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Analysis of the *gooseberry* locus in *Drosophila* embryos: *gooseberry* determines the cuticular pattern and activates *gooseberry neuro*

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SUMMARY

The segment-polarity class of segmentation genes in *Drosophila* are primarily involved in the specification of sub-segmental units. In addition, some of the segment-polarity genes have been shown to specify cell fates within the central nervous system. One of these loci, *gooseberry*, consists of two divergently transcribed genes, *gooseberry* and *gooseberry neuro*, which share a paired box as well as a *paired*-type homeobox. Here, the expression patterns of the two *gooseberry* gene products are described in detail. The gooseberry protein appears in a characteristic segment-polarity pattern of stripes at gastrulation and persists until head involution. It is initially restricted to the ectodermal and neuroectodermal germ layer, but is later detected in mesodermal and neuronal cells as well. The gooseberry neuro protein first appears during germ band extension in cells of the central nervous system and also, much later, in epidermal stripes and in a small number of muscle cells. P-element-mediated transformation with the *gooseberry* gene has been used to demonstrate that *gooseberry* transactivates *gooseberry neuro* and is sufficient to rescue the *gooseberry* cuticular phenotype in the absence of *gooseberry neuro*.

Key words: *gooseberry*, *gooseberry neuro*, transactivation, *gooseberry* rescue, embryonic expression, *Drosophila*

INTRODUCTION

During early *Drosophila* embryogenesis, a number of developmental programs unfold, including segmentation, the generation of the germ layers, and neurogenesis. While segmentation may be viewed as a process beginning with the specification of position along the anteroposterior axis of the embryo (reviewed by Akam, 1987; Ingham, 1988), the germ layers are established by the division of the embryo, along the dorsoventral axis, into longitudinal regions (Hartenstein et al., 1985; Mayer and Nüsslein-Volhard, 1988; reviewed by Govind and Steward, 1991). Later, during germ band extension, the process of neurogenesis begins. The founder cells of the central nervous system (CNS), the neuroblasts, begin to delaminate from the neuroectoderm and migrate inwards (Hartenstein and Campos-Ortega, 1984; Hartenstein et al., 1987). Most neuroblasts divide asymmetrically several times to generate a string of progeny known as ganglion mother cells. Each ganglion mother cell then divides once symmetrically to generate a pair of sibling neurons. Thus, the approximately twenty neuroblasts per hemisegment that leave the ectoderm give rise to about 250 neurons. During germ band retraction, the CNS continues to differentiate and neurons send out their axons (Goodman et al., 1984).

From a genetic and molecular analysis of these early developmental events, it has become increasingly clear that they are directed by relatively small groups of genes, which interact with each other in complex hierarchical regulatory networks. For example, the segmentation genes direct the proper establishment of the metameric organization of the embryonic body plan. The hierarchical activation of three classes of segmentation genes - the gap, pair-rule, and segment-polarity genes - defines position along the anteroposterior axis in progressively smaller units (Nüsslein-Volhard and Wieschaus, 1980). Similarly, a set of hierarchically acting genes has been described, which controls dorsoventral patterning and thus determines the anlagen of the germ band. Subsequently, the proneural and neurogenic gene sets specify which cells become neuroblasts and which remain on the surface of the embryo and become epidermal cells (reviewed by Campos-Ortega and Knust, 1990).

Interestingly, some of the genes involved in segmentation are redeployed in other developmental processes such as neurogenesis (reviewed by Doe and Scott, 1988). For example, many of the segment-polarity genes are expressed in the neuroectodermal region at the onset of neurogenesis and are also expressed later by subsets of neurons. Detailed analysis of neural development in segment-polarity mutants suggests that certain segment-polarity genes indeed play a specific role in neurogenesis (Patel et al., 1989a).

One of the segment-polarity loci, *gooseberry*, is unique

for several reasons. It encodes two transcripts that share extensive sequence homology with each other and with the pair-rule gene *paired* (*prd*). The homologous regions comprise two domains, the paired-domain and the *prd*-type homeodomain (Bopp et al., 1986). Moreover, the 5′ ends of the two *gooseberry* transcription units face each other, being separated by about 10 kb (Baumgartner et al., 1987; Li et al., 1993), raising the intriguing possibility that both transcripts share common *cis*-regulatory elements. Finally, two independent mutagenesis screens failed to produce point mutations for either of the two *gooseberry* genes (Nüsslein-Volhard et al., 1984; Côté et al., 1987). All known *gooseberry* mutants are the result of deficiencies. If indeed point mutations of the *gooseberry* locus cannot be obtained, an explanation might be that either of the two *gooseberry* products functionally substitutes for the other.

Here we report, in detail, the developmental expression of the two *gooseberry* genes, *gooseberry* (*gsb*; previously called *gsb-BSH9* or *gsb-d*) and *gooseberry neuro* (*gsbn*; the former *gsb-BSH4* or *gsb-p*). In addition, we demonstrate that *gsb* is sufficient to rescue fully the *gooseberry* cuticular phenotype and that *gsb* activates *gsbn* in *trans*.

MATERIALS AND METHODS

Construction of expression and rescue plasmids

Plasmids expressing gsb or gsbn protein, pAR-gsb.fl and pAR-gsb-neuro.fl, in bacteria were constructed as follows. To obtain pAR-gsb.fl, an *Eco*RV-*Eco*RI *gsb*-cDNA fragment of BSH9c2 (Baumgartner et al., 1987) was subcloned with blunt ends into the *Bam*HI site of the bacterial expression vector pAR3039 (Studier and Moffat, 1986). Since the *Eco*RV site of BSH9c2 is 40 bp downstream of the *gsb* start codon, the bacterially expressed gsb protein lacks the 15 N-terminal amino acids of the full-length gsb protein (427 amino acids). To obtain pAR-gsb-neuro.fl, a *Nco*I-*Nsi*I fragment of the *gsbn*-cDNA BSH4c4 (Baumgartner et al., 1987) was subcloned with blunt ends into the *Bam*HI site of the bacterial expression vector pAR3040. As the *Nco*I site in BSH4c4 contains the start codon of the gsbn protein, the bacterially expressed protein contains the full-length gsbn protein (452 amino acids).

The P-element plasmid containing the *gsb* gene, gsb-pKSpL2, was constructed by subcloning a 20 kb genomic fragment of the *gsb* region (Fig. 6B), obtained from a partial *Eco*RI digest of the genomic clone P920 (in EMBL 4), into pKSpL2. The vector pKSpL2 was constructed as follows. The *Not*I site of Bluescript pKS^+ was destroyed by filling in the cleaved ends with Klenow enzyme and subsequent religation, a short stretch of the polylinker between *Hin*dIII and *Xho*I was removed (ligation of the filled up sites restores the *Hin*dIII site), and a *Not*I site was introduced into the cleaved *Eco*RV site of the polylinker by blunt end ligation of (GCGGCCGC). The newly created polylinker was confirmed by sequencing. The final *gsb* rescue plasmid, BSH9-16.18, was constructed in two steps. First, a 17 kb *Xba*I-*Not*I fragment of gsb-pKSpL2 was subcloned into cp20.2, which had been constructed by removing the *Kpn*I-*Sal*I *lacZ* fragment from HZ50pL (Hiromi et al., 1985), and second, the 3.1 kb *Xba*I fragment of gsb-pKSpL2 was inserted to generate BSH9-16.18.

