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

Krzysztof Jagla, Teresa Jagla, Pascal Heitzler, Guy Dretzen, François Bellard and Maria Bellard*

Institut de Génétique et de Biologie Moléculaire et Cellulaire, CNRS/INSERM/ULP, Collège de France, B.P. 163, 67404 Illkirch Cedex, C.U. de Strasbourg, France

*Author for correspondence

**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 (Wildier 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*, *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 AZap 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 pSGS-*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-Evenant antibodies. Stained embryos were dehydrated, mounted in Canada balsam and photographed using a Nomarski 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 EcoRI site of the P-element vector pCaSpeR-hs and injected into *w1118* embryos according to standard procedure (Rubin and Spradling, 1982). From several transformants, the hs-*lbe*4A and hs-*lbl*5/I insertions on chromosone X were used in most experiments. Double hs-*lbe*-hs-*lbl* transgenic flies were generated by recombination of hs-*lbe*-transgenes on hs-*lbl*-transgene 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 *w1118* (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*Δ1144 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*Δ20, *en*Δ880 and hypomorphic eveΔD10 were kindly provided by W. Gehrng (Biozentrum, Basel), *hh*Δ35, *ptc*ΔJN108, *wg*ΔX4, *wg*Δ114, *nkd*ΔH16, Df(2R)gsbΔIX62, and *y arm*Δ22 mutants were obtained from the Tubingen stock center. Df(2R)gsbΔIX62 (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 Bloomington stock center. *bap*Δ08 and *tin*C40 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 are inactivated by the Df(3R)eF1 deletion.
Fig. 1. Molecular organization of the \( lbe \) locus. (A) Chromosomal extents of four deficiencies from the 93 region (Mohler and Pardue, 1984). (B) Chromosomal walk along the \( lbe \) locus and EcoRI 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 (Dohmann et al., 1990). Arrows indicate the directions and the extents of 93E gene transcripts. Black boxes, within \( lbe \) and \( lbl \) coding sequences (numbered from 1 to 3) correspond to the regions amplified by PCR. Exon/intron organization of the \( lbe \) genes is depicted below the restriction map. The \( \lambda \) 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 \( lbe \) 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 \( lbe \) 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. 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 homeo-domain in the carboxy-terminal part and two poly-Ala stretches on either side of the protein (Fig. 2A,B). The lbe cDNA clones lbi20 (1477 bp), lbi5 (1867 bp) and lbi6 (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 (LbH6) differs from the others at the carboxy-terminal region (Fig. 2B). The common features of lbi 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 lbi 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 lbi 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 lbi transcripts (not shown) and Lbl protein (Fig. 4G,H). This is consistent with a spatial distribution of lbi 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 lbi gene expression takes place between 5 and 7 hours AEL, although the most prominent region of lbe activity remains the anal plate (Fig. 4C,E,I,K). At about 5 hours AEL, both lbe and lbi 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 lbe 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 lbe genes is detected in the dorsal epidermal cells of the most posterior abdominal (A8 and A9) segments (Fig. 4C,F,L). At the late extended germ band stage lbe transcripts also appear in the ventral, but not the lateral epidermis (Fig. 4D,E). These ventral lbe stripes are weaker than dorsal patches (Fig. 4D) and in the case of lbi 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 lbi 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, lbe 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 lbe and wg genes are co-expressed in the trunk epidermis and in the terminal regions of the body**

The distribution of lbe genes products in the epidermis of the trunk is particularly reminiscent of the late wg expression pattern. To localise more precisely the lbe 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 lbe expression that overlaps the cells expressing wg and gsb, located just anteriorly to the cells expressing en. The posterior-most row of lbe-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 lbi (not shown) remain co-expressed with wg. In addition to the expression in the trunk, we also observed common epidermal domains of lbe and wg activity in the...
Epidermal function of the ladybird genes

95

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 wg1114 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
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 lbe expression.

**gsb activity maintains both wg and lbe 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 lbe 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 lbe gene products.

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

In order to test the influence of lbe 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 lbe (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)eGC14 (Mohler and Pardue, 1984, see Fig. 1A) which delete together the same genomic region but retain bap and lbe loci. This suggests that the phenotype observed in Df(3R)eF1 embryos is due to a loss of either bap or lbe 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 lbe was shown by rescue experiments in which continuous expression between 4 and 9 hours AEL of lbe 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 lbe gene products and cannot be fully rescued by one of them. Together this data indicates that lbe genes maintain late wg expression in dorsal and terminal epidermis and that a wg-lbe 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, lbe genes

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**Fig. 5.** Co-expression of lbe, wg and gsb genes in the embryonic epidermis. Both lbe genes are co-expressed with wg in dorsal and ventral epidermis and in terminal regions of the body. lbe-gsb co-expression is limited to ventral stripes. The positions of lbe-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 lbe activity. To further confirm the dependence of lbe 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).
Epidermal function of the ladybird genes

Seem to be required for the broadening of the \( \text{wg} \) expression domain in this region during germ band retraction (Fig. 8B).

**Ubiquitous expression of lbe and lbl causes ectopic \( \text{wg} \) expression in dorsal epidermis**

Ubiquitous \( \text{lb} \) expression was induced by heat-shock treatment of transgenic embryos carrying \( \text{lbe}, \text{lbl} \) or both coding sequences under an hsp70 promoter (see Materials and methods). Since \( \text{lb} \) genes are required for late \( \text{wg} \) 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 \( \text{lbe} \) (not shown) or \( \text{wg} \) expression (Fig. 9A) and dorsal cuticle pattern (Fig. 9B). The same treatment of \( \text{hs-lbe} \) embryos led to uniform \( \text{lbe} \) expression and induced ectopic activation of \( \text{wg} \) transcripts in dorsal epidermal cells (Fig. 9C, arrows). Since \( \text{lb} \) genes are required for late \( \text{wg} \) 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 \( \text{lbe} \) (not shown) or \( \text{wg} \) expression (Fig. 9A) and dorsal cuticle pattern (Fig. 9B). The same treatment of \( \text{hs-lbe} \) embryos led to uniform \( \text{lbe} \) expression and induced ectopic activation of \( \text{wg} \) transcripts in dorsal epidermal cells (Fig. 9C, arrows). Moreover, \( \text{hs-lbe} \) embryos displayed abnormalities in the dorsal denticle pattern (Fig. 9D). Similar, although stronger, ectopic expression of \( \text{wg} \) (Fig. 9E) and cuticle alterations (Fig. 9F) were observed after simultaneous heat-shock induction of both \( \text{hs-lbl} \) and \( \text{hs-lbe} \) transgenes. The ectopic dorsal expression of \( \text{wg} \) is reminiscent of that induced by \( \text{hs-gsb} \) in the ventral region (Li and Noll, 1993). However, unlike the ventral cells after \( \text{hs-gsb} \) treatment, the dorsal cells do not form ectopic \( \text{wg} \) stripes. They are arranged anteriorly to the most lateral \( \text{wg} \) expressing cells from the dorsal stripes (Fig. 9C,E). The \( \text{hs-lb} \)
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 lb1 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 lb1 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, filtzkörper; 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 cuticle 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 lb1, 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 lb1 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 lb1 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 lb 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 lb 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
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
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 lbi 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 lbi are functionally redundant. As a consequence of this redundancy, we failed to identify null lbe and lbi 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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