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Molecular organization and embryonic expression of the *hedgehog* gene involved in cell-cell communication in segmental patterning of *Drosophila*

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Summary

***hedgehog* is a segment polarity gene necessary to maintain the proper organization of each segment of the *Drosophila* embryo. We have identified the physical location of a number of rearrangement breakpoints associated with *hedgehog* mutations. The corresponding *hh* RNA is expressed in a series of segmental stripes starting at cellular blastoderm in the posterior portion of each segment. This RNA is localized predominantly within nuclei until stage 10, when the localization becomes primarily cytoplasmic. Expression of *hh* RNA in the posterior compartment is independent of most other segment polarity genes, including *en*, until the late extended germ-band stage (stage 11). Sequence analysis of the *hedgehog* locus suggests the protein product is a transmembrane protein, which may, therefore, be directly involved in cell-cell communication.**

Key words: *Drosophila* embryogenesis, segmentation, *hedgehog*, segment polarity genes, pattern formation, *su(w^a)* suppression.

Introduction

The segmental pattern of the *Drosophila* embryo becomes defined at the cellular blastoderm as the spatially reiterative expression of various segment polarity class segmentation genes along the anterior-posterior axis. This reiterative pattern of segment polarity gene expression is derived from two maternal morphogenic gradients of *bicoid* and *nanos* proteins (Driever and Nüsslein-Volhard, 1988; Wang and Lehmann, 1991) by a regulatory cascade of segmentation genes (gap and pair-rule), which sequentially restrict the embryo along the anterior-posterior axis into progressively smaller transcriptional domains (see reviews by Akam, 1987; Ingham, 1988 and Pankratz and Jäckle, 1990). Some of the segment polarity genes are transcription factors and presumably act to define subsegmental domains for the cells expressing those genes. For example, *engrailed (en)*, a homeobox transcription factor, and *ci^D*, a zinc finger transcription factor, (Kornberg et al., 1985; Orenic et al., 1990) are expressed in the posterior and anterior compartments, respectively, of each segment.

Following the blastoderm stage, the embryo undergoes two massive sets of cellular movements: gastrulation, involving invagination of the mesoderm and midgut primordia, and germ-band extension, involving the expansion of the segmented germ-band along its length and constriction along its width by a factor of two (Sonnenblick, 1950). Following these movements, during the extended germ-band stage (stage 10), the subsegmental domains of segment polarity gene expression are refined, aligned and maintained and errors in either the initial establishment of the subsegmental domain or in misdirected cell movement during germ-band extension are corrected by interactions between the cells of the various domains (DiNardo et al., 1988; Martinez-Arias et al., 1988). This "editing" process is regulated by a second set of segment polarity genes. For example, continued expression of *en* in the posterior compartment requires the proper expression of *wingless (wg)*, *armadillo (arm)*, *fused (fu)* and *hedgehog (hh)* (DiNardo et al., 1988), whereas the maintenance of *wg* expression in the immediately anterior cell requires the proper function of the *en*, *hh* and *fu* genes (Martinez-Arias et al., 1988; Hidalgo and Ingham, 1990; Limbourg-Bouchon et al., 1991). This intercellular closed-circuit regulatory loop insures the correct juxtaposition of these two cell types across the parasegmental boundary. Many of the genes of this second class of segment polarity genes encode proteins that are either secreted (*wg*, van den Heuvel et al., 1989), are positioned at the cell membrane (*patched (ptc)*, Hooper and Scott, 1989; Nakano et al., 1989; *armadillo (arm)*, Riggleman et al., 1990; Peifer and Wieschaus, 1990), or are putative signal transduction proteins (serine kinases, *fused (fu)*, Preat et al., 1990; *l(1)zw3*, Siegfried et al., 1990) and are believed to play a role in the cell-cell communication of positional identities. Many of the "editing" class of segment polarity genes are transcriptionally activated in their appropriate domains at the cellular blastoderm (*wg*, Baker, 1987, 1988; *ptc*, Hooper and Scott, 1989; Nakano et al., 1989) or are supplied at least in part maternally (*arm*, Wieschaus and Noell, 1986; Klingensmith et al., 1989; Riggleman et al., 1990; *fused (fu)*, Counce, 1956; Martinez-Arias, 1985; *dishevelled (dsh)*, Perrimon and Mahowald, 1987; *l(1)zw3*,

Perrimon et al., 1989), yet do not appear to be active in respecifying cell fate until the extended germ-band during stage 10, approximately ninety minutes after the cellular blastoderm.

The morphological manifestations of segmentation start with the formation of a shallow parasegmental furrow that divides the anterior and posterior compartments of each trunk segment during stage 10. This is followed in stage 11 by the formation of a tracheal pit midway between the parasegmental furrows and of the segmental grooves (separating the posterior compartment of one segment from the anterior compartment of the next) between the parasegmental furrow of one segment and the tracheal pit of the next (Martinez-Arias and Lawrence, 1985). Mutations in many of the segment polarity genes result in alterations of these initial segmentation events. Embryos mutant for *wg*, *fu*, *arm* or *dsh* fail to form segmental grooves in stage 11 and possess extensive areas of cell death in each segment, including the cells of the posterior compartment (Perrimon and Mahowald, 1987; Klingensmith et al., 1989). Ventral larval cuticle from these mutant embryos consists of a lawn of denticles with no segmental manifestations; this type of segment polarity gene is referred to as belonging to the "lawn" class. In contrast, embryos mutant for *ptc*, a gene required to restrict *wg* expression to the posterior margin of the anterior compartment (Martinez-Arias et al., 1988), develop an ectopic "patched furrow" (associated with an ectopic domain of *en* expression) between the normal segmental grooves in stage 11 (DiNardo et al., 1988). Larval cuticle from these *ptc* embryos possesses mirror-image duplicated structures from the region around the segmental boundaries.

We have identified the physical locus encoding another segment polarity gene of the "lawn" class, *hedgehog (hh)*. Sequence analysis of cDNA clones indicates the *hedgehog* product contains a putative transmembrane domain and suggests that it may, therefore, be localized at the cell surface and act directly in cell-cell communication. The RNA from this locus is expressed in the posterior compartment of each embryonic segment, consistent with its hypothesized locus of activity derived from mosaic analysis of imaginal discs (Mohler, 1988). Finally, *hedgehog* RNA shifts from primarily nuclear to a cytoplasmic localization immediately prior to the apparent time of gene function. This delayed entry of *hedgehog* RNA into the cytoplasm may be important in the regulation of the timing of the "editing" phase of segmental gene activity.

Materials and methods

hedgehog *mutants*

The isolation of all *hedgehog* mutations has been previously described (Jürgens et al., 1984; Mohler, 1988; Mohler et al., 1992), except for hh^{H90}, which is an insertion of a Pl-wB transposon into the *hedgehog* and was a gift of L. Higgins.

Southern and northern analysis

Drosophila DNA was prepared by homogenization of adult flies in 100 mM Tris pH 9, 20 mM EDTA pH 8, 5% sucrose, 1% SDS. The homogenate was extracted with equal volumes of phenol twice, and of $CHCl_3$ twice. The DNA was precipitated from the extracted homogenate with ethanol. Phage and plasmid DNAs were isolated as described by Sambrook et al. (1989). *Drosophila* RNA was prepared by homogenization of 0.5 g of *Drosophila* embryos in a mixture of 25% phenol, 25 % $CHCl_3$, 50 % homogenization buffer (0.15 M sodium acetate, 5 mM EDTA, 1% SDS, 50 mM Tris, pH 9). The aqueous homogenate was recovered and extracted once with a 50:50 mixture of phenol and $CHCl_3$, then extracted twice with $CHCl_3$. Nucleic acid was recovered by precipitation with ethanol. PolyA+ and polyA– RNA was prepared by fractionation over oligo-dT cellulose columns.

For *Drosophila* DNA, either 1 μg or the equivalent of 1 fly was digested with an appropriate restriction enzyme overnight in the buffer supplied by the enzyme manufacturer. For phage or plasmid clone DNAs, 0.1 μg of DNA was similarly digested. Digested DNA was electrophoresed at 25 V overnight in 0.7% agarose gels in TAE buffer and blotted to Nylon 66 membrane (Micron Separation) as described by Sambrook et al. (1989).

For *Drosophila* RNA, 5 μg of polyA+ and 50 μg polyA– RNA was loaded onto formaldehyde denaturing gels and run at 150 V for two hours. The gel was blotted overnight to Nylon 66 membrane as described by Sambrook et al. (1989).

Filters were hybridized to plasmid or phage cloned DNAs labelled with ^{32}P by random priming (Sambrook et al., 1989) at 70ºC in 0.3 M NaCl overnight, and non-hybridized label was removed with four half-hour washes at 70ºC in 0.1 M NaCl. Hybridized filters were autoradiographed for 15 minutes to 2 weeks depending on the intensity. Northern filters probed with DNAs from the *hh* region that showed no distinct bands, were exposed for at least 2 weeks, then reprobed for another embryonic RNA.

Embryological preparations

In situ hybridization to embryos was performed according to the procedure of Tautz and Pfeifle (1989) using digoxygenin-labelled probes. Because the genomic probes (N6-3A, N6-11, N6-4, BB1, PS1.5 and Sac2) and the Z3 cDNA yielded similar hybridization patterns, the N6-11 genomic probe was used as a standard probe for hybridization. Hybridizations were performed on embryos collected overnight at 18ºC from Oregon-R, $arm^{YD35}/FM7$, en^{SFX31}/CyO, en^{IK57}/CyO, ptc^{IF85}/CyO, wg^{IG22}/CyO, $hh^{GS1}/TM3$, $hh^{13C}/TM3$, $hh^{HL3}/TM3$ and $ci^{D}/Df(4)G$ parents. These flies were raised under altered day/night cycles, with nightfall set approximately six hours before egg harvest.