Preparation of purified antisera and immunocytochemistry on whole-mount embryos

Rabbit antisera were generated and purified essentially as described previously for the anti-prd antiserum (Gutjahr et al., 1993) with the following modifications. Both antisera were directed against the full-length proteins and cross-reacted on western blots with bacterially expressed gsb and prd proteins. The anti-gsb (anti-gsbn) antiserum was depleted of such cross-reactive antibodies by passing it over a column to which a crude bacterial extract containing gsbn (gsb) protein had been bound. Subsequently, the antisera were further affinity-purified (positive adsorption) as described previously for the anti-prd antiserum (Gutjahr et al., 1993). The specificity of both antisera was confirmed by staining embryos homozygous for the deficiency *Df(2R)IIX62*, which removes both *gsb* genes. In these embryos, no staining was observed using either antiserum (not shown). The specificity of the anti-gsb antiserum was further corroborated by staining embryos homozygous for the deficiency $Df(2R)Kr^{SB1}$, which removes only the *gsb* gene (Bopp et al., 1986; Côté et al., 1987). In such embryos, *gsbn* was expressed at high levels in the head region but only at extremely low levels in very few cells of the CNS whereas no staining was detected using the gsb antiserum. Finally, the specificity of the gsbn antiserum is inferred from the fact that during gastrulation, when *gsb* is expressed at high levels, no staining was seen with the anti-gsbn antiserum. Staining of fixed embryos with 100-fold diluted anti-gsb and anti-gsbn antisera and photography on a Zeiss Axiophot with Nomarski optics (unless otherwise indicated) were as described (Patel et al., 1989b; Gutjahr et al., 1993).

Embryos of a *wingless lacZ*-enhancer trap line, *17en40/CyO* (kindly provided by Norbert Perrimon), were stained with mouse anti-β-galactosidase (Promega) and either anti-gsb or anti-en antiserum (mAb 4D9; Patel et al., 1989b) to determine the relative positions of the wingless (wg), engrailed (en), and gsb domains in the ectoderm and in neuroblasts. The relationship of wg and en domains in the neuroblast map (Fig. 4) was also checked by examining embryos that had been both hybridized in situ with a digoxigenin-labeled *wg* probe and immunostained for en expression and were kindly provided by Armen Manoukian. The relative positions of en and gsb or gsbn protein were also determined by double labeling embryos with mAb 4D9 and anti-gsb or anti-gsbn antiserum.

Neuroblast patterns were initially sketched by hand and are schematically illustrated in Fig. 4. The particular stages illustrated were chosen because they are easily recognizable. The stage in Fig. 4A is characterized by the appearance of the first row 6 neuroblast, and the stage shown in Fig. 4B by the appearance of the extremely medially located row 5 neuroblast. We note that the general neuroblast patterns shown in Fig. 4 closely match those drawn by Doe (1992). In some cases, however, we are not sure of a one-to-one correspondence between specific neuroblasts in our map and the numbered neuroblasts of Doe's map. Therefore, we have not attempted to use Doe's numbering system at present. Future double-labeling experiments using the additional neuroblast markers described by Doe (1992) should allow a precise integration of the two maps.

Transgenic fly stocks

To generate transgenic flies, the *gsb* rescue plasmid was injected into ry^{506}/ry^{506} embryos. Four independent transformed lines were obtained from which the following genotypes were generated: $Df(2R)Kr^{SB1}/CyO$; $P[ry^+; gsb^+]$ and *Df(2R)IIX62/CyO*; $P[ry^+; gsb^+]$. Eggs of these lines or from crosses of these lines with each other were collected and either fixed for antibody staining or allowed to age for 48 hours for cuticle preparation.

RESULTS

Both gsb and gsbn proteins are localized in the nucleus, as is the case for other paired- and homeodomain containing

