody (brown). (B,D-F) stage 11 null mutant embryos: (B) en^{IIB86}, (D) nkd^{7H16}, (E) ptc^{IN108}, (F) *y* arm^{XK22} and (C) wg^{IL114} thermosensitive mutant embryo of late stage 12. The dorsal domains of *lbe* expression in *nkd* and *ptc* mutants follows ectopic expansion of *wg* domain and disappears in en^{-}, wg^{IL114} and arm^{-} embryos. (G-I) analysis of *hs-gsb* embryos displying ectopic *wg*-dependent expression of both *gsb* and *lbe* genes. (G) Ventral, and (H,I) lateral views of the terminal abdominal segments; (G,H) *hs-gsb*, (I) *hs-gsb/wg*$^{-}$ embryos stained for: (G) Gsb protein, (H) *lbe* RNA (I) Lbe protein. Ectopic Gsb (G) and *lbe* (H) stripes (arrows) induced by ubiquitous *gsb* expression are *wg*-dependent since they do not appear in wg^{-} context (I). Whole-mounts are oriented and photographed as in Fig. 5.

seem to be required for the broadening of the *wg* expression domain in this region during germ band retraction (Fig. 8B).

Ubiquitous expression of *lbe* and *lbl* causes ectopic *wg* expression in dorsal epidermis

Ubiquitous *lb* expression was induced by heat-shock treatment of transgenic embryos carrying *lbe*, *lbl* or both coding sequences under an hsp70 promoter (see Materials and methods). Since *lb* genes are required for late *wg* expression in dorsal epidermis (Fig. 8) we focused our analysis on this region. Triple heat-shock treatments (15 minutes each) administered between 4 and 9 hours AEL on control wild-type embryos had no effect on *lbe* (not shown) or *wg* expression (Fig. 9A) and dorsal cuticle pattern (Fig. 9B). The same treatment of *hs-lbe* embryos led to uniform *lbe* expression and induced ectopic activation of *wg* transcripts in dorsal epidermal cells (Fig. 9C, arrows). Moreover, *hs-lbe* embryos displayed abnormalities in the dorsal denticle pattern (Fig. 9D). Similar, although stronger, ectopic expression of *wg* (Fig. 9E) and cuticle alterations (Fig. 9F) were observed after simultaneous heat-shock induction of both *hs-lbl* and *hs-lbe* transgenes. The ectopic dorsal expression of *wg* is reminiscent of that induced by *hs-gsb* in the ventral region (Li and Noll, 1993). However, unlike the ventral cells after *hs-gsb* treatment, the dorsal cells do not form ectopic *wg* stripes. They are arranged anteriorly to the most lateral *wg* expressing cells from the dorsal stripes (Fig. 9C,E). The *hs-lb*