Single-strand in situ hybridization was performed according to the methods of Ingham et al. (1985) using ^{35}S-labeled RNA probes. The Z3 cDNA and Sac2 genomic fragment were cloned in pGem3Z(f(–)) and labelled RNA probes were transcribed from the T7 and Sp6 promoters. In each case only labelled transcripts made corresponding to the proximal-to-distal chromosome orientation yielded a striped pattern of expression, indicating the *hh* locus is transcribed in a distal-to-proximal direction.

Early embryos (devitillinized and hybridized) were dehydrated in ethanol prior to mounting in methyl salicylate/canada balsam and were staged according to Campos-Ortega and Hartenstein (1984). Cuticle preparations were prepared of dechorionated, unhatched larvae cleared in Hoyer's media (Wieschaus and Nüsslein-Volhard, 1986).

Isolation of cDNA clones

The cDNA library of M. Noll, constructed of cDNAs derived from 0-4 hour embryonic RNA inserted into λgt10 (Frigerio et al., 1986) was screened by hybridization with ^{32}P-labelled *Drosophila* DNA in Bluescript KS– according to the procedure of Sambrook et al. (1989). Three different genomic probes were used: N6-3, N6-4 and N6-11. cDNA clones of two plasmid cDNA libraries of

N. Brown, constructed of cDNAs derived from 0-4 hour and 4-8 hour embryonic RNA inserted into pNB40 (Brown and Kafatos, 1988), were screened with the ^{32}P-labelled restriction fragments of either the N6-3 (0-4 hour), N6-4 (0-4, 4-8 hour), N6-11 (0-4 hour) and PS1.5 (4-8 hour) genomic clones that were recovered from an agarose gel according to the procedures of Sambrook et al. (1989). Approximately 150,000 clones were screened in each library with each probe, except the 450,000 clones of the Noll library were screened with N6-11. The Z3 cDNA was isolated from the Noll library with the N6-11 probe. The other 5 cDNAs were isolated from the Brown libraries with the N6-11 (h1a, h7a - 0-4 hour) and PS1.5 (5a, 10a, 11a - 4-8 hour) probes.

Sequence analysis

The cDNA clones and *Eco*RI fragments of genomic DNA were recloned into *Eco*RI sites Bluescript KS– (Stratagene) or pGEM3z(f(–)) (Promega Biotech) for sequencing. The ends of the Z3, h1a and h7a cDNA clones were sequenced, following rescue of single-stranded DNA by f1R408 helper phage, using oligonucleotide primers corresponding to the T7 and T3 promoters in Bluescript KS–, Sp6 and T7 promoters in pGEM3z(f(–)) and the Sp6 promoter from pNB40 retained in the recloning of the Brown cDNAs with the Sequenase Kit (USB) according to procedures of the kit. The ends of the 5a, 10a and 11a cDNA clone were sequenced from T7 and Sp6 promoter primers in the original pNB40 clones using the double stranded sequencing procedures of Ausubel et al. (1987).

Sets of nested deletions were prepared in both directions for genomic *Eco*RI fragments, in the proximal-distal direction for the PS1.5 *Pst*I-*Sac*I fragment, in both directions for the Z3 cDNA clone and in the 5′ to 3′ direction for the 5a, h1a and h7a cDNA clones according to the procedure of Henikoff (1984), after double digestion of the plasmids with *Kpn*I or *Apa*I and *Hin*dIII (*Eco*RI fragments in Bluescript KS–) or *Sph*I and *Pst*I (N6-PS1). Deletion constructs were sequenced using the T3 promoter primer (*Eco*RI fragments and cDNA clones in Bluescript KS−) or the Sp6 promoter primer (N6-PS1 and Z3 in pGEM3z(f(–))) of either rescued single-strand DNA (N6-4, Z3, h1a and h7a) or double-strand DNA (all others and one strand of N6-4). The sequence was analyzed using HIBIO DNASIS and PROSIS software (Hitachi).

PCR amplification of cDNA

First strand cDNA was prepared from 2 μg RNA from 0-8 and from 5-12-hour embryos using 20 ng of the appropriate downstream primer incubated for 1 hour at 42ºC with AMV reverse transcriptase (ART) in 25 μl ART buffer (Sambrook et al., 1989) and 0.5 mM dNTPs. Amplification reactions were prepared by the addition of 20 ng of the appropriate upstream primer, 0.5 mM dNTPs, and Vent Polymerase (NEBiolabs) in 50 μl of manufacturer-supplied buffer to the first strand synthesis reaction. Amplification was done through 35 cycles of a 1 minute denaturation (94ºC), 1 minute annealing (55ºC) and 2 minute polymerization (72ºC), followed by an additional 5 minute polymerization.

The names of the following primers, used in these amplifications, indicate the site of the 5′ nucleotide in the *hh* genomic sequence and whether the primer corresponds to the transcribed strand (T, upstream primer) or reverse strand (R, downstream strand). T3087 (start-ORF1): GGAATTCATATGGATAACCACAGCTCAGTGCCTTGG; T3325 (mid-ORF1): GCCCCGGCTCACAGCTGCGGT; T11506 (mid-ORF2): GTCCAAATACGGCATGCTCG; R11531 (mid-ORF2): GCGAGCGAGCATGCCGTATT; R12090 (mid-ORF3): TCTTTCAGATCTTGAGAGTC; R12206 (end-ORF3): CGGGATCCGGGTTCCAGCCCAGTCTTCAGT; polyA-tail primer: GGGAGATCTTTTTTTTTTTTTTTT.

Results

hh *rearrangement breakpoints map to the –48 to –61 region*

In two previous studies (Mohler, 1988; Mohler et al., 1992), *hh* mutations associated with rearrangement breakpoints were isolated to aid in the identification of the *hedgehog* locus on the chromosome walk of the 94DE region of Mohler et al. (1991). Southern analysis mapped these *hh*-associated rearrangements within a 13 kb region from –48 to –61 on this chromosome walk, as shown in Fig. 1. Six strong *hedgehog* rearrangement mutations were isolated by Mohler (1988) by their failure to complement an adult viable allele of *hedgehog*, *bar-3*. The *bar-3* mutation itself is associated with a small deficiency of approximately 2 kb around the *Eco*RI site at –58. Three of these strong alleles, hh^{GW1}, hh^{HL1} and hh^{GS1}, associated with inversion breakpoints were found to map in a 5.5kb *Eco*RI fragment between –52 and –58. Two other strong alleles, generated by hybrid dysgenesis, hh^{HL2} and hh^{HL3}, were found to be associated with small deletions at about –51 and –59 respectively. Finally, the distal breakpoints of two cytologically visible hh^{-} deficiencies that extend proximally were mapped in this region: *Df(3R)GW2* (94D3-8,94D10-E2) had a distal breakpoint between –55 and –58, and *Df(3R)GR2* (94F11-14,94D10-E5) had a distal breakpoint between –45 and –48.

Several small deficiencies extending into the *hedgehog* locus from the adjacent *unk* gene have been produced by loss of a w^{+} transposon (P(w,ry)A, Hazelrigg et al., 1984) inserted in the *unk* gene following hybrid dysgenesis (Mohler et al., 1992). One deficiency, *Df(3R)r90b*, is genetically deficient for *hedgehog*, producing a strong phenotype, and has a proximal breakpoint between –56 and –60. Another deficiency, *Df(3R)r94a*, is embryonic lethal and fails to complement *hh* mutations, but produces no segmental defects in the larval cuticle, and has a breakpoint between –48 and –53. The unk^{r98b} deletion complements *hedgehog* lethal mutations and has a proximal breakpoint between –45 and –48. The unk^{r98b} deletion also has a unique adult dominant segment polarity phenotype, loss of the anterior portion of middle abominal tergites and duplication of posterior tergite bristles, that is indicative of a possible misregulation of *hh* imaginal expression. Finally, the site of insertion of an "enhancer trap" in *hedgehog* (hh^{H90}, P-1wB, Wilson et al., 1989) was also mapped between –48 and –50; this enhancer trap insertion fails to complement *hh* lethal alleles, but shows only a head defect and no segmental abnormalities. The *hedgehog* phenotypes of these latter three mutations (*Df(3R)r94a*, hh^{H90} and unk^{r98b}) do not fall in the normal hypomorphic series of other *hedgehog* mutations (Mohler, 1988); because these mutations disrupt or delete material near the 5′ end of the transcribed region (see below), we suspect these mutations disrupt upstream regulatory sequences.

The localization of these rearrangement mutations of *hedgehog* indicates that *hedgehog* must lie proximal to –45 and that the region between –50 and –61 is essential for *hedgehog* activity.