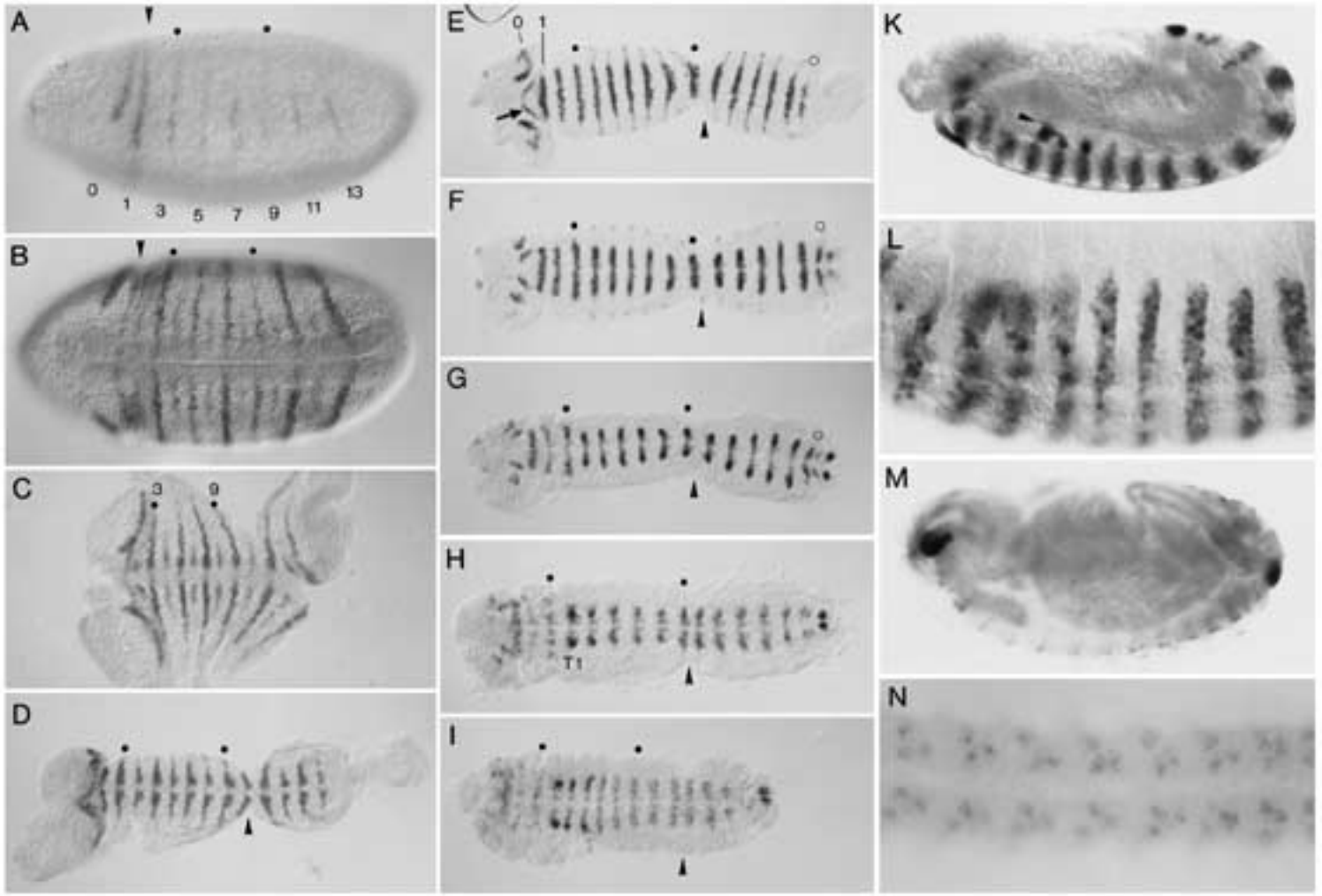

Fig. 1. Expression of gsb protein in wild-type *Drosophila* embryos. Whole-mount preparations of wild-type embryos were stained with anti-gsb antiserum. After clearing in glycerol, germ band extended embryos (C-I) were cut along the amnioserosa, unfolded and flattened so that the entire germ band could be photographed in a single focal plane. Stages of embryos are (A) onset of gastrulation (stage 6; Campos-Ortega and Hartenstein, 1985); (B) mid-gastrulation (stage 7); (C,D) rapid phase of germ band extension (early and late stage 8); (E) slow phase of germ band extension (stage 10); (F-H) extended germ band stage (early, mid, and late stage 11), (I-L) mid-germ band retraction (stage 12), and (M, N) head involution (stage 14). Whole mounts show lateral (A,K,M), ventrolateral (L), or ventral views (B,N). All embryos are oriented with their anterior to the left and, in the lateral or ventrolateral views (A,K-M), dorsal side up. The antennal stripe 0 and the odd-numbered stripes 1-13 are labeled in panel A. The stripes are numbered according to the corresponding RNA stripes (Baumgartner et al., 1987), applying the same system used to number the en stripes (DiNardo and O'Farrell, 1987) - stripe 1 is in the mandibular segment, stripe 4 in the first thoracic segment T1, stripe 14 in the eighth abdominal segment A8, etc. The arrow in E points to the intercalary *gsb* stripe appearing during the fast phase of germ band extension. The dots mark stripes 3 and 9, and stripe 15 is labeled by an open circle. Note that stripe 16 appears (G) after stripe 17 (F). Arrowheads indicate the position of the cephalic furrow (A,B), the most posterior region of the embryo prior to unfolding (D-I), or three spots of late mesodermal *gsb* expression (K). K and M are focused on the CNS whereas L, which shows a higher magnification of an embryo at a stage similar to that shown in K, is focused on the epidermis. N shows an enlarged ventral view of the CNS. Note that at the stage shown in L, gsb protein is expressed at low levels in epidermal cells of the posterior portion of each segment and strictly limited in its lateral extent. To reveal very faint staining which would not have been visible otherwise, the preparations in M and N were photographed in bright-field illumination, in contrast to all other preparations which were photographed with Nomarski optics.

proteins, and are expressed in a typical, segmentally reiterated, segment-polarity pattern (Figs 1, 2). Although *gsb* and *gsbn* expression overlap, the two proteins predominate at different stages of development and in different tissues.

Expression of *gooseberry*

The gsb protein is initially expressed in a segmentally reiterated pattern of stripes with a pair-rule modulation of intensity. The first set of stripes is detectable at the end of cellularization and includes the odd-numbered stripes 1-13 plus an anterior stripe 0 (Fig. 1A) that probably corresponds to the antennal segment of the head (Jürgens et al., 1986). The antennal stripe and stripe 1 are the first to appear, followed after a short delay by stripes 3, 13, 7, 11, and finally 5 and 9. At mid gastrulation, the even-numbered stripes 2-12 emerge simultaneously (Fig. 1B). Stripe 14 appears at the onset of germ band extension (Fig. 1C,D). All the stripes quickly reach equal levels during the rapid phase of germ band extension (Fig. 1D). At the same time, the shape of the stripes changes, acquiring a distinct triangular appearance.

Towards the end of germ band extension, stripes 4-14 become laterally restricted to the neuroectodermal portion of the ectoderm (Fig. 1E,F). At this stage, gsb protein reaches its highest levels and is detectable in the maximum number of segments, including 14 body stripes, 4 regions anterior to the mandibular segment, and 3 regions posterior to the eighth abdominal segment (Fig. 1F,G). The gsb stripes assume a barbell shape as the more medial areas of expression narrow. This exposes a pair of distinct *gsb*-expressing cells which are located close to the mesectodermal region (Fig. 3G) and are probably the most medial neuroblasts of row 5 (Fig. 4B). Subsequently, gsb protein levels begin to decrease in the head and later in the trunk segments (Figs 1G,H, 3D), and gsb stripes broaden at the end of the extended germ band stage and during germ band retraction (Fig. 1H,I). Towards the end of germ band retraction, *gsb* expression increases again in the ectoderm (Figs 1L, 3E) albeit to levels lower than the preceding peak of ectodermal *gsb* expression. This low ectodermal *gsb* expression (stage 13) is no longer detectable by the time of head involution (stage 14).

During germ band extension (stage 9), neuroblasts begin to delaminate and the gsb ectodermal stripes narrow (compare widths of stripes in Fig. 1D,E). Those *gsb*-expressing ectodermal cells that become neuroblasts maintain *gsb* expression. Eventually, all neuroblasts of row 5 and 6 express *gsb*, and transient *gsb* expression is also seen in the most medial neuroblast of row 7 (Fig. 4B). In addition, *gsb* appears to be weakly and transiently expressed by three midline cells directly anterior to the median neuroblast (Fig. 4B). Based on their position, they may be the precursors to the VUM neurons (Klämbt et al., 1991). Expression of *gsb* persists at low levels in a few neuroblasts and ganglion mother cells until germ band retraction (Fig. 3D,E). Very low levels of gsb protein also remain detectable until head involution in large cells at the extreme ventral surface of the CNS, which may be the remnants of the embryonic neuroblasts (Fig. 1M,N).

It should be noted that during its initial expression, gsb protein is mostly excluded from the mesoderm, the mesectodermal cells and the region generating the amnioserosa (Fig. 1A,B). At mid germ band extension, gsb protein appears in the mesoderm (Fig. 3A,B) where it seems to persist until the end of germ band retraction (Fig. 3C-E) when it is most prominent in three patches of mesodermal cells in the thoracic segments (Fig. 1K). Later these patches appear to merge into a single patch, before *gsb* expression disappears from the thoracic mesoderm during head involution.

The patterns of *gsb* expression in the tail and, particularly, in the head region are more complex. At mid germ band extension, the antennal stripe divides into two independent regions, and a novel stripe, intercalated between the antennal stripe 0 and the mandibular stripe 1 and corresponding to the anlagen of the intercalary segment (Jürgens et al., 1986), begins to express gsb protein (Fig. 1D,E). During the slow phase of germ band extension, a bilaterally symmetric pair of patches expressing *gsb* emerges in the dorsal region of the clypeolabrum, and a small number of cells express *gsb* in the non-segmented pre-antennal region of the head (Fig. 1E,F). Expression in the posteriormost abdominal region starts with the appearance of stripe 15 in A9 at the end of the rapid phase of germ band extension (Fig. 1E). Subsequently, *gsb* expression begins in 'stripe' 17 (Fig. 1F) as a pair of bilateral patches of cells in the central region of the anal pads (A11; Jürgens, 1987). Finally, 'stripe' 16 emerges as a very narrow string of cells that initially abuts stripe 15, but separates from it during the extended germ band stage (Fig. 1F,G). The late appearance and reduced size of stripes 15-17 reflect the rudimentary nature of the terminal abdominal segment anlagen, A9-A11, in *Drosophila* (Baumgartner et al., 1987; Jürgens, 1987). During head involution, *gsb* is transiently expressed at high levels in a subset of cells of the pharynx and anal pads (Fig. 1M).

Expression of *gooseberry neuro*

As shown in Fig. 2A, gsbn protein first appears at stage 10 in a small number of neuroblasts, ganglion mother cells, and neurons. As neurogenesis proceeds, gsbn protein levels rise, an increasing number of ganglion mother cells and neurons express *gsbn*, and a low level of gsbn protein persists in some neuroblasts (Figs 2A-E, 3H,I). The ganglion mother cells and neurons that express *gsbn* are predominantly, though perhaps not exclusively, the progeny of the *gsb*-expressing neuroblasts. By the end of stage 11, *gsbn* is clearly expressed in a segmentally reiterated neural pattern from the mandibular to the tenth abdominal neuromere. In the trunk segments, *gsbn* expression in the CNS forms a typical L-shaped pattern in each hemisegment (Fig. 2C,D,I).