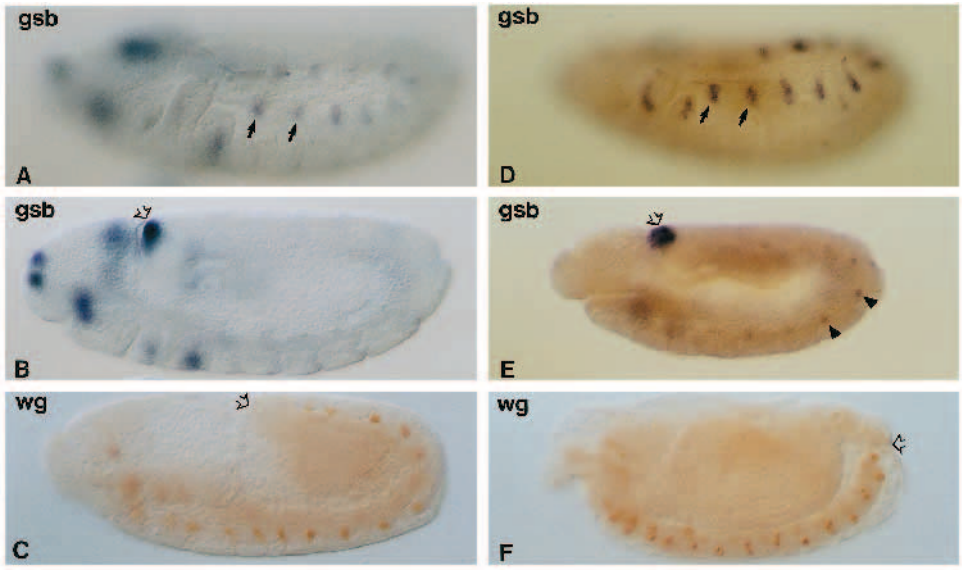

Fig. 7. *wg* and *lb* gene expressions in dorsal and terminal epidermis are *gsb*-independent. In *gsb*$^{-}$ embryos, *wg* and *lbe* expression is missing only from the ventral region, but is unaffected in dorsal epidermis (black arrows) and anal plate (open arrows). (A,B,D,E) Homozygous Df(2R) gsb^{IIX62} stage 11 embryos immunostained for Wg (A,B) or hybridized with *lbe* probe (D,E). The arrowheads point to *lb*-expressing cells in the CNS. (C,F) wg^{CX4} embryos during early (C) and late (F) germ band retraction stained for Lbe (C) or Lbl protein (F). Whole-mounts are oriented and photographed as in Fig. 5.