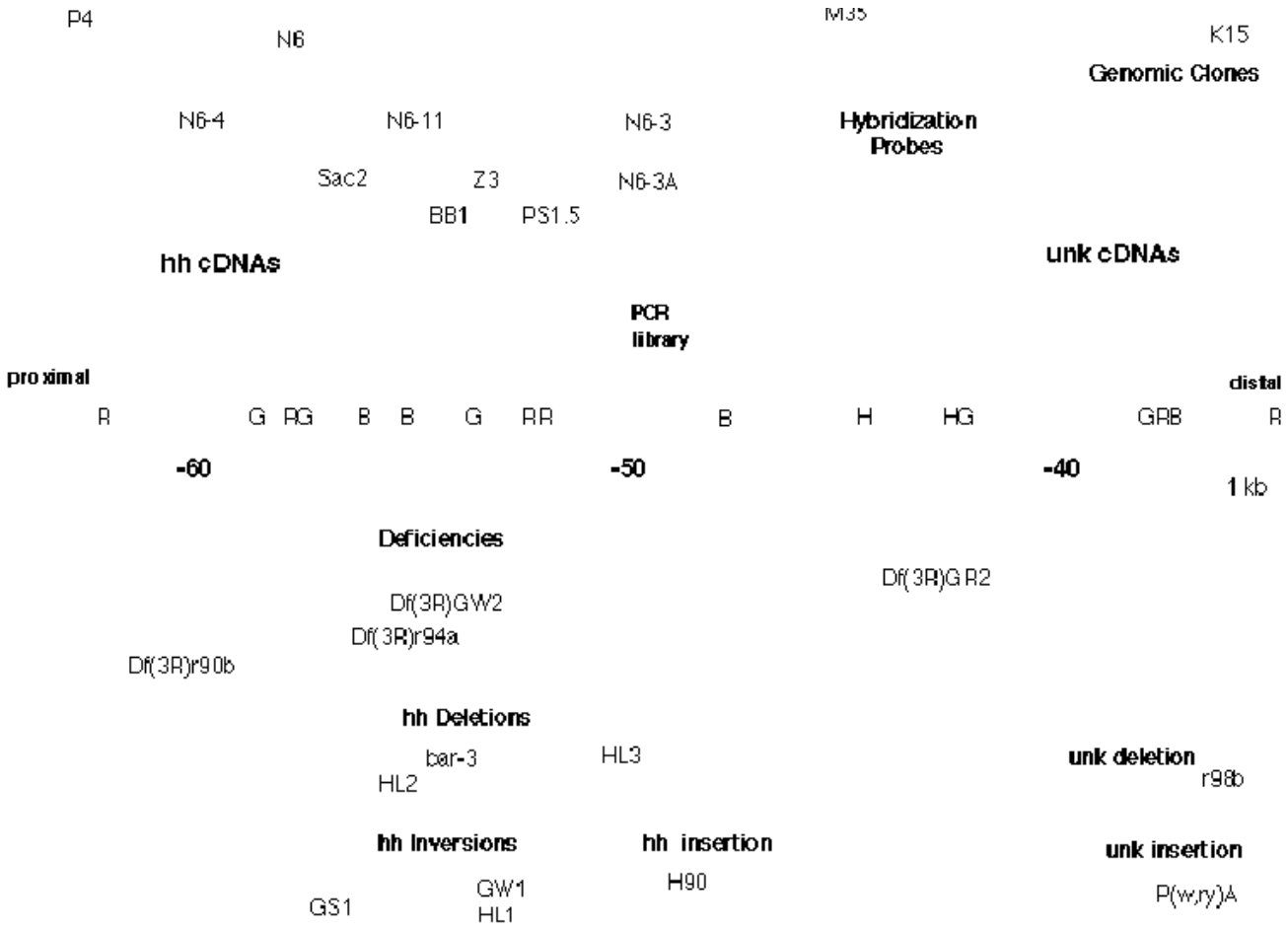


Fig. 1. Genomic Map of the *hedgehog* region. The region of the chromosome walk of Mohler et al. (1991) from −32 to –63, including the *hedgehog* and *unkempt* loci is shown. On top are the genomic lambda clones isolated in the chromosome walk and subclones of the N6 genomic clones used as hybridization probes for the characterization of the *hedgehog* region. Shown above the restriction map of genomic clones for this region is the combined extent of the six *hh* library cDNAs isolated in this study, the structure of the PCR-generated *hh* cDNA and the extent of the *unk* cDNAs (Mohler et al., 1992). Shown below the restriction map are the breakpoints of genomic rearrangements used in this study to map the extent of the *hh* region (thick lines indicate regions definitely deleted, thin lines denote the limits of uncertainty in breakpoint position). Three deletion derivatives of the P(w,ry)A insertion in *unk* (P(w,ry)A^RO20(1C), Levis et al., 1985) are shown, two of which fail to complement *hh* lethal mutations (*Df(3R)r94a* and *Df(3R)r90b*) and one of which complements the lethality of many *hh* mutations (unk^{r98b}). Three small deletions within the *hh* gene (*HL3*, *HL2* and *bar-3*), two deletions extending from *hh* proximally (*Df(3R)GW2* and *Df(3R)GR2*), three inversions broken in the gene (*GS1*, *GW1* and *HL1*), and the site of insertion of an enhancer trap (*H90* (P-lwB, Wilson et al., 1989)) are shown. Most *Drosophila* strains differ from this restriction map and contain an additional large stretch of DNA (of variable size but usually greater than 15kb) at –45 not found in the M35 genomic clone. This additional DNA does not contain restriction sites for most restriction enzymes and consists in part of a tandem repeat of 0.3kb *Eco*RV fragment. Unlike most of the genomic clones of this chromosome walk, which are derived from Oregon-R flies (Pirrotta et al., 1983) that contain this additional DNA, the M35 genomic clone was obtained from a library of DNA clones prepared from *Drosophila* DNA of "heterogeneous" sources by P. Schedl.

hh *RNA is localized to the posterior compartment*

Various DNA probes from the –48 to –58 region revealed similar patterns of RNA expression as detected by in situ hybridization. RNA is first detectable at cellular blastoderm stage in 17 segmental stripes in the embryo: 14 one-cell stripes from 10 to 70% egg-length flanked by two three-cell wide stripes at 5% and 75% egg-length and a dorsal anterior spot at 97% egg-length (Fig. 2B). Initiation of the segmental stripes is asynchronous: even parasegmental-numbered one-cell stripes are activated before adjacent odd-numbered stripes and anterior stripes are activated before more posterior stripes (Fig. 2A). These stripes are activated circumferentially around the entire embryo, but disappear from the amnio-serosa and mesoderm soon after gastrulation.

Striped expression persists through embryogenesis. Segmental stripes are prominent in the extended germ-band stage and are located in the stage 11 embryo just posterior to the parasegmental furrow (Fig. 2H) and spaced one cell anterior to the tracheal pit in each segment (Figs. 2D, I). Segmental expression persists after germ-band retraction and is localized to the posteriormost portion of the lateral

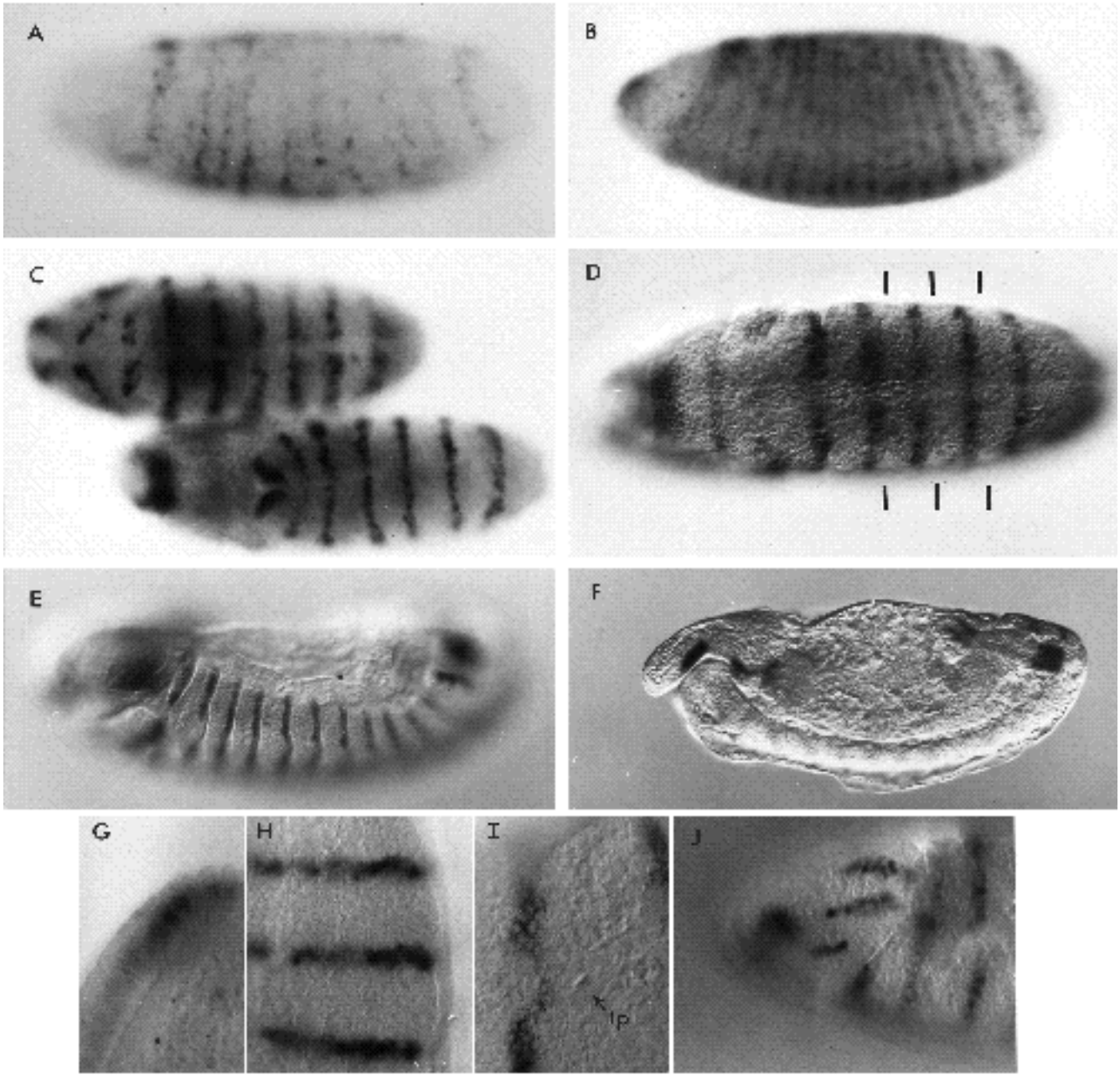


Fig. 2. Expression of *hedgehog* in *Drosophila* embryos as assayed by in situ hybridization. All figures show the results of hybridization with the N6-11 genomic probe. (A) Initiation of striped *hedgehog* expression in cycle 14 blastoderm. (B) Early gastrula, lateral view. (C) Ventral and dorsal views of stage 10 extended germ-band embryos. (D) Ventral view of stage 11 embryo, lines indicated tracheal pits of T2, T3 and A1. (E) Lateral view of stage 12 embryo. (F) Mid-sagittal view of stage 14 embryo. (G) Nuclear localization of *hedgehog* RNA in dorsal anterior cap region of cycle 14 blastoderm. (H) Registration of the stripes with respect to parasegmental furrow. Anterior is up. (I) Registration of the stripes with respect to the tracheal pit and cytoplasmic localization of RNA in stage 11 embryo. Anterior is left, tp, tracheal pit. (J) Head stripes in stage 10 embryo (lateral view)