Similarly to *gsb*, *gsbn* is expressed in the terminal regions. In the head, gsbn protein is detected in neurons of the brain (Fig. 2F,G) while, in the tail region, it appears in neurons of A9 and in cells of the anal pad (Fig. 2C,D). Finally, *gsbn* is also expressed in a small number of neurons lying between A9 and the anal pads. These neurons are derived from 'stripe' 16 of gsb and may be evidence for a rudimentary tenth abdominal neuromere (Fig. 2C,D).

During subsequent stages of development, gsbn protein persists in a subset of neurons until nerve cord retraction during stage 17 (Fig. 2F,G,I,L). Furthermore, after germ

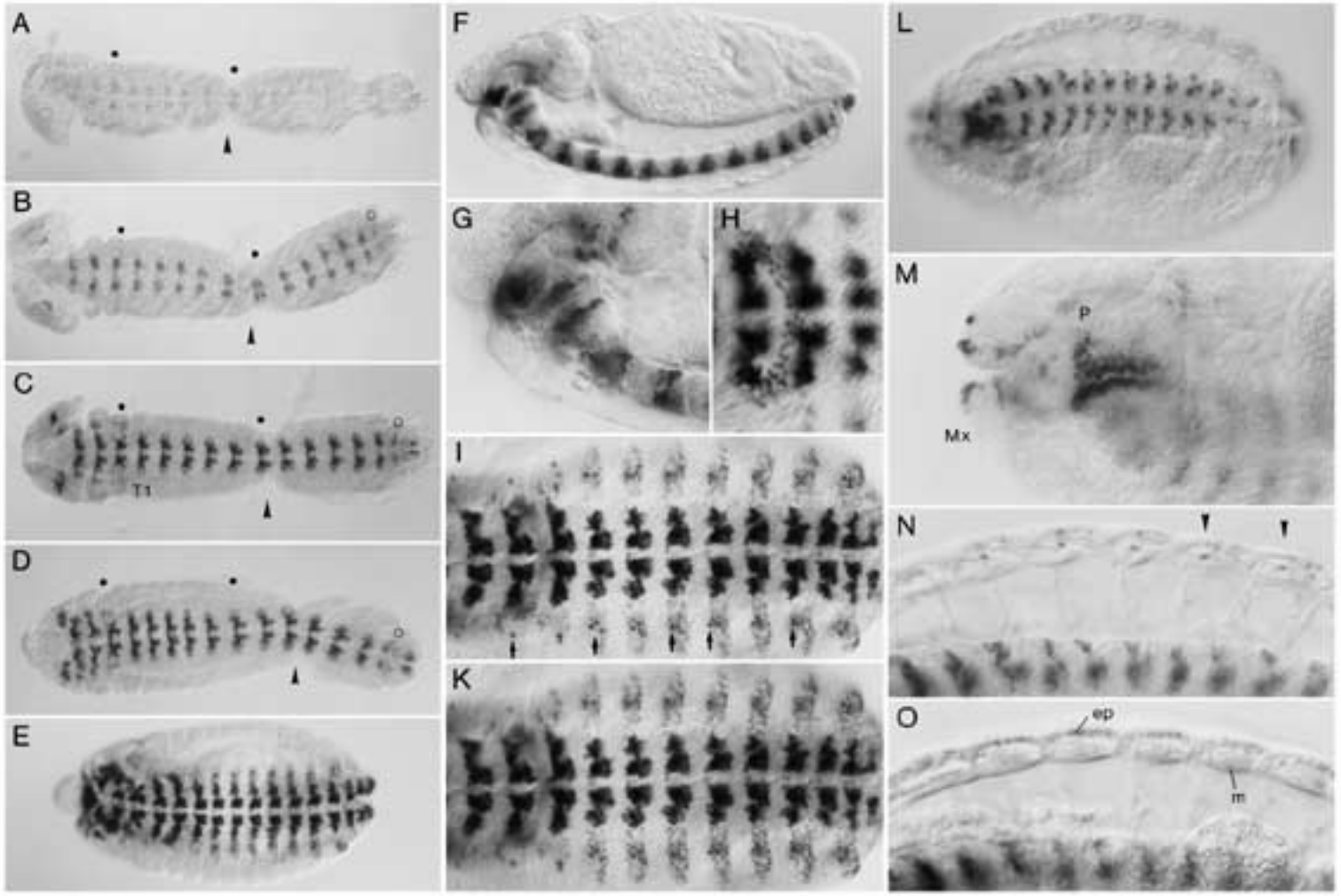

Fig. 2. Expression of gsbn protein in wild-type *Drosophila* embryos. Whole-mount preparations of wild-type embryos were stained with anti-gsbn antiserum. Embryos shown in A-D were unfolded as in Fig. 1. Stages of embryos are (A) slow phase of germ band extension (stage 10); (B, C) extended germ band stage (early and late stage 11); (D) mid-germ band retraction (stage 12); (E) after completion of germ band retraction (stage 13); (F-K) head involution (stage 14); (L-O) ventral cord retraction (stage 17). All embryos are oriented with their anterior to the left and, in lateral views, dorsal side up. Whole mounts show lateral (F,G) or ventral views (E,I,L) focused on *gsbn* expression in the CNS, except for K which is focused on the epidermis. In unfolded preparations (A-D), stripes 3 and 9 are marked by dots and stripe 15 by an open circle, and the most posterior region prior to unfolding is indicated by an arrowhead. G is an enlargement of the anterior portion of the embryo shown in F. H-K are photographs of the same embryo focused on the CNS and PNS (I) or the epidermis (K), or displaying an enlarged thoracic view focused on the ventral mesoderm (H). Arrows point at some of the gsbn-positive cells that presumably belong to the PNS. (M) An enlargement of the head region, shows gsbn protein expression in a few cells of the maxillary lobe (Mx) and in a T-shaped patch of cells at the entry of the pharynx (labeled P; compare to G). N and O are focused on the ventrolateral ectoderm and mesoderm of the embryo shown in L. Note that *gsbn* is expressed in a few epidermal cells (ep in O) and ventral superficial muscle cells (arrowheads in N) which are just adjacent to the more prominent, non-*gsbn*-expressing, ventral internal muscles (m in O).