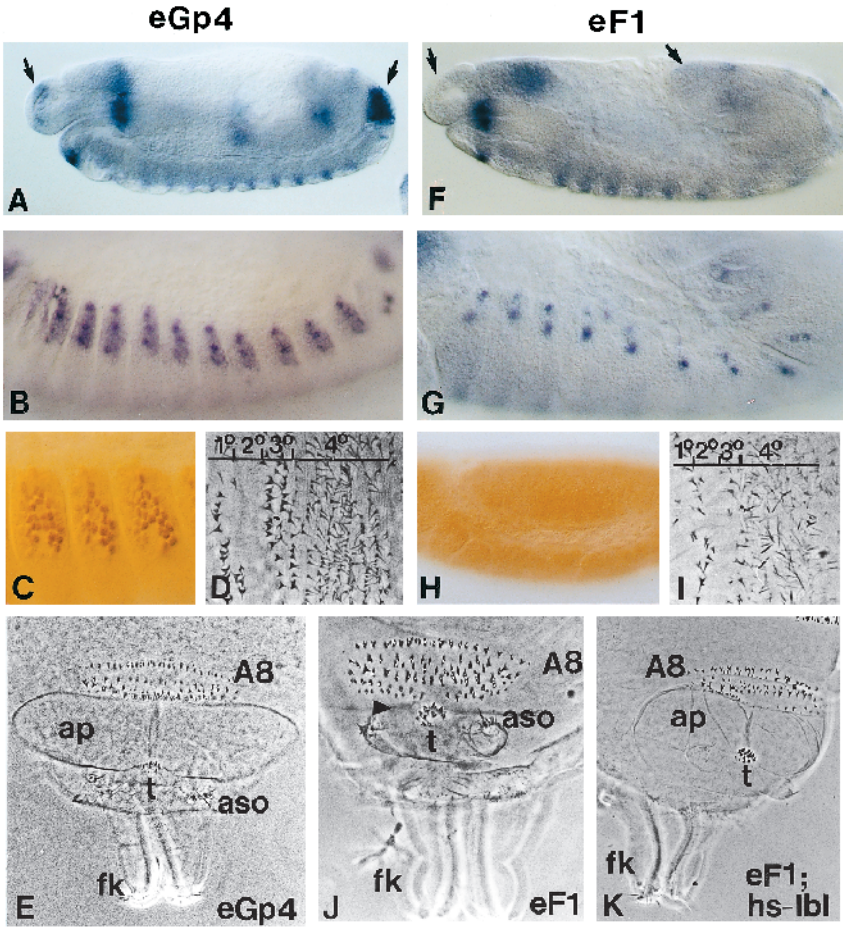

Fig. 8. *lb* gene activity maintains late *wg* expression in dorsal and terminal epidermis and is required for anal plate formation. Comparison of embryos homozygous for Df(3R)eGp4 (A-E) and Df(3R)eF1 (F-J) deficiencies with Df(3R)eF1/*hs-lbl* embryo after rescue experiment (K). (A,B,C) Late, (F,G) early stage 12 and (H) stage 11 whole-mount embryos immunostained (A,F,G) for Wg, (C) Lbe, (H) both Lb proteins and (B) hybridized with *wg* probe. (D,E,I,J) Phase contrast views of (D,I) dorsal and (E,J,K) tail cuticle. Late *wg* expression is missing from labrum and anal plate (arrows) and reduced in the dorsal region of Df(3R)eF1 (F,G), but not Df(3R)eGp4 (A,B) embryos. *lbe* and *lbl* gene activity is abolished in Df(3R)eF1 (H) but not in Df(3R)eGp4 mutation (C). Df(3R)eF1 embryos do not develop the anal plate (arrowhead in J) which is restored in Df(3R)eF1/*hs-lbl* embryos after heat-shock induction of *lbl* activity (K). In addition, Df(3R)eF1 embryos have defects of *wg*-dependent type 4° denticles as well as changes in type 3° hairs in dorsal cuticle (I). In Df(3R)eGp4 embryos, the cuticular pattern of dorsal (D) and terminal (E) regions is unaffected. According to Bokor and DiNardo, (1996) a particular shape of secreted dorsal cuticle, labeled 1° to 4°, depends on the position of epidermal cells within the segment. The dorsal epidermal domains of *wg* (B) and *lbe* (C) broaden during germ band retraction up to the segmental borders. In (A-D) and (F-I) anterior is left, whereas in E,J anterior is up. A8, abdominal segment 8; ap, anal pads; aso, anal sensory organ; fk, filtzkorper; t, tuft.

dorsal cuticle is affected in the region overlapping the segmental border row of cells (row 1°) and cells just posterior to the row 1° (for a definition of cell types see Bokor and DiNardo, 1996). We observe that smooth cuticule adjacent to the row 1° is absent and fate of type 3° cells altered and difficult to distinguish from that of type 1° (Fig. 9D,F). This cuticle phenotype of *hs-lb* embryos may result from ectopic expression of *wg* and in consequence from alterations in Hh signaling.

DISCUSSION

In this report we show that *lbe* and *lbl*, members of the 93E homeobox gene cluster, code for highly related nuclear proteins. They play an important role in tail development and maintain late *wg* expression in dorsal epidermis. The high similarity of the predicted DNA-binding domains strongly suggest that *lbe* and *lbl* may recognize common target sequences and collaborate in the regulation of downstream genes.

Wg is required for activation and maintenance of *lb* expression

Although *lbe* and *lbl* genes have a common epidermal expression domain, the *lbe* transcripts appear earlier and are more abundant. Like the majority of segment polarity genes, *lb* genes display specific expression in the underlying CNS. In contrast to the segment polarity genes which are activated at the blastoderm stage (for review see Perrimon, 1994), epidermal expression of *lb* genes appears later at the extended germ band stage. We have found a striking similarity between the expression patterns of *wg* (Baker, 1987; Ingham and Hidalgo, 1993; van den Heuvel et al., 1993) and *lbe* gene. The expression domains coincide in the terminal regions of the body (labrum and anal plate), and are both restricted to laterally interrupted stripes in the trunk. The *wg* and *lb* epidermal expression domains differ in the most posterior abdominal segments (A8 and A9) where *lb* activity is absent in the dorsal cells. In an attempt to understand the regulatory events that direct *lbe* gene expression we have analysed a set of embryos carrying mutations affecting *wg* function. We found, that embryos lacking functional products of *wg*, *en* and the other components of Wg and En/Hh signaling pathways (Perrimon, 1994) show misexpression of the *lb* genes that follows changes in *wg* pattern. As determined by analysis of *hs-gsb* and *hs-gsb/wg*$^{-}$ embryos, the induction of ectopic *lbe* gene expression is *wg* dependent and cannot be detected in *wg*$^{-}$ context. Since Arm, a component of Wg signaling (Perrimon, 1994), is required for *lb* activity, we conclude that the Wg signal activates and maintains the epidermal expression of *lb* genes.

wg and *lb* genes form an autoregulatory loop in the embryonic tail and dorsal epiderm

Embryos carrying a null mutation of both *lb* genes, do not show *wg*-like cuticle phenotype in the ventral epidermis, probably because *wg* expression in this region is maintained by *gsb* (Li and Noll, 1993) and does not require *lb* function. However, the anal plate does not develop and dorsal cuticle shows alterations