ectoderm of each segment (Fig. 2E). This localized expression pattern is consistent with restricted expression in the posterior compartment similar to that observed for *engrailed* (Kornberg et al., 1985; DiNardo et al., 1985). RNA is also persistent in the fore- and hindguts following gastrulation and germ-band elongation, where expression can be seen anterior and lateral to the developing anterior midgut and in a ring around the posterior midgut invagination. In the stage 15 embryo, expression is limited to four distinct regions of the gut: pharynx roof, esophagus, a ring in the hindgut just posterior to the malpighian tubules and a ring in the posterior portion of the hindgut (Fig. 2F). Hybridization with single-strand probes to sectioned embryos indicated that transcription is in the distal to proximal direction (data not shown).

Expression of this RNA in the cephalic region of the embryo is consistent with the proposed segmentation plan of Cohen and Jürgens (1990). In the stage 10 embryo, in

addition to stripes corresponding to the three gnathal segments (mandibular, maxillary and labial) and the clypeolabrum, three stripes of expression ("epaulets") are seen in the ventral-lateral regions anterior to the cephalic furrow (Fig. 2J). These stripes correspond to compartments of the hypopharyngeal (intercalary) segment, the antennal lobe and the procephalic lobe. During stage 12, expression can be seen to be limited to the posterior portions of the labial lobe, maxillary lobes, mandibular segment, hypopharyngeal primordium, antennal lobe, procephalic lobe and clypeolabrum (Fig. 2E).

During early embryogenesis, up to stage 10, the intensity of staining is heavier in the nucleus than in the cytoplasm of the cell, as can be seen most clearly at late blastoderm stage (Fig. 2G). After stage 10, RNA staining is predominantly cytoplasmic (Fig. 2I). The nuclear to cytoplasmic shift occurs first during mid-stage 10 in the epidermal cells of each gnathal, thoracic and abdominal segment, and subsequentally during early stage 11 in the cephalic segments and the ventral neuroblasts of each body segment.

Cellular effects of hedgehog *mutants are coincident with cytoplasmic* hh *RNA localization*

hedgehog is a member of the "lawn"-class of segment polarity genes (Mohler, 1988). Embryos mutant for strong alleles of *hedgehog* develop larval cuticle consisting of a lawn of denticles in the ventral abdominal region of the embryo, devoid of the naked cuticle which is usually spaced between the anterior denticle rows of each segment (Mohler, 1988). In strong mutants of *hedgehog*, the first morphological defect is during stage 11, when the segment grooves fail to form. Instead deep pits form at the site of the parasegmental furrows, interdigitated between the tracheal pits, and necrotic cells appear at the base of the these pits (Fig. 3A). As the germ-band retracts during stage 12, large patches of necrotic cells appear roughly halfway between the tracheal pits of each segment (Fig. 3B), which are unaffected in *hedgehog* mutants (Fig. 3C). In slightly weaker mutations, occasional segmental grooves are present in random places along the length of the embryo (not shown). This pathology differs markedly from mutants in other segment polarity genes of the "lawn" class (*wg*, *dsh* and *arm*; Perrimon and Mahowald, 1987; Klingensmith et al., 1989), in which the first morphological defects are seen in the tracheal pits, which enlarge to form similar deep pits of cell necrosis, and eventually fuse to form a mid-lateral groove along the length of the embryo during early stage 12. Thus, while extensive cell death is involved in the genesis of the similar denticle "lawn" phenotype of both *hedgehog* mutants and the other "lawn"-class segment polarity mutants, the early embryonic phenotype of *hedgehog* mutants during stage 11 and 12 is strikingly different from the other "lawn" class mutants, especially in the anterior portion of the anterior compartment.

Altered *hedgehog* expression patterns were observed in embryos for three different *hedgehog* mutants: hh^{GS1}, a strong, apparent null, rearrangment-associated mutation located near the 3′ end of the transcribed region; hh^{13C}, a strong, apparent null "point" mutant; and hh^{HL3}, a strong hypomorphic mutation associated with a small 0.5 kb deletion located within the transcribed region. Expression in hh^{GS1} is strongly reduced as compared with wild-type levels, but appears in an apparently normal pattern through stage 11, when the mutant pathology becomes manifest. Thus, this mutation appears to affect either the transcription or stability of the *hedgehog* RNA.

Striped expression in hh^{13C} and hh^{HL3} is activated at normal levels in a normal pattern at blastoderm and persists normally to stage 10. During stages 10 and 11, expression of these mutant RNAs becomes greatly reduced (disappearing somewhat more quickly in hh^{HL3} than hh^{13C}). RNA levels become greatly reduced in all of the ectodermal tissues during stage 10, but consistently persist at normal levels in the four regions of the gut. The staining decreases first in the epidermal cells of each segment, when the RNA localization in these cells shifts from nuclear to cytoplasmic. In contrast, normal RNA levels persist in the neuroblasts on ventral mid-line cells of each stripe (Fig. 4A) and the cephalic segments to stage 11, during which time the RNA localization is nuclear. During stage 11, the deep necrotic pits form anterior to the disappearing region of hybridization. This decrease in RNA levels in the epidermal tissues corresponds in time to the shift in RNA localization from the nucleus to cytoplasm, and precedes any morphological manifestation of the *hedgehog* mutation. In the apparent null mutant, hh^{13C}, no *hh* RNA remains in the epidermal regions after stage 11. In contrast, hh^{HL3} embryos continue to express cytoplasmic *hh* RNA at normal levels in a fused cephalic expression domain and in a number of variable sized pockets in the lateral and dorsal epidermal region (Fig. 4B), infrequently comprising an intact segmental stripe of *hh* RNA expression associated with a segmental groove. This residual *hh* RNA expression at normal cytoplasmic levels, albeit only in limited regions, indicates that the decay of the *hh* RNA in hh^{HL3} mutant embryos at stage 10 is not due to an inherent cytoplasmic instability of this RNA. Instead, this premature RNA decay must reflect an altered expression state of these cells, although whether this alteration is specific to *hh* expression or is representative of a general cessation of transcription prior to eventual cell necrosis is unclear. The apparent coincidence of this decay in *hh* RNA levels in *hh* mutants with the transition in RNA localization suggests that this nuclear-cytoplasmic shift may play an important role in control of *hh* expression.

Spatial expression of hedgehog *RNAs is altered in mutants of other segment polarity genes*

The expression pattern of *hedgehog* RNA was also examined in mutants of five other segment polarity genes that potentially regulate or interact with *hedgehog*. Embryos mutant for loss-of-function alleles of *wg*, *arm* and *en* have similar effects on *hh* expression to those observed in *hh* mutant embryos. In these mutant embryos, the expression of *hedgehog* RNA is activated normally and persists through germ-band extension, but RNA levels fall during stages 10 and 11 of embryogenesis (not shown). In mutants of *wg* and *arm*, residual expression of *hh* has disappeared prior to the formation of the segmental grooves in stage 11. In contrast, in embryos mutant for *en* or deficient for both

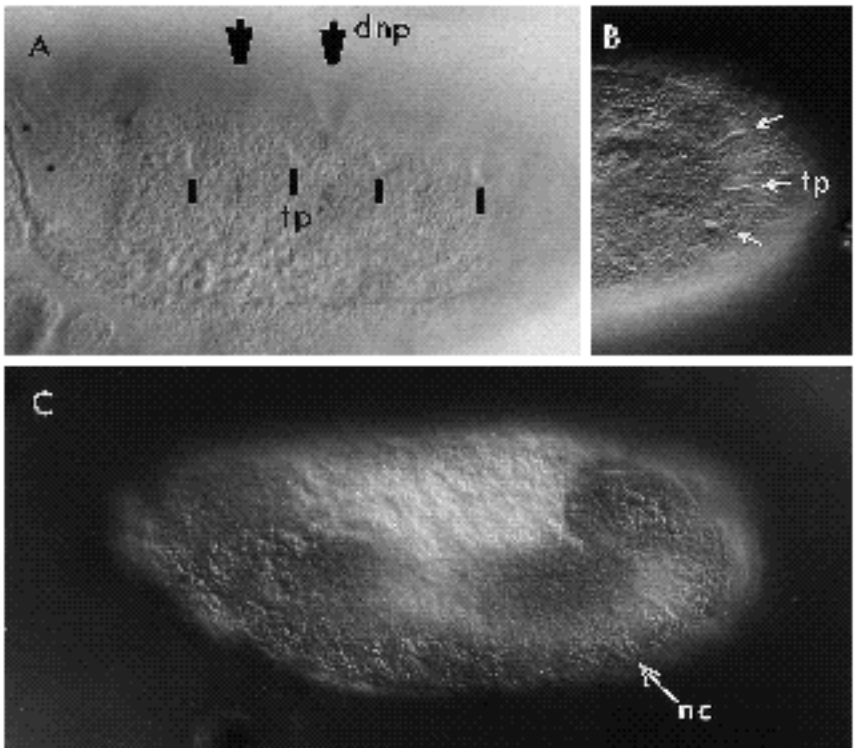


Fig. 3. Developmental pathology of *hedgehog* embryos. (A) stage 11 hh^{GS1} embryo. Lines indicate tracheal pits (tp) of four abdominal segments, arrows demark deep necrotic pits (dnp). (B) Tracheal pits in mid-abdominal segments of a stage 12 hh^{13C} homozygous embryo (tp, tracheal pit). (C) Stage 13 hh^{13C} embryo (nc, necrotic cells).

en and *inv*, expression of *hedgehog* becomes patchy only in late stage 11, with aberrant stripes at most one cell wide, and expression completely disappears during germ-band retraction. Because the effects of *wg*, *arm*, *en* and *hh* mutants on *hh* expression are similar, this common pattern of *hh* expression (normal activation followed by loss during germ-band extension) probably reflects the effect on *hh* expression (either specifically or as a transcript of a cell fated for necrosis) of the general failure of the cell-cell communication loop between the cells of the posterior compartment and the anterior adjacent cells.