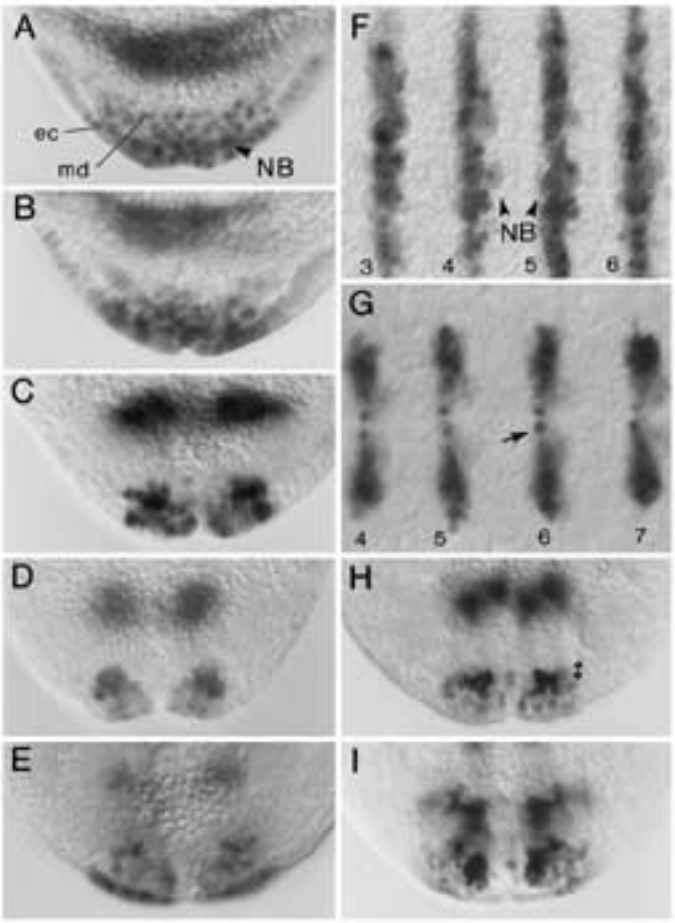

Fig. 3. Expression of *gsb* and *gsbn* in different germ layers. Mid-sagittal optical sections of the portion of the germ band curved around the posterior end (A-E,H,I) or ventral superficial optical sections of the developing thoracic neuromeres (F, G) of embryos stained with anti-gsb (A-G) or anti-gsbn antiserum (H, I) are shown at different stages of germ band extension and germ band retraction: stage 10 (A,B,F), stage 11 (C,D,G,H), and stage 12 (E,I). Early neuroblasts, easily recognized by their large size, are indicated by arrowheads (A and F). The arrow in G points to the most medial of the row 5 neuroblasts. Stripes 3-7 of *gsb* expression (corresponding to posterior labial to first abdominal segments) are labeled in F and G. The asterisks in H are next to sibling ganglion mother cells and neurons. Note that *gsb* expression in the mesoderm and in some neuroblasts is only transient (compare A-C) and that ectodermal gsb protein expression decreases transiently (C,D). Abbreviations: ec ectoderm; md mesoderm; NB neuroblast.

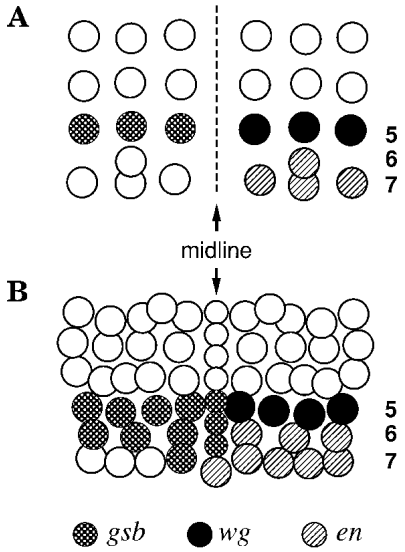

Fig. 4. Map of *gsb*, *en*, and *wg* expressing neuroblasts. The relative arrangement of neuroblasts is schematically illustrated (A) during stage 9 [shortly after the division 1 stage of Hartenstein and Campos-Ortega (1984); between stages S1 and S2 of Doe (1992)], and (B) during the middle of the extended germ band stage [stage 11; between stages S4 and S5 of Doe (1992)]. For both parts, the left hemisegment shows the pattern of *gsb*-expressing neuroblasts and the right hemisegment the pattern of *wg*- and *en*-expressing neuroblasts. *en* is expressed by all neuroblasts of rows 6 and 7, *wg* is expressed by all neuroblasts of row 5, and *gsb* protein appears in the neuroblasts of rows 5 and 6 and the most medial neuroblast of row 7. At the midline, *gsb* is weakly expressed by three cells anterior to the median neuroblasts although only the most anterior of the three maintains *gsb* expression when the cells move inward. Based on the data of Klämbt et al. (1991), these three cells may be the precursors to the VUM neurons. Also at the midline, *en* is expressed by the median neuroblast. As neurogenesis proceeds, *en* is expressed by many of the progeny of the row 6 and 7 neuroblasts, by some of the progeny of the median neuroblast, and by the four support glia cells that come to lie immediately posterior to the median neuroblast. In addition, at least three non-*en*-expressing neuroblasts generate a few *en*-expressing neurons. *wg* transcripts do not seem to accumulate in any progeny of neuroblasts whereas gsb and gsbn protein appear at least transiently in many, if not all, of the progeny of the neuroblasts that express *gsb*. We have not yet been able to map the expression in neuroblasts of *gsbn* as thoroughly as that of *gsb* because of the relatively late timing and low levels of *gsbn* expression.

band retraction *gsbn* becomes expressed in a few lateral cells per hemisegment that might belong to the muscle founder cells or the PNS (arrows in Fig. 2I), as well as in a striking stripe of mesodermal cells of T2 (Fig. 2F,H). Similarly to *gsb*, after germ band retraction *gsbn* is also expressed in ventral ectodermal stripes in the posterior region of each segment (Fig. 2K). However, the gsbn stripes persist until much later in development (up to stage 17; Fig. 2O) than the gsb stripes. In addition, gsbn protein is detectable in a number of patches of epidermal cells, or derivatives thereof, in the head region, including the pharynx (Fig. 2L,M). Finally, gsbn protein appears in the nuclei of one of the ventral superficial oblique muscles (Fig. 2N,O).

Coexpression of *gooseberry* and *gooseberry neuro* with *engrailed*

To determine the relative positions of *gsb*- and *gsbn*-expressing cells with respect to the parasegmental and the segmental boundaries, double-labeling experiments were performed with an anti-gsb or anti-gsbn antiserum and an anti-en monoclonal antibody (Patel et al., 1989b). As is apparent from Fig. 5A-C, the anterior border of a *gsb* stripe is anterior to that of *en*-expressing cells by one to two rows of cells at the extended germ band stage. Similarly, the posterior boundary of *en* is one to three cells posterior to that