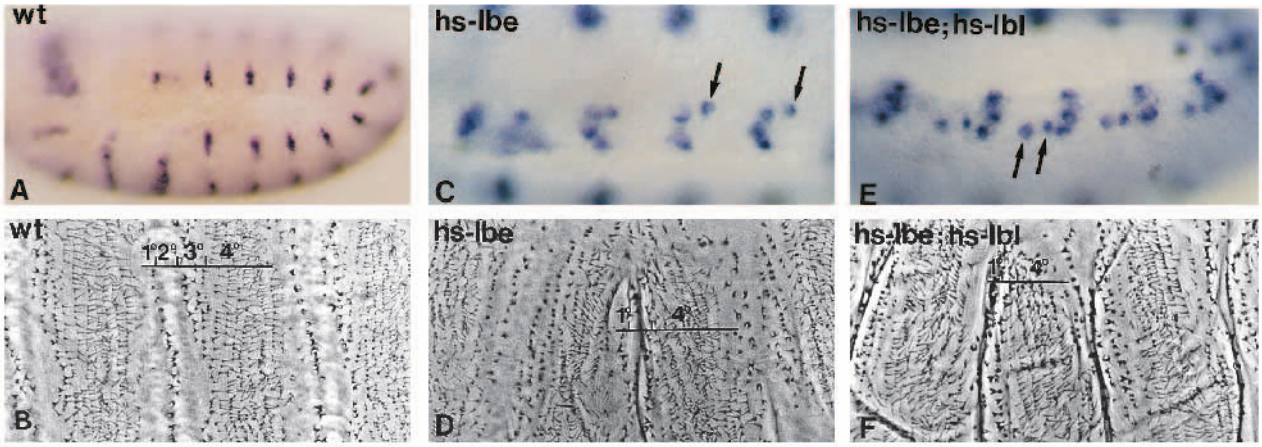

Fig. 9. Ectopic dorsal *wg* activity and defects in dorsal cuticle pattern are induced by ubiquitous expression of *lb* genes. Uniform *lbe* and *lbl* expression was maintained between 4 and 9 hours AEL by 3 heat-shocks of 15 minutes. (A,B) Wild-type w^{1118}, (C,D) *hs-lbe* and (E,F) *hs-lbe/lbl* transgenic embryos. In heat-shock treated wild-type embryos, neither *lbe* (not shown) or *wg* (A) expressions, nor cuticular pattern (B) were affected. Uniform expression of *lbe* or both *lb* genes was found to recruit new dorsally located *wg* expressing cells (arrows in C and E) and defects in dorsal denticle pattern (D,F). Dorsal cell types 1°-4° are as in Bokor and DiNardo (1996).

of *wg*-dependent hairs (Bokor and DiNardo, 1996). In these regions, Wg protein decays suggesting a mutual requirement for *lb* and *wg* genes. The *wg-lb* regulatory feedback loop in the anal plate (Fig. 10) appears at the same time (about 6 hours AEL) as the *wg-gsb* loop in the ventral epidermis (Li and Noll, 1993) and seems to supply the information required to specify the anal plate cells from the non-differentiated epidermal tail cells. *lbe* and *lbl* gene activity in the anal plate is likely to be activated by the homeotic gene *fork head (fkh)* (Weigel et al., 1989) which governs terminal development. Alternatively, the homeobox gene *caudal (cad)* required for the anal pads, tuft and anal sense organ formation (Macdonald and Struhl, 1986), could be part of the genetic circuitry that switches on the *wg-lb* autoregulatory loop in the terminal region.