In embryos mutant for ci^D, the stripe of *hh* expression is enlarged to two-cells wide at blastoderm with a two-cell interstripe and, in extended germ-band embryos, can be seen to be expanded posteriorly from the wild-type domain of expression, so that it now abuts the tracheal pit (Fig. 4C). Expression of this RNA also disappears in ci^D embryos by mid-stage 11. This expression suggests that ci^D gene is required for the proper activation of the *hedgehog* gene to prevent expression in the cells fated to give rise to the anterior compartment. The failure to maintain expression of this RNA during stage 11 may be due to a coordinate displacement and misexpression of other segment polarity genes normally active in the anterior compartment.

Embryos mutant for loss-of-function alleles of *ptc* develop normally through stage 10. In stage 11, the proper segmental grooves fail to form and "patched" furrows develop ectopically in the middle of each anterior compartment, equidistant from the cells that express *en*. Expression of *hedgehog* RNA in *ptc* mutant embryos is normal through stage 10, and in stage 11, the "patched" furrow appears equidistant between the expression stripes. In a few *ptc* embryos, a second stripe of expression transiently appears during stage 11 in each segment at the base of the novel "patched furrow" (Fig. 4D), but does not appear to persist into stage 12.

In general, the effects of these other segment polarity genes on the spatial expression of the *hedgehog* RNA are similar to their effects on the other posterior compartment segment polarity gene, *engrailed*, as reported by DiNardo et al. (1988). Because *hedgehog* is expressed normally in *engrailed* mutants into stage 11 and because of subtle differences in the expression patterns of these two genes, it is likely that the expression of these genes in the posterior compartment is in response to a common set of regulatory controls during early embryogenesis (see Discussion).

Open reading frame analysis of hedgehog *genomic DNA suggests possible spliced exons*

In order to determine the nature of the product of this locus, the sequence of the genomic region from –48 to –62 of the chromosome walk, containing all the rearrangement breakpoints associated with amorphic mutations, was determined. Fig. 5 shows 13757 bp of genomic sequence in the distal-to-proximal chromosome orientation, in keeping with the direction of transcription determined by in situ hybridization using single-strand probes and with the directionally cloned cDNAs (see below). Conceptual translation of this genomic DNA revealed 16 open reading frames of greater than 300 nucleotides on the transcribed strand and 6 such open reading frames on the opposite strand. The largest open reading frame is 579 nucleotides, which is on the transcribed strand (labelled ORF1 in Fig. 6). None of these open reading frames have significant similarity to any proteins of the PIR (R25.0) or SWISS-Prot (R14.0) databases. Three of these open reading frames, indicated as ORF1, ORF2 and ORF3 in Fig. 6, possess non-random codon usage that is significantly correlated with known codon

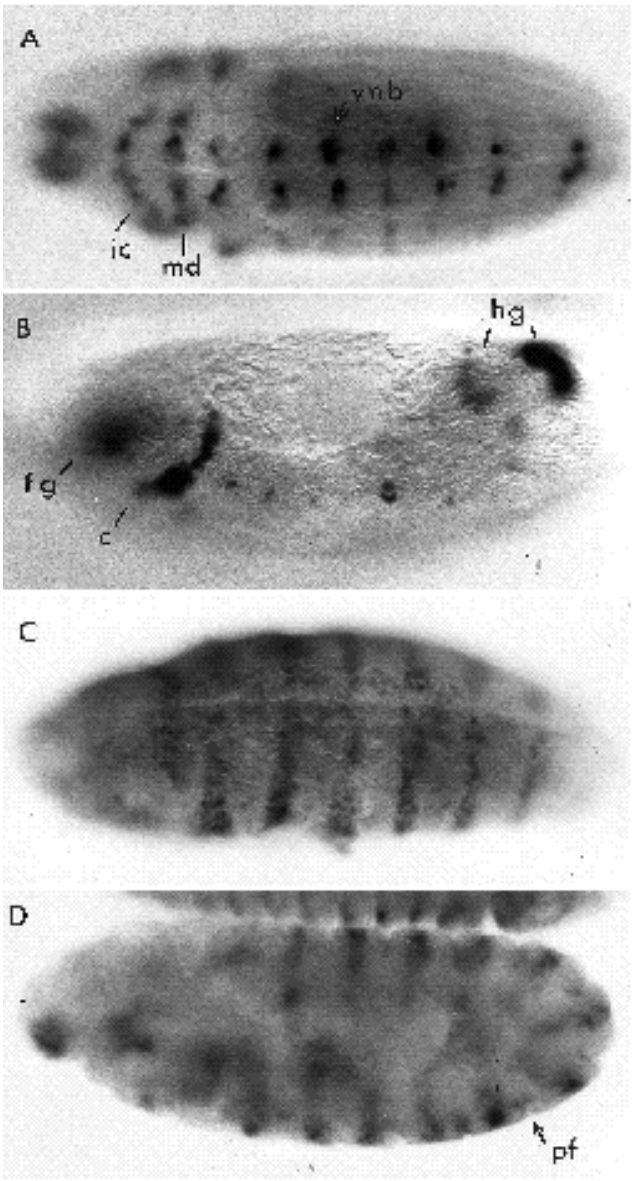


Fig. 4. *hedgehog* expression in mutants for segment polarity genes, assayed by in situ hybridization. (A,B) hh^{HL3} embryos. (A) stage 10, ventral view. ic, intercalary segment; md, mandibular segment; vnb, ventral neuroblasts. (B) stage 13, lateral view. fg, foregut; hg, hindgut; c, fused cephalic segment. (C) ci^{D} homozygote, stage 11, ventral view. (D) ptc^{IF85} homozygote, stage 11, midsagittal view (pf, ectopic "patched furrow").

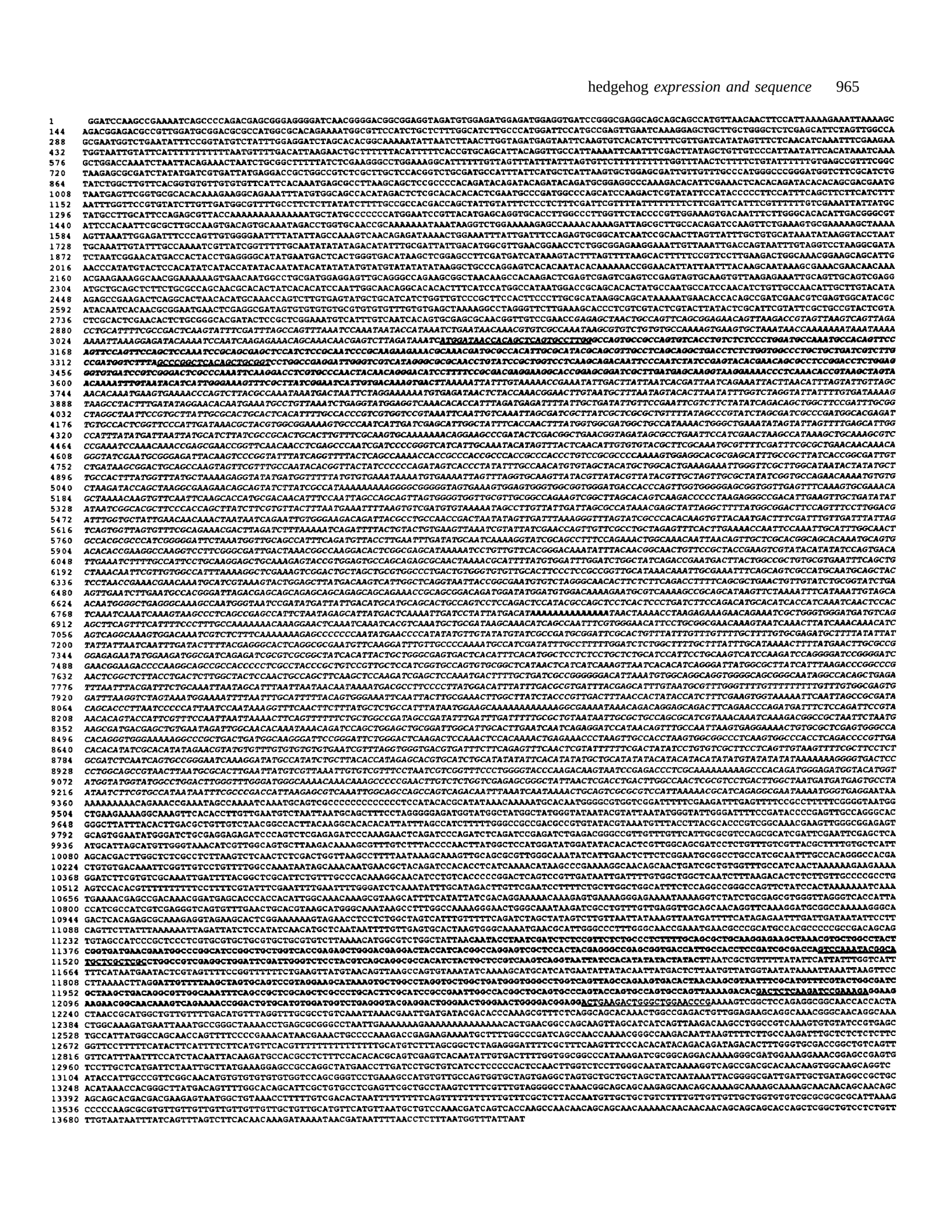
usage in *Drosophila* (Ashburner, 1989). (Correlation coefficients: ORF1, r=0.49 [P<0.001]; ORF2, r=0.64 [P<0.001]; ORF3, r=0.40 [P=0.001]). In the transcribed strand there are three sequences possessing an eight-out-of-nine nucleotide match to the consensus 5′ splice junction sequence (Mount, 1982); two of these potential 5′ splice junctions are located in ORF1 and ORF2. Near the 5′ end of ORF2 is a potential 3′ splice junction sequence with a good match to consensus lariat branchpoint and 3′ junction sequences (Mount, 1982; Ruskin et al., 1984) that would create an in-frame splice with the 5′ splice junction in ORF1. There is no obvious candidate 3′ splice junction sequence in ORF3. Because all of the strong amorphic mutants either delete ORF1 or ORF2 or are chromosomal breakpoints between ORF1 and ORF2 (Fig. 6), the genetic mapping of *hedgehog* mutants is consistent with a spliced RNA between ORF1 and ORF2 as the functional coding RNA.