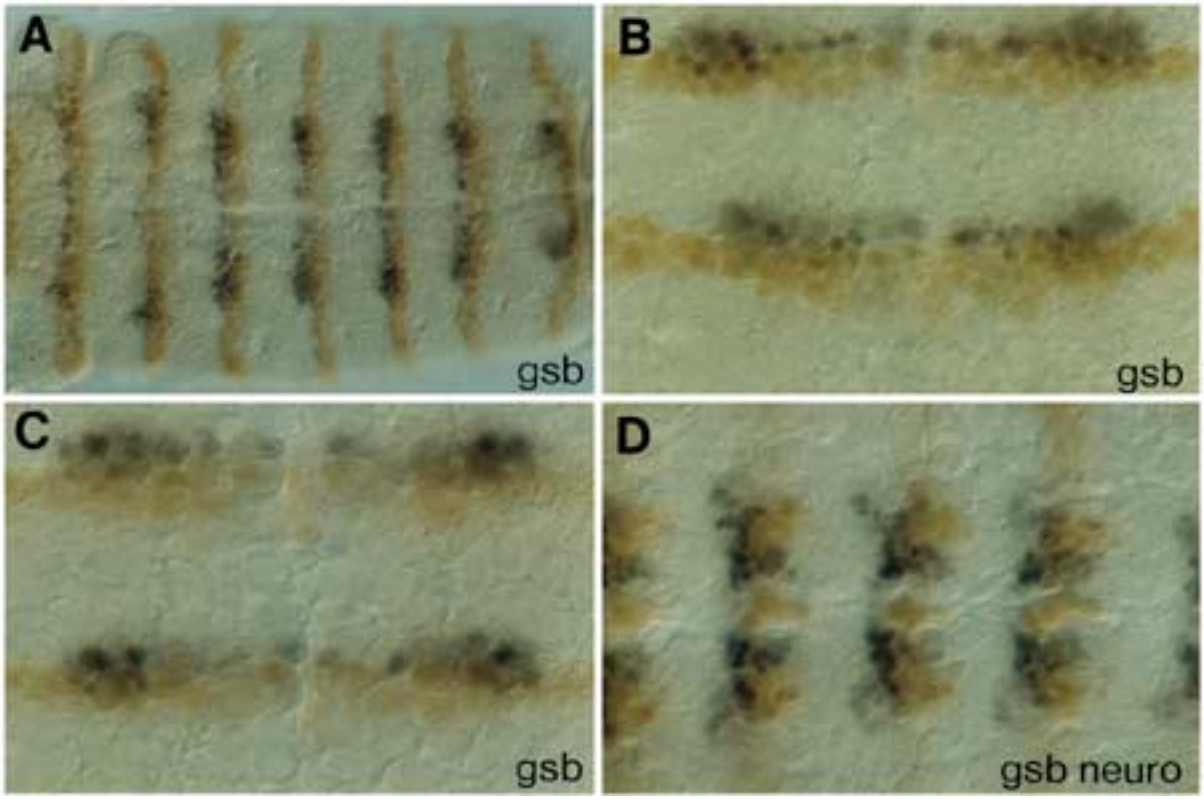

Fig. 5. Coexpression of *gsb* and *gsbn* with *en*. Embryos at mid (A-C) or late stage 11 (D), first stained with either anti-gsb (A-C) or anti-gsbn antiserum (D) and subsequently with monoclonal anti-en antibody, are shown with their anterior to the left (A, D) or up (B,C). Portions of embryos shown correspond to the maxillary to second abdominal segment (A), posterior T3 to anterior A2 (B,C), and posterior labial segment to anterior A1 (D). Planes of focus are in the epidermis (A,B), the underlying neuroblast layer (C) or the layer of ganglion mother cells and neurons (D). Note that expression of *gsb* is anterior to that of *en* by one to two rows of cells.

of *gsb* expression. The greatest extent of overlap between gsb and en protein is seen in the widest and most lateral regions of the gsb stripes. Furthermore, gsb protein is expressed by all neuroblasts of rows 5 and 6, and transiently by the most medial neuroblast of row 7, while en is expressed by all neuroblasts of rows 6 and 7 (Figs 4B, 5C).

Extensive overlap of gsbn and en is seen in the CNS. During the early extended germ band stage, *en* is expressed by a large number of ganglion mother cells and neurons derived from the neuroblasts of rows 6 and 7 as well as from the median neuroblast (Fig. 4B). At this stage, *gsbn* is expressed in ganglion mother cells and neurons derived from neuroblasts of row 5 and 6, and from the most medial neuroblast of row 7 as evident from the overlap between the *en* and *gsbn* expression patterns (Fig. 5D). Further details of *gsb* and *gsbn* expression in the CNS will be discussed in the context of the specific role of the *gooseberry* locus in neural development (Patel, Li, Gutjahr, Ferres-Marco, Noll and Goodman, unpublished data).

We further examined the overlap of gsb with wg (see Materials and methods). This analysis revealed that the wg domain coincides with the anterior gsb domain during the extended germ band stage (not shown). In the CNS, *wg* is expressed by the neuroblasts of row 5 (Fig. 4). Thus, in both the ventral ectoderm and the underlying neuroblasts, *gsb* expression includes most, if not all, of the wg domain, plus part of the anterior portion of the en domain. Since the boundary between the ectodermal en and wg domains demarcates adjacent parasegments, it follows that *gsb* expression spans the parasegmental boundary at the extended germ band stage.

Rescue of the *gooseberry* cuticular phenotype

In order to test the contributions of *gsb* and *gsbn* to the cuticular (Nüsslein-Volhard and Wieschaus, 1980) and CNS *gsb*-phenotypes (Patel et al., 1989a) and to detect a potential transregulation of *gsbn* by *gsb*, we generated transgenic flies carrying a 20 kb genomic DNA fragment harboring the intact *gsb* gene, the region separating the two *gsb* transcripts, and the 5′ portion of the *gsbn* gene comprising the paired-domain and the first two introns of the *gsbn* gene (Fig. 6B). Conceivably, this construct permits the expression of a functional gsb protein (see below), yet only of a truncated gsbn protein (consisting of the paired-domain fused to vector sequences). Four independent transgenic lines were crossed into *gsb* mutant backgrounds to test the ability of the *gsb* gene to rescue the *gsb* cuticular phenotype.

We first analyzed the cuticles of embryos transheterozygous for the deficiencies $Df(2R)Kr^{SB1}$ and *Df(2R)IIX62* that carried one copy of the exogenous *gsb* gene ($Df(2R)Kr^{SB1}/Df(2R)IIX62;\ P[ry^{+},\ gsb^{+}]/ry^{506}$). The deficiency *Df(2R)IIX62* removes both the *gsb* and the *gsbn* gene while $Df(2R)Kr^{SB1}$ removes *gsb*, but not *gsbn* (Fig. 6B;

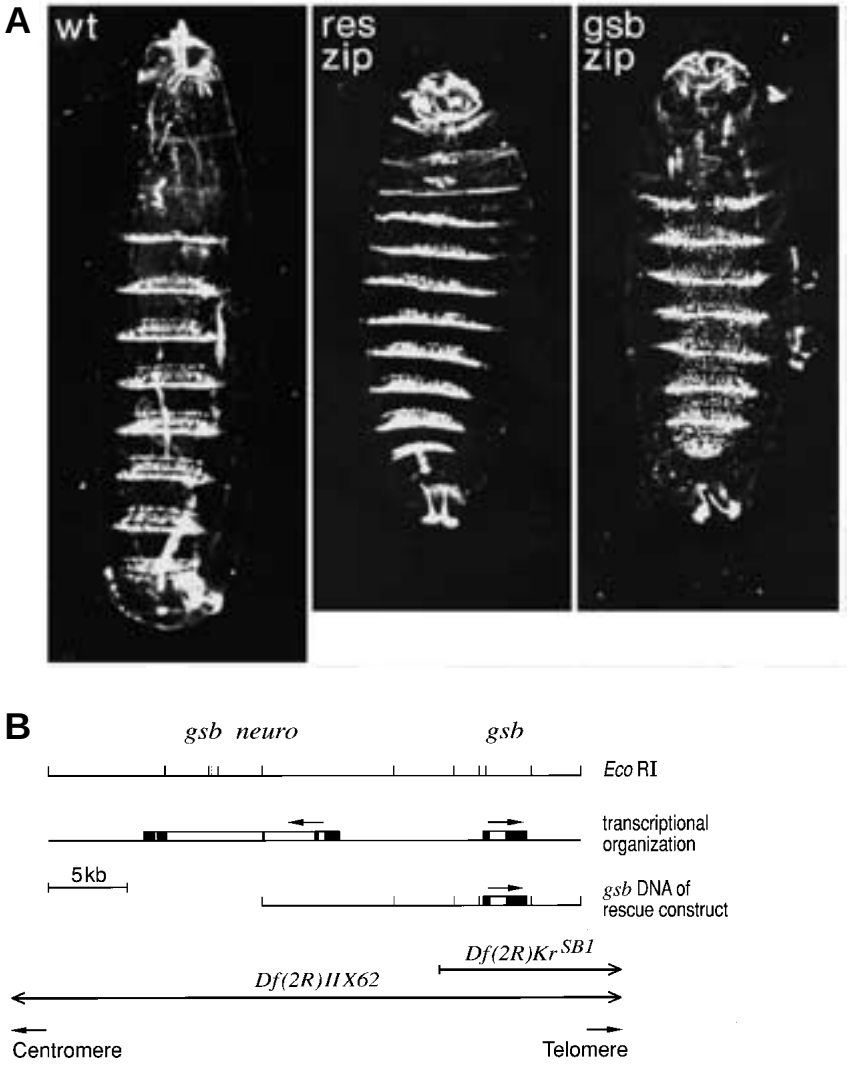

Fig. 6. Rescue of the *gsb* cuticular phenotype. (A) Cuticle preparations of a wild-type embryo in the left panel (wt), of a *Df(2R)IIX62/ Df(2R)IIX62*; *P[ry*$^{+}$*, gsb*$^{+}$*]/P[ry*$^{+}$*, gsb*$^{+}$*]* embryo in the middle panel (res, zip), and of a *Df(2R)IIX62/Df(2R)IIX62* embryo in the right panel (gsb, zip) are shown under dark-field illumination. Note that the difference between the embryo in the middle panel and the embryo in the right panel is only the *P[ry*$^{+}$*, gsb*$^{+}$*]* which rescues fully the *gsb* cuticular phenotype. Cuticles were prepared essentially as described by Wieschaus and Nüsslein-Volhard (1986). (B) Shown are, from top to bottom, an *Eco*RI restriction map of the *gsb* locus, the organization of the *gsb* and *gsbn* transcription units (arrows indicate the directions of transcription, open and closed bars the extent of introns and exons; Baumgartner et al., 1987; Li et al., 1993), the *gsb* DNA used in the rescue construct (cf. Materials and methods), and the DNA deleted by the deficiencies *Df(2R)Kr*^{SB1} and *Df(2R)IIX62* (Baumgartner et al., 1987; Côté et al., 1987).