Mutual activation of *lb* and *wg* genes was observed at distinct times in the anal plate and dorsal epidermis (Fig. 10). In the dorsal epidermis, *wg* becomes dependent on *lb* genes during germ band retraction (at about 8 hours AEL). Temporal asymmetry between the appearance of the *wg-gsb* autoregulatory loop in the ventral epidermis (Li and Noll, 1993) and that of *wg-lb* in the dorsal epidermis suggests that other factors, as yet unknown, may maintain *wg* expression in dorsal epidermis between 6 and 8 hours AEL. Since the late *wg* function (after 9 hours AEL) in the dorsal region is unknown, we can only speculate about a role for the *lb-wg* autoregulatory loop in this region. The most attractive possibility is that the late Wg signal in the dorsal epidermis, like the early one (Wu et al., 1995), is required for differentiation of the underlying heart mesoderm. This hypothesis seems to be supported by the observation that *lb-wg* interactions are restricted only to the seven abdominal segments in which heart develops. Following the *wg-lb* regulatory interactions, the dorsal *lb*-dependent *wg* expression domain broadens anteriorly and posteriorly, suggesting self-propagation of the *lb-wg* autoregulatory loop. In this case, the secreted Wg protein (van den Heuvel et al., 1993) might activate *lb* in neighbouring cells where *lb* gene products, in turn, may switch on *wg* expression. In contrast, the ventral *wg* expression domain, maintained by the *wg-gsb* autoregulatory loop, is restricted anteriorly by the repressing activity of *ptc*, and posteriorly the activity of *en* (Li and Noll, 1993). As a consequence of this dorsal *wg* expansion, only cells at the segment boundaries do not express both *wg* and *lb* genes. The ectopic expression of Lb

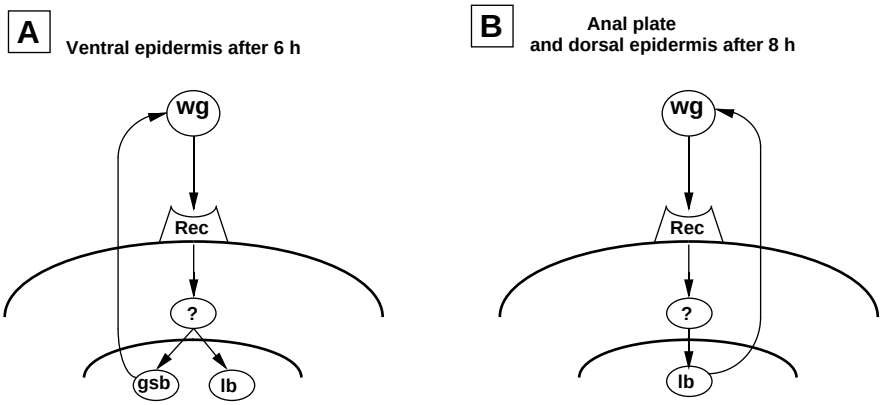

Fig. 10. Graphic representation of the *wg-lb* interactions in embryonic epidermis. (A) In ventral epidermis (after 6 hours AEL) a *wg-gsb* autoregulatory loop is required to initiate and maintain *lb* expression. Since *gsb* cannot initiate *lb* expression in wg^{-} embryos, *lb* activity is dependent on Wg signaling. (B) In the anal plate and dorsal epidermis, *wg* is still required for *lb* expression but its own activity is *gsb*-independent. Instead, in these regions the homeodomain-containing Lb proteins maintain *wg* expression by a regulatory feedback loop. The *wg-lb* autoregulatory loop in the anal plate appears earlier than in dorsal region and results from distinct regulatory events specific for terminal regions. In the dorsal epiderm the *wg-lb* interactions occur later (about 8 hours AEL) and appear to promote self-propagation of both *lb* and *wg* domains.

proteins in these segment boundary cells, after heat-shock induction, results in severe defects of the dorsal cuticle pattern.

The significance of *lb* gene duplication in *Drosophila*

The tandem organisation of *lb* genes appears to be specific for *Drosophila* and was not evolutionarily conserved, since in mouse and human we detected only one orthologous *lb* gene per locus (Jagla et al., 1995). This could indicate a particular protection for developmental decisions involving these two Lb proteins in *Drosophila*. Here we show that *lbe* and *lbl* are related by their structure and almost identical expression patterns. Our analysis of phenotypes generated by the ubiquitous expression of both *lb* genes (unpublished observations) and rescue experiments in which *lbl* gene product was sufficient to replace two lacking Lb proteins have led to the conclusion that *lbe* and *lbl* are functionally redundant. As a consequence of this redundancy, we failed to identify null *lbe* and *lbl* alleles among lethal 93E EMS (Azpiazu and Frasch, 1993) and insertional P-element mutants (Spradling et al., 1995). In this report, we focused our analysis on the epidermal *lb* functions and showed that they are required for late Wg signaling in the anal plate and dorsal epidermis. Although tail development requires *lb* function, we presume that *lb* genes are also involved in differentiation of mesodermal and CNS lineages in which they are specifically expressed. This aspect remains to be investigated.

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