Fig. 5. Sequence of *hedgehog* region genomic DNA (from –48 to –62). DNA found in library cDNA clones is indicated in italics. Sites corresponding to 8/9 nucleotide matches to the 5' splice junction consensus ($^{C}/_{A}$AGGTAAGT, Mount, 1982) are underlined and the three open reading frames with possible *Drosophila* codon usage and the A_{14} genomic site that appears to be the priming site of 5 out of 6 library cDNA clones are indicated in boldface. The nucleotide extent of each of the six cDNAs is: 5a (2823-6850), 11a (3364-6850), 10a (4064-6850), h1a (4381-6850), Z3 (4580-6821) and h7a (6855-9355). This sequence has been submitted to the EMBL sequence data base (accession number Z11840).

hh *cDNA clones are colinear with genomic DNA*

Six cDNA clones were isolated from three early embryonic cDNA libraries (from 0-4 hour and 4-8 hour RNAs) using probes that collectively span the region from –46 to –62. For four of these cDNAs (Z3, h1a, h7a and 5a), their position on the genomic map was assigned by comparison of their sequence with the genomic sequence. For the other two cDNAs (10a and 11a), their position on the genomic map was determined by sequencing the ends and alignment of internal restriction sites. All six cDNAs appear to be colinear with genomic DNA; their combined extent is 7534 bp (Figs 6 and 7) and includes ORF1 near the 5′ end (Fig. 7A). Neither of the two cDNAs isolated with a probe containing ORF1, and which overlap ORF1, utilize the potential 5′ splice junction in ORF1. Directional cloning of five of these cDNAs (Brown and Kafatos, 1988) confirms the direction of transcription determined by in situ hybridizations using single-strand probes. Hybridization of these cDNAs or of genomic DNA from various genomic regions to northern blots of embryonic RNA consistently failed to detect RNA species of defined sizes (data not shown), although subsequent rehybridization of these blots with probes to *Antp* (Garber et al., 1983; Scott et al., 1983), *ftz* (Kuroiwa et al., 1984; Wiener et al., 1984), CNC (Mohler et al., 1991), *unk* (Mohler et al., 1992), dFRA or dJRA (Perkins et al., 1989) yielded bands of appropriate size and temporal expression.

These cDNAs do not appear to encompass the entire *hh* transcription unit, as a genomic DNA probe (N6-4) entirely downstream from the 3′ end of these cDNAs hybridizes weakly to an RNA with a segmentally reiterated pattern of expression. Because the 3′ ends of five of the six cDNAs are located in either of two genomic tracts of polyA, these cDNAs appear to have been primed from an internal site within the transcript from which they were derived. (The sixth cDNA, which ends just 27 nucleotides upstream from one of these tracts of polyA, was obtained from a library



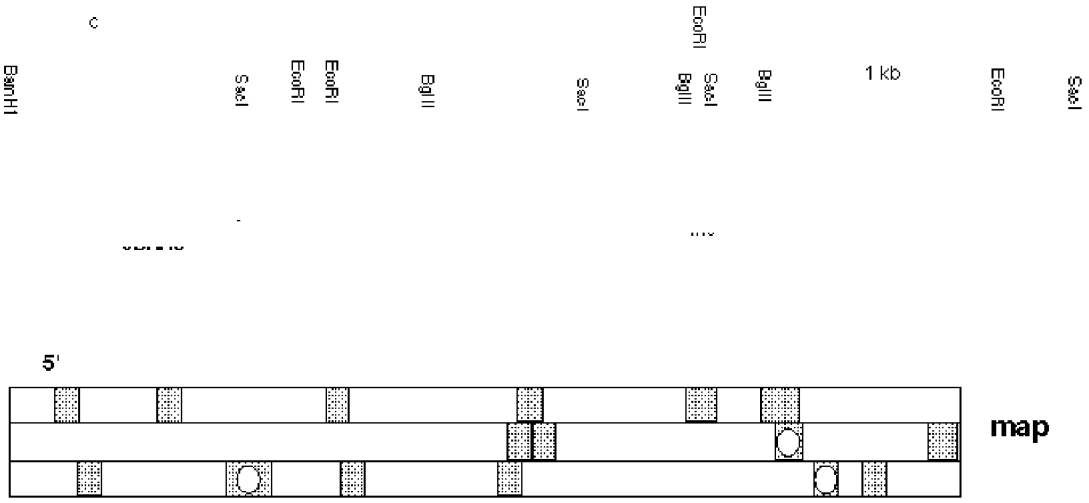


Fig. 6. The ORF map of the *hedgehog* region, oriented in the direction of transcription. The open reading frames of greater than 100 amino acids from the three possible frames of the translated strand of the genomic sequence of the *hedgehog* region from –48 to –62 are shown. The three open reading frames with *Drosophila* codon usage (ORF1, ORF2 and ORF3) have been indicated. The extent of each of the six library cDNAs is also indicated. The location of strong and weak rearrangement mutations have been redrawn from Fig. 1 and aligned beneath the open reading frames.

whose clones rarely extend as far 3′ as the oligo-dT primer site; see, for example, Frigerio et al., 1988 and Mohler et al., 1991.) Thus the RNA from which these cDNAs were derived would appear to have an unusual primary structure for a mRNA, with a 0.6kb potential protein coding region and at least 6.5kb of 3′ trailer.

PCR analysis reveals a spliced hedgehog *RNA encoding a putative transmembrane protein*

To determine whether transcripts spliced between ORF1, ORF2 and ORF3 were present in the early embryo, cDNA derived from RNA from early embryos was amplified by PCR using primers corresponding to sequences in the middle of each of the three ORFs and to the 5′ and 3′ ends of ORF1 and ORF3, respectively, and an oligo-dT primer for the potential 3′-polyA tail. Productive amplifications were only observed between primers for either the middle or the initiation codon of ORF1 and the primer corresponding to the middle of ORF2 (Table 1). The sizes of the fragments generated between these primers were consistent with use of the predicted splice-junction sequences. Sequence analysis of the larger PCR fragment confirms this splicing as is shown in Fig. 7B. Because the 5′ primer that generates this fragment includes the potential initiation codon of ORF1 (which is six nucleotides downstream of a termination codon), this sequence corresponds to the N-terminal portion of a potential protein. Unfortunately, because we were not able to determine what the subsequent exon to ORF2 is or whether the 5′ splice junction sequence at the 3′ end of ORF2 is utilized, it is not clear if this sequence corresponds to all or to only the N-terminal portion of a potential *hedgehog* protein.

The potential proteins generated either from the unspliced ORF1 or the spliced ORF1-ORF2 RNAs both contain a potential transmembrane domain, which is indicated in boldface in Fig. 7. Fig. 8 shows a hydrophobicity plot of the amino acids of the spliced ORF1-ORF2 protein as a function of position in the peptide. The region from 63 to 81 shows a nineteen-amino acid hydrophobic region, flanked by basic hydrophilic domains, typical of transmembrane domains (Rao and Argos, 1979).