Bopp et al., 1986; Baumgartner et al., 1987; Côté et al., 1987). Therefore, *Df(2R)Kr*^{SB1}*/Df(2R)IIX62; P[ry*$^{+}$*, gsb*$^{+}$*]/ ry*^{506} embryos carry one copy each of the endogenous *gsbn* and of the exogenous *gsb* gene. All four transgenic *gsb* lines tested were able to reverse the *gsb* cuticular phenotype (similar to the embryo shown in the central panel of Fig. 6A).

To exclude the possibility that *gsbn* contributes to the cuticular rescue, we also tested embryos homozygous for *Df(2R)IIX62*. These embryos exhibit both the *zipper* phenotype, characterized by defects in the head skeleton, and the *gsb* cuticular phenotype (right panel of Fig. 6A; Nüsslein-Volhard et al., 1984). Again, all four transgenic *gsb* lines were able to completely rescue the *gsb* cuticular phenotype of *Df(2R)IIX62* homozygotes but still displayed the *zipper* phenotype (middle panel of Fig. 6A). Since several loci are deleted in addition to *gsb* in both *Df(2R)Kr*^{SB1} and *Df(2R)IIX62* deficiencies (Côté et al., 1987), embryonic lethality was not rescued. The rescue of the *gsb* cuticular phenotype does not depend on the *gsbn* sequences that are also present in the rescue construct and encode a truncated gsbn protein because complete rescue is also achieved by a shorter construct carrying no *gsbn* sequences (not shown). We conclude that the *gsb* gene is able to rescue fully the *gsb* cuticular phenotype in the absence of *gsbn*. Moreover, as shown below, relatively low levels of gsb protein appear to be sufficient to rescue completely the *gsb* cuticular phenotype.

Transactivation of *gooseberry neuro* by *gooseberry*

The observed general overlap of *gsbn* and *gsb* expression in the CNS suggests a possible activation of *gsbn* by *gsb*. To test this possibility, we examined whether *gsbn* protein, which is undetectable in the trunk of *Df(2R)Kr*^{SB1}*/ Df(2R)IIX62* embryos, is expressed in transgenic *Df(2R)Kr*^{SB1}*/Df(2R)IIX62*; *P[ry*$^{+}$*, gsb*$^{+}$*]/ P[ry*$^{+}$*, gsb*$^{+}$*]* embryos carrying two exogenous *gsb* genes and one copy of *gsbn*. In such embryos, *gsbn* is clearly expressed in ganglion mother cells and neurons (Fig. 7A), and later in the epidermis (not shown), of the same regions as in wild-type embryos although at much lower than wild-type levels. Since no gsbn protein was observed in *Df(2R)IIX62/ Df(2R)IIX62*; *P[ry*$^{+}$*, gsb*$^{+}$*]/P[ry*$^{+}$*/gsb*$^{+}$*]* embryos (not

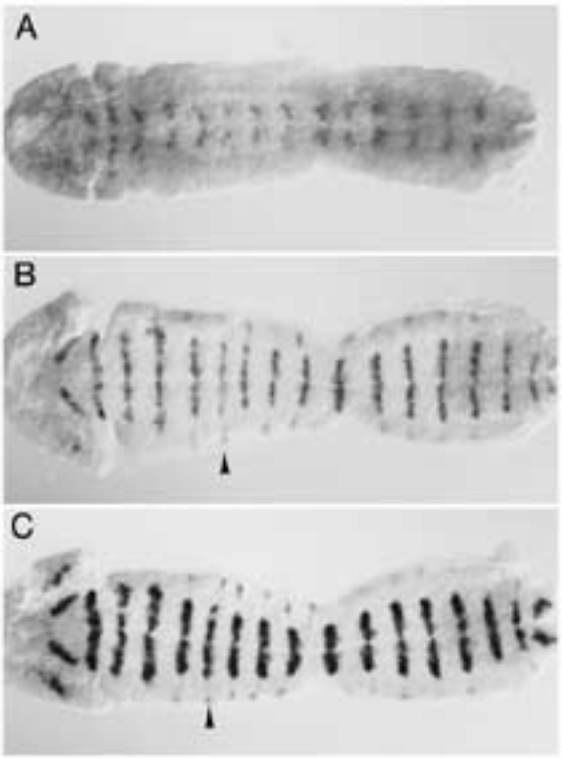

Fig. 7. Transactivation of *gsbn* by *gsb*. Unfolded embryos at the early extended germ band stage (early stage 11) of the genotype *Df(2R)IIX62/Df(2R)Kr^{SB1}*; *P[ry$^+$, gsb$^+$]/P[ry$^+$, gsb$^+$]* (A, B) or *Df(2R)Kr^{SB1}/CyO*; *P[ry$^+$, gsb$^+$]/P[ry$^+$, gsb$^+$]* (C) have been stained with anti-gsbn (A) or anti-gsb antiserum (B,C). The embryos are oriented with their anterior to the left. Photographs are focused on the epidermis (B,C) or on the underlying developing CNS (A). Arrowheads point at a dominant reduced expression of *gsb* in T2 of heterozygous *Df(2R)Kr^{SB1}* embryos.

shown), expression of the transgenic truncated *gsbn* gene (paired domain) is undetectable with the cross-absorbed anti-gsbn antiserum (see Materials and methods), demonstrating that the gsbn protein detected in *Df(2R)Kr^{SB1}/Df(2R)IIX62*; *P[ry$^+$, gsb$^+$]/ P[ry$^+$, gsb$^+$]* embryos must be attributed to the activation of the intact endogenous *gsbn* gene. Hence, these results demonstrate that the exogenous *gsb* gene is indeed able to activate *gsbn* expression in the transgenic transheterozygous *gsb* mutants. The relatively low expression of *gsbn* in these embryos probably reflects two different effects. First, these embryos contain only one copy of the *gsbn* gene. In fact, higher levels of *gsbn* expression were observed in homozygous *Df(2R)Kr^{SB1}/Df(2R)Kr^{SB1}*; *P[ry$^+$, gsb$^+$]/P[ry$^+$, gsb$^+$]* embryos, which carry two copies of *gsbn*, both in the CNS and the epidermis (not shown). Second, expression of the exogenous *gsb* gene is weaker than that of the endogenous *gsb* gene, especially in the CNS (compare Fig. 7B with Figs 7C and 1F). It should be noted that the low, but easily detectable, expression of *gsbn* in the pharynx and anal pads in *Df(2R)Kr^{SB1}/Df(2R)IIX62* embryos (not shown; Ouellette et al., 1992) indicates that not all cell types require *gsb* to activate *gsbn* expression.