Suppression of weak hedgehog *mutants by* su(w^a) *is consistent with inefficient splicing*

The preceding data suggest there are at least two types of transcripts from the *hedgehog* locus: an unspliced RNA (corresponding to cDNA isolated from cDNA libraries) and an RNA spliced between ORF1 and ORF2 (isolated from PCR-amplified cDNA). A similar situation occurs normally at the *su(w^a)* locus and at the *w* locus with a *w^a* mutation, where the coding RNA is less abundant than noncoding forms retaining specific introns (Pirrotta and Bröckl, 1984; Chou et al., 1987). At both loci, splicing efficiency is increased in mutants of *su(w^a)*, an RNA-binding protein putatively involved in splicing regulation (Zachar et al., 1985; Zachar et al., 1987). If the spliced *hh* RNA is the

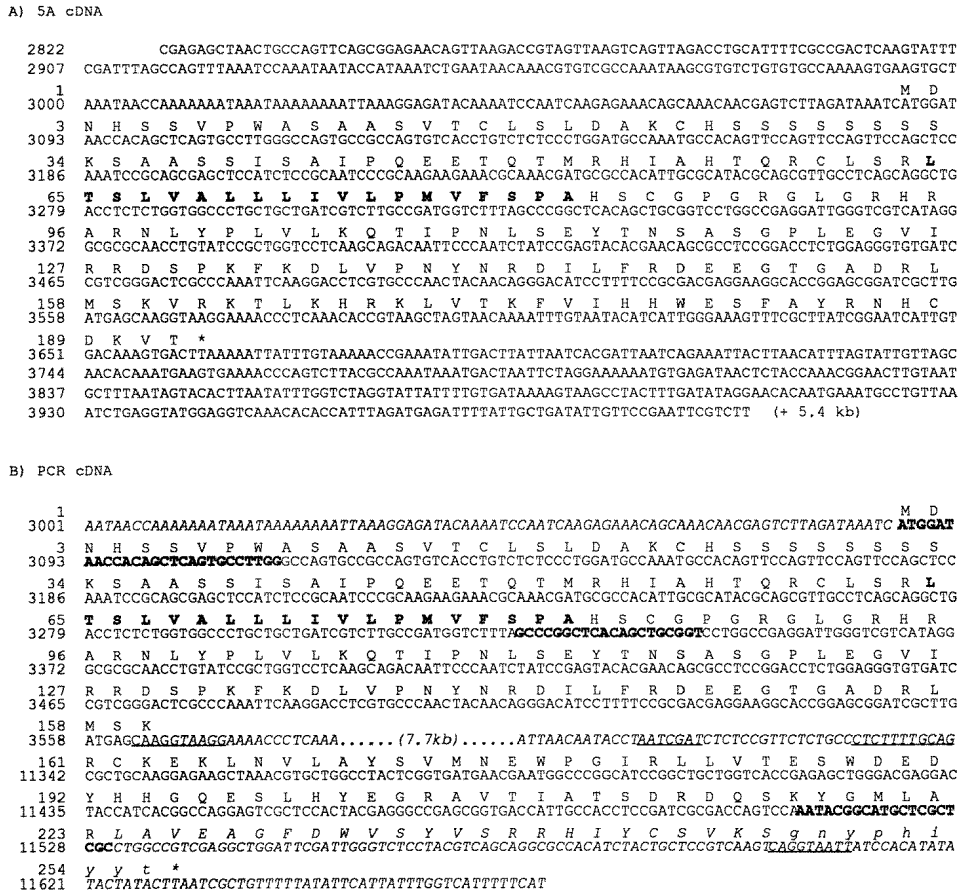


Fig. 7. Coding regions of possible *hedgehog* RNAs. The possible transmembrane domain in each potential product is indicated in boldface. (A) ORF1, coding region of library cDNAs. (B) Coding region of spliced PCR cDNA. Nucleotides and amino acids corresponding to PCR cDNA are in regular face, flanking and intronic genomic DNA and coding amino acids are in italics, and predicted amino acids C-terminal to the predicted splice junction at the 3′ end of ORF2 are in lower case. Predicted splice junction sequences are underlined, primer sequences are in bold-face.

Table 1. *PCR amplification products from primers within open reading frames with* Drosophila *codon usage*

5′ Primer	3′ Primer	Products
Start-orf1 3087	Mid-Orf2 11531	0.7 kb
	Mid-orf3 12090	-
	End-orf3 12206	-
Mid-orf1 3325	Mid-orf2 11531	0.4 kb, 0.2 kb*
	Mid-orf3 12090	-
	End-orf3 12206	-
	Oligo-T	-
Mid-orf2 11506	Mid-orf3 12090	-
	End-orf3 12206	-
	Oligo-T	-

Three 5′ primers, corresponding to the initiation codon of orf1 and internal sites of orf1 and orf2, and four 3′ primers, corresponding to internal sites in orf2 and orf3, the termination codon of orf3 and a polyA primer, were utilized. The position within the genomic sequence of the 5′ (end) nucleotide for each primer is indicated below the primer name. For each combination of 5′ and 3′ primers, the size of detected fragments is shown.

*The 0.4 kb product, but not the 0.2 kb product, hybridized to *hedgehog* DNA on Southern blots; sequence analysis of the 0.2 kb product indicated that it was derived from an mRNA of the *awd* locus (Biggs et al., 1988).

primary coding RNA, partial loss-of-function alleles of *hh* might be suppressed or ameliorated by an increase in splicing efficiency. Because the *su(w^a)* mutation could potentially cause an increase in splicing efficiency of *hh* RNAs as well, the effect of the *su(w^a)* mutation on the phenotype of *hh* alleles was investigated.

Effects of the *su(w^a)* mutation on the phenotype of *hh* mutations can be observed only for the two weakest *hh* alleles causing segmentation defects: *hh^{9K}* and *hh^{10B}* (Mohler, 1988). The *hh^{9K}* allele is a very weak allele that is embryonic viable and normal at 18ºC and lethal at 25ºC with partial denticle-belt fusions, principally of A1 and A2 and of A6, A7 and A8 at 25ºC (RANK 1 of Mohler, 1988). In a *su(w^a)* background *hh^{9K}* is embryonic viable and normal at both 18ºC and 25ºC. The *hh^{10B}* mutation is a slightly stronger allele which is lacking naked cuticle between most denticle belts of almost all segments at 25ºC, but shows little reversal of denticle polarity (RANK 2 of Mohler, 1988). In a *su(w^a)* background, *hh^{10B}* mutant embryos at 25ºC possess many denticle bands of normal morphology and the corresponding regions of naked cuticle between those bands, especially in the anterior abdominal region (RANK 1). At 18ºC, the range of phenotype of *hh^{10B}* embryos, both in the presence and absence of *su(w^a)$^+$*, are overlapping and display a RANK 1 phenotype (partial denticle-belt fusions principally of A6, A7 and A8 and of A1 and A2) at 18ºC, although *su(w^a) hh^{10B}* embryos generally contain greater areas of naked cuticle. Because a similar shift in phenotypic rank (from RANK 2 to RANK 1) can

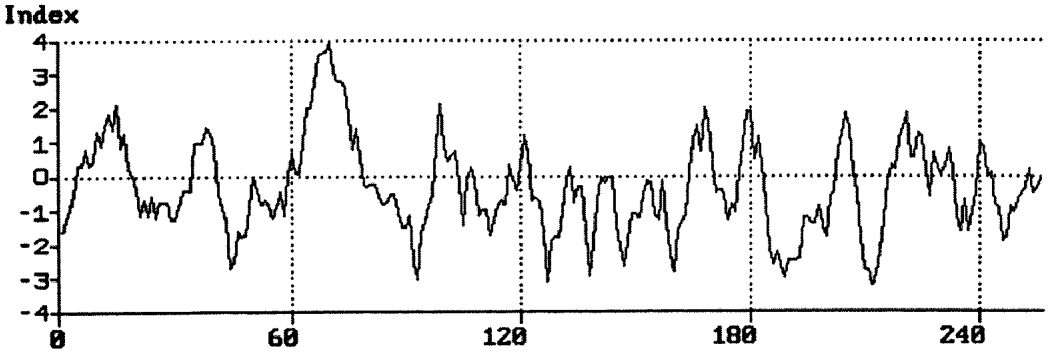


Fig. 8. Hydrophobicity blot of the predicted protein from the spliced RNA derived from PCR cDNAs. The hydrophobicity is determined using the parameters of Kyte and Doolittle (1982), with a window size of 6 amino acids.

be caused by doubling the gene dose of weak *hedgehog* alleles (i.e. between hh^{9K} homozygotes and hh^{9K}/*null* heterozygotes at 25°C or between hh^{10B} homozygotes and heterozygotes with *hh* nulls at 18°C; J. Mohler, unpublished results), we estimate the effect of $su(w^a)$ on the phenotype of these alleles is roughly equivalent to a two-fold increase in the mutant gene product at 25°C and somewhat less at 18°C. This effect of $su(w^a)$ on *hedgehog* mutants is, therefore, comparable in magnitude to its effect on the splicing efficiency of the *w* gene in w^a mutant flies (Zachar et al., 1985).

Discussion

In the results reported above, we have found that the *hedge - hog* gene appears to be expressed in the cells of the posterior compartment of the early *Drosophila* embryo. The predicted protein product apparently contains a transmembrane domain, which may allow the *hedgehog* protein to function directly in a cell communication loop involving the cells of the posterior compartment with the adjacent cells of the anterior compartment, both of which require *hedgehog* function (DiNardo et al., 1988; Martinez-Arias et al., 1988).

Coregulation of hedgehog *and* engrailed *in the posterior compartment*

The temporal and spatial expression pattern of *hedgehog* is remarkably similar to that of *engrailed* (Kornberg et al., 1985; DiNardo et al., 1985; DiNardo et al., 1988). Both genes are activated in 14 one-cell wide stripes during late blastoderm stages in the segmented portion of the embryo. For both genes, these stripes are activated in roughly an anterior-to-posterior order, with activation of the even-numbered parasegmental stripes preceding the odd-numbered stripes. After germ-band extension, expression of both genes are found located just posterior to the parasegmental furrow and anterior to the cell on the anterior side of the tracheal pit. After germ-band retraction, both genes are expressed on the posterior margin of the segment adjacent to the segmental groove. In addition, both genes respond to mutations in other segment polarity genes in a similar fashion: in mutants of *wg* and *arm*, both *hh* and *en* are activated normally but expression stops in the late extended germ-band embryo, and in *ptc* mutants, ectopic expression of both *hh* and *en* is activated in the late extended germ-band midway between their normal expression domains (comparing the results of *hedgehog* in this study with those of *engrailed* done by DiNardo et al., 1988).

There are some notable differences between the expression patterns of *hh* and *en*. The most obvious difference is the activation of *hedgehog* transcription outside the segmented body region during blastoderm stages. In contrast, *en* is activated in these regions later, during the late extended germ-band (Kornberg et al., 1985). A second difference is the failure to maintain the second ectopic stripe of expression in *ptc* mutant embryos: *en* is activated in the ectopic *ptc* furrow and that expression is maintained during subsequent embryogenesis, whereas *hh* expression in this ectopic stripe disappears before germ-band retraction. These differences suggest that the early expression pattern of *hh* is not defined through the sole action of the *en* homeobox protein. This conclusion is confirmed by the observation that activation of these stripes is normal in embryos mutant for *en* or for a deficiency for both *en* and the related *inv* genes, and is only affected after stage 11.

Identity of the functional hedgehog *RNA*

In the course of this analysis, we have recovered two classes of cDNAs, suggesting the existence of two types of *hh* mRNAs: an unspliced mRNA (corresponding to cDNA isolated from cDNA libraries) and a spliced mRNA (corresponding to PCR-amplified cDNAs). Both classes of mRNA encode possible protein products that include a transmembrane domain, suggesting that the *hedgehog* protein, which may be involved in cell-cell communication, may be located on the cell surface. Although both RNAs may encode functional *hedgehog* RNA, genetic evidence suggests that both the first and second exons of the spliced mRNA are required for *hedgehog* function and that, therefore, the spliced mRNA may be the only coding mRNA.

The spliced mRNA contains a splice from the first open reading frame in the genomic sequence utilizing appropriate *Drosophila* codon usage (ORF1) to a second one (ORF2) about 9 kb downstream. cDNA corresponding to

this class of RNA was isolated by PCR amplification from primers located within these open reading frames. This PCR cDNA can be generated using primers that include the initiation codon of the first open reading frame, suggesting that this corresponds to the N-terminal end of the encoded polypeptide. Primers for possible exons further downstream than the second open reading frame (including oligo-dT) failed to generate PCR products, so the C-terminal extent of this polypeptide encoded by this RNA is not known. However, because there is a potential 5′ splice junction with a good match to the consensus sequence (8 out of 9 nucleotides) near the end of the second open reading frame (ORF2), it is likely that this RNA is spliced to an additional exon or exons further downstream that have not yet been identified.

We believe the functional *hedgehog* product is encoded by the spliced RNA identified from the PCR cDNA for two reasons. First, the rearrangement mutations that break between ORF1 and ORF2 or remove ORF2 show a strong, essentially amorphic, *hedgehog* phenotype. Although these rearrangement mutations also affect the 3′-trailer region of the unspliced RNAs (i.e. those corresponding to library cDNAs) and could possibly affect RNA stability or nuclear/cytoplasmic partitioning of such RNAs, it is noteworthy that a small deletion (*bar-3*) removing the apparent 3′-terminus of these RNAs, but not affecting ORF2, is adult viable with an effect predominantly on eye facet number. Second, because of the apparent ability of the *su(w^a)* mutation to increase splicing efficiency of poorly spliced RNAs, the suppression effect of *su(w^a)* on the phenotype of weak *hedgehog* alleles is consistent with an increase in amount of a partially functional protein mediated through an increase of a spliced messenger RNA encoding that protein. We believe the unspliced cDNAs correspond to either unspliced nuclear precursor RNAs or to cytoplasmic RNA from transcripts that have been prematurely polyadenylated.

Possible roles of the hedgehog *protein in cell communication across the parasegmental boundary*

The presence of a transmembrane domain in the putative *hedgehog* product suggests that the *hedgehog* product may be present on the surface of the posterior compartment cell where it could potentially mediate the interaction with the adjacent anterior cell. There are two possible roles for such a cell surface *hedgehog* protein in the communication loop in which *hedgehog* is involved: as part of the signaling pathway from the posterior compartment cell to the adjacent *wg*-expressing cell, or as part of a receiving network in the posterior compartment cell of the signal(s) from the adjacent anterior cell. Because the communication between these two cells is a closed-circuit loop, in which each cell is required to maintain the transcription state of the other, and because mutations in *hedgehog* have profound effects on both cells, there is no clear evidence for which process the *hedgehog* product functions.

hedgehog function is required in the extended germ-band stage for continued expression of *en* in the posterior compartment and of *wg* in the immediately adjacent anterior cell. In *hedgehog* mutants, *wg* expression decays during stage 10 of embryogenesis (Hidalgo and Ingham, 1990), whereas *en* expression decays during stage 11 (DiNardo et al., 1988). Because the effect on the *wg*-expressing cell is apparent first, the primary effect of *hedgehog* is more likely to be in the signaling by the *en*-expressing cell of the *wg*-expressing cell. The further fact that mutants in *ptc* (a membrane protein expressed in anterior compartment cells required to repress *wg* expression from more anterior cells in the anterior compartment) can suppress the lawn phenotype of strong *hh* mutants, has led to a simple model in which a *hedgehog* signal from the posterior compartment inactivates the *ptc* protein in the adjacent anterior compartment cell allowing continued *wg* expression (Hidalgo and Ingham, 1990; Ingham et al., 1991). While there is no substantiation of this model at a biochemical level, the possible cell-surface location of the *hedgehog* protein implied by its transmembrane domain is consistent with this model, in which the membrane bound *hedgehog* protein may act as a non-diffusible signal on the anteriorly adjacent cell. Alternatively, it is equally possible that *hedgehog* might be necessary for generation or release of a diffusible signalling molecule from the posterior compartment cell to the adjacent anterior cell.

While such a model for the role of the *hedgehog* product is formally consistent with the known data, a number of slightly more complex models cannot be ruled out. For instance, if a *wg* signal were required to elicit generation of a responding signal from the neighboring posterior compartment cell, *hedgehog* could function in the reception of the *wg* signal and still be formally required for signaling the adjacent *wg*-expressing cell. However, if *wg* is also required for the correct patterning of anterior compartment cells at a later stage in embryogenesis, as is suggested by cuticular phenotypes following late temperature-shift experiments of a *wg^{ts}* allele, in which only cells of the anterior compartment have adopted an altered fate (Baker, 1988; Bejsovec and Martinez-Arias, 1991), the expression pattern of *hedgehog* would be inconsistent with an expected pattern for a single *wg* receptor, which should be present at some stage in all the epidermal cells. Similarly, the observation that the first morphological manifestation of *wg* mutations is the enlargement of the tracheal pits in the anterior portion of the anterior compartment (Perrimon and Mahowald, 1987), which are essentially unaffected by *hh* mutations, indicates that the response to the *wg* signal is not mediated exclusively through *hedgehog*. Thus, if *hedgehog* does mediate the reception of the *wg* signal in the posterior compartment cell, it must do so in a compartment-specific fashion.

Finally, it should be noted that these two possible roles for the *hedgehog* protein are not mutually exclusive. For example, the effects of mutations of *armadillo*, a *Drosophila* homolog of plakaglobin/β-catenin (Peifer and Wieschaus, 1990; McCrea et al., 1991), suggest that cadherin-mediated cell adhesion might play a role in this closed-circuit loop between the *wg*-expressing cells and cell of the posterior compartment. If the role of *hedgehog* is to modulate formation or positioning of a cadherin/plakoglobin complex by the cells of the posterior compartment, the potential effects of *hedgehog* mutations on such an intercellular adhesive complex could directly affect functions in both adhering cells.

Possible levels of post-transcriptional regulation of hedgehog *expression*

The behavior of the *hedgehog* RNA during early *Drosophila* embryogenesis suggests two possible levels of control that may regulate the timing of expression of the *hedgehog* product: splicing efficiency and RNA nuclear export. The existence of inefficient, and possibly regulated, splicing of *hedgehog* RNAs is evident from the different types of cDNA: unspliced cDNAs obtained from cDNA libraries and PCR cDNAs generated from distant primers, as is noted above. The possibility of regulated nuclear export of *hedgehog* RNA is inferred from shifts in the intracellular localization of RNA detected by in situ hybridization using genomic *hedgehog* DNA as probe, which is predominantly nuclear prior to stage 10 and cytoplasmic by stage 11.

Transcripts of *hedgehog* first appear at the blastoderm stage and are restricted to the nucleus. High nuclear and low cytoplasmic concentrations of this RNA persist to the middle of the extended germ-band stage (stage 10), 60 to 90 minutes later, when the RNA localization becomes predominantly cytoplasmic. This developmentally regulated shift in *hedgehog* RNA localization does not appear to be dependent on splicing of ORF1 and ORF2, since in situ hybridization with probes (N6-11 and the Z3 cDNA) for sequences between ORF1 and ORF2 also shows this shift in nuclear/cytoplasmic localization. This nuclear localization appears to be general throughout the nucleus, unlike the nuclear localization to one locus observed for nascent transcripts of large transcription units (Shermoen and O'Farrell, 1991).

Post-transcriptional regulation delaying translation of *hedgehog* products might be functionally significant in delaying the "editing" phase of segment polarity gene activity to a time when cell-cell contacts have been stabilized. The segmentation pattern of the embryo is established in the unilayer blastoderm as the spatially reiterative pattern of transcription states (reflected in the expression of such genes as *en*, *gsb*, *wg*, *ptc*, ci^D and *hh*) along the anterior-posterior axis (see review by Ingham, 1988; also Kornberg et al., 1985; Bopp et al., 1986; Baker, 1987; Hooper and Scott, 1989; Nakano et al., 1989; Orenic et al., 1990). The embryo then undergoes two major morphogenetic movements, gastrulation and germ-band extension, to reorganize the embryo. Following these movements, the known segment polarity genes are thought to provide key regulatory functions in the "editing" process (DiNardo et al., 1988), that result in either respecification or death of inappropriately positioned cells. Given this scenario, premature activation of these "editing" genes might be expected to have dire consequences for subsequent development. Premature activation during these extensive cell movements would occur at a time when cellular neighbors are labile and might result in inappropriate respecification of cell fate, if the appropriate cell neighbors are transiently misaligned. It is reasonable, therefore, that mechanisms should exist that delay expression of critical cell-specific "editing" genes, from the time of the establishment of a specific segmental transcription state until the end of germ-band extension. These potential post-transcriptional controls on *hedgehog* expression (inefficient splicing and delayed nuclear export) might provide such a mechanism.

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