DISCUSSION

Both *gsb* genes, *gsb* and *gsbn*, encode transcriptional regulators whose N-terminal halves consist of a paired-domain and a *prd*-type homeodomain (Bopp et al., 1986). Their extreme structural conservation suggests that the function of the gsb and gsbn proteins are probably very similar at the molecular level. The difference in function between the two genes might then consist of a difference in their expression patterns rather than in their specificity of molecular action. We have shown here that gsb protein is continuously expressed in a typical segment-polarity pattern in the epidermis until head involution, transiently in the developing CNS and mesoderm, and finally in specific structures of the head and tail region. The epidermal and CNS expression of *gsb* in segmentally repeated stripes strikingly parallels the delayed expression of *gsbn* in these tissues, which suggests a possible dependence of *gsbn* expression on *gsb*.

Gooseberry functions in the specification of the cuticular pattern

Since in all presently known *gsb* alleles the *gsb* gene is deleted and expression of the neighboring *gsbn* gene is entirely eliminated or at least reduced to undetectable levels in most parts of the embryo, it was not clear which of the two genes is responsible for the cuticular phenotype. Their patterns of transcripts, however, suggested that *gsb* rather than *gsbn* specifies the cuticular pattern (Bopp et al., 1986; Baumgartner et al., 1987). Due to the late expression of *gsbn* in the epidermis (Fig. 2K), however, the possibility remained that *gsbn* is also involved in the specification of the cuticle. The rescue of the *gsb* cuticular phenotype by a *gsb* transgene in the absence of both *gsb* and *gsbn* demonstrates that *gsb* is sufficient while *gsbn* is dispensible for proper development of the cuticle (Fig. 6).

Similarly to other segment-polarity genes, *gsb* is first activated by pair-rule gene products (Baumgartner, 1988). For example, prd and odd-paired (opa) are required for the activation of *gsb* in odd- and even-numbered stripes, respectively (Bopp et al., 1989; Li et al., 1993). Activation by *prd* is further reflected in the initial pair-rule pattern of gsb (Fig. 1A) which precisely parallels that of the prd protein (Gutjahr et al., 1993). In other words, the prd bands appear in the same order as and immediately precede the corresponding gsb bands, suggesting that the prd protein probably activates the *gsb* gene directly by binding to the corresponding *gsb cis*-regulatory elements (Li et al., 1993). The later ectodermal expression of *gsb*, accompanied most notably by the lateral restriction of the gsb stripes to the neuroectodermal region of the extended germ band, is activated and maintained in response to the wg signal (Li et al., 1993). The cuticular pattern only clearly depends on the *wg* product before germ band retraction (Bejsovec and Martinez-Arias, 1991), exhibiting a *wg*-dependent mutant phenotype very similar to that of *gsb*. Since only *gsb* but not *gsbn* is expressed during the temperature-sensitive period of the temperature-sensitive *wg* allele, it is not surprising that *gsb* rather than *gsbn* is responsible for the determination of the cuticular pattern (Fig. 6). Moreover, by the same argument the late epidermal expression of *gsbn* does

not influence the cuticular pattern. Since this late epidermal expression of *gsbn* depends on *gsb*, which is activated by wg, the wg signal is also required for the late epidermal expression of *gsbn*. Hence, specification of the cuticular pattern by *gsbn* would also be in conflict with the observed temperature-sensitive period of the temperature-sensitive *wg* allele (Bejsovec and Martinez-Arias, 1991). The function of the late epidermal *gsbn* expression remains to be elucidated.

Gooseberry activates *gooseberry neuro* in *trans*

The observation that *gsbn* is not expressed in transheterozygous *Df(2R)Kr^{SB1}/Df(2R)IIX62* embryos, in which one copy of the *gsbn* gene is retained but both copies of the *gsb* gene are deleted, could be explained by inactivation of the remaining *gsbn* gene in *cis* or *trans*. Since we could show that *gsbn* is expressed in transgenic embryos into which an exogenous *gsb* gene had been introduced, we conclude that the inactivation occurs in *trans* and that gsb protein is required for the activation of *gsbn*.

The expression patterns of both *gsb* and *gsbn* are altered in pair-rule mutants in the same manner (Bopp et al., 1989; X. Li, unpublished observations). A possible explanation would be that both genes are regulated by the same combinations of pair-rule gene products that interact with the *cis*-regulatory region of each gene to activate its transcription. Alternatively, one of the two *gsb* gene products could activate the other gene in *trans*. Our finding that the expression of *gsbn* depends on the expression of *gsb* favors the second alternative. In all cells and tissues expressing *gsbn*, expression of *gsb* immediately precedes that of *gsbn*, indicating that the transactivation of *gsbn* by *gsb* might be direct. In the CNS, for example, gsb protein appears in those neuroblasts and ganglion mother cells that subsequently express *gsbn* and apparently give rise to *gsbn*-expressing neurons. Also in the epidermis, where *gsbn* expression is initiated during stage 13, it is preceded by and dependent on *gsb* expression. However, *gsbn* expression does not always completely depend on *gsb* activity as suggested by the expression of *gsbn* in the pharynx and anal pads of the transheterozygous *gsb* embryos.

Expression of *gsb* does not persist in cells and tissues that continue to express *gsbn*, as for example in the CNS or epidermis. Therefore, *gsbn* expression is maintained by (a) protein(s) different from *gsb*. The simplest mechanism for *gsbn* to maintain its expression would be by autoregulation.

Role of *gooseberry* genes in neurogenesis

The expression of *gsb* and *gsbn* in the CNS suggests that both genes play a role in the development of the CNS. In fact, the known *gsb* deficiencies also exhibit a CNS phenotype in which *even-skipped*-expressing cell lineages are altered and the posterior commissures are missing (Patel et al., 1989a). The redeployment of segmentation genes in neurogenesis seems to be a general phenomenon as most of them are reexpressed in the developing CNS at various stages. This expression in the CNS is crucial for the proper specification of neuronal fates as demonstrated for the pair-rule genes *fushi tarazu*, *even-skipped*, and *runt* (Doe et al., 1988a,b; Duffy et al., 1991). Our studies shown here suggest that one evident function of *gsb* is the activation of *gsbn* expression in the CNS. In addition, we have found that an exogenous copy of *gsb* rescues the neural defects seen in *Df(2R)Kr^{SB1}/Df(2R)IIX62* embryos and that both *gsb* and *gsbn* are required for a complete rescue of all neural phenotypes (Patel, Li, Gutjahr, Ferres-Marco, Noll, and Goodman, unpublished data).

Are there no point mutants of *gooseberry*?

Two independent screens for *gsb* mutations failed to produce point mutants but generated only deletions (Nüsslein-Volhard et al., 1984; Côté et al., 1987). Hence, the question arose whether point mutations have not been obtained because both *gsb* genes need to be inactivated to observe the *gsb* cuticular phenotype. Our results argue against such an assumption for two reasons. Our demonstration that *gsb* is sufficient to specify the cuticle renders the *gsbn* gene dispensible with respect to cuticular patterning. Moreover, since we have shown that *gsbn* expression depends on a functional gsb protein, inactivation of the *gsb* product by point mutations is expected to inactivate both genes. Therefore, we expect that it should be possible to generate point mutations in the *gsb* gene that result in a cuticular phenotype.

Is there an ancestral *gooseberry* gene?

The organization of the *gsb* locus and the sequence homology between the two genes suggest that the two *gsb* genes have originated from a common ancestral gene through gene duplication. If this interpretation is correct, the question arises whether the two genes of the *gsb* locus exert specialized and separate functions which were previously the task of a single gene. It may thus be possible to isolate the *gsb* gene from a more distantly related insect or arthropod in which only one *gsb* gene exists which performs both functions in segmentation and neurogenesis.

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