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Control of germ-band retraction in *Drosophila* by the zinc-finger protein HINDSIGHT

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SUMMARY

***Drosophila* embryos lacking *hindsight* gene function have a normal body plan and undergo normal germ-band extension. However, they fail to retract their germ bands. *hindsight* encodes a large nuclear protein of 1920 amino acids that contains fourteen C_2H_2-type zinc fingers, and glutamine-rich and proline-rich domains, suggesting that it functions as a transcription factor. Initial embryonic expression of *hindsight* RNA and protein occurs in the endoderm (midgut) and extraembryonic membrane (amnioserosa) prior to germ-band extension and continues in these tissues beyond the completion of germ-band retraction. Expression also occurs in the developing tracheal system, central and peripheral nervous systems, and the ureter of the Malpighian tubules. Strikingly, *hindsight* is not expressed in the epidermal ectoderm which is the tissue that undergoes the cell shape changes and movements during germ-band retraction. The embryonic midgut can be eliminated without affecting germ-band retraction. However, elimination of the amnioserosa results in the failure of germ-band retraction, implicating amnioserosal expression of *hindsight* as crucial for this process. Ubiquitous expression of *hindsight* in the early embryo rescues germ-band retraction without producing dominant gain-of-function defects, suggesting that *hindsight*'s role in germ-band retraction is permissive rather than instructive. Previous analyses have shown that *hindsight* is required for maintenance of the differentiated amnioserosa (Frank, L. C. and Rushlow, C. (1996) *Development* 122, 1343-1352). Two classes of models are consistent with the present data. First, *hindsight*'s function in germ-band retraction may be limited to maintenance of the amnioserosa which then plays a physical role in the retraction process through contact with cells of the epidermal ectoderm. Second, *hindsight* might function both to maintain the amnioserosa and to regulate chemical signaling from the amnioserosa to the epidermal ectoderm, thus coordinating the cell shape changes and movements that drive germ-band retraction.**

Key words: *hindsight*, zinc finger, germ-band retraction, *Drosophila*, morphogenesis

INTRODUCTION

In metazoa morphogenetic cell shape changes and movements are important in the formation of complex three-dimensional body structures (Bard, 1994). The genetic approach — particularly in *Drosophila* — is beginning to yield important insights into the mechanisms that control and coordinate these morphogenetic processes. Such genetic strategies have previously illuminated the mechanisms by which cells are assigned positional values and are fated to contribute to particular tissue types (Lewis, 1996; Nüsslein-Volhard, 1996; Wieschaus, 1996). During *Drosophila* embryogenesis, several coordinated morphogenetic processes occur shortly after blastoderm cellularization is completed (Campos-Ortega and Hartenstein, 1985; Costa et al., 1993; Martinez Arias, 1993). First, mesoderm is internalized through the formation of the ventral furrow. The endoderm then invaginates from each end of the embryo as the anterior and posterior midgut. Concurrent with posterior midgut invagination, germ-band extension drives cells around the posterior tip of the embryo, converting it into a 'U-shape' folded upon itself dorsally. Several hours later, the germ band retracts back around the posterior tip, repositioning the caudalmost body parts at the posterior end of the embryo.

The cellular events and the genetic control of several of these morphogenetic processes are now under study. For example, it has been shown that mesodermal and posterior midgut invaginations occur in two phases (Kam et al., 1991; Sweeton et al., 1991). The first is slow and stochastic, with isolated cells within the presumptive mesoderm and endoderm initiating apical constriction. The second phase is rapid with all of the unconstricted cells in these domains simultaneously undergoing apical constriction (Kam et al., 1991; Sweeton et al., 1991). Two known loci — *concertina* (*cta*) and *folded gastrulation* (*fog*) — are required for the transition from the first to the

second phase (Zusman and Wieschaus, 1985; Parks and Wieschaus, 1991; Sweeton et al., 1991; Costa et al., 1994). *cta* encodes a G_α-like protein that is produced maternally and deposited in the egg and early embryo (Parks and Wieschaus, 1991). *fog* encodes a novel putative secreted protein that is expressed in the invagination primordia in a pattern that precisely prefigures the pattern of apical cell constrictions (Costa et al., 1994). It has been suggested that these two gene products constitute part of a signaling pathway that coordinates the group behaviour of the cells undergoing morphogenetic alterations. Neither *cta* nor *fog* mutations affect the assignment of positional values to cells; rather they specifically disrupt coordinate cell shape changes.

In contrast to the mesodermal and endodermal invaginations, germ-band extension is not driven by cell shape changes but by cell rearrangements (Irvine and Wieschaus, 1994): extensive intercalation of cells results in a decrease in cell number along the dorsoventral (D-V) axis of the embryo with a concomitant increase in cell number along the anteroposterior (A-P) axis.

Germ-band retraction is attained through a combination of cell shape changes and cell rearrangements, with the former playing a more important role than the latter (Campos-Ortega and Hartenstein, 1985; Martinez Arias, 1993). Cell shape changes in the epidermal ectoderm account for a 40% reduction along the A-P axis and an 85% increase across the D-V axis relative to the unretracted state (Martinez Arias, 1993). Local cell rearrangements produce a further 10% decrease along the A-P axis with a concomitant 15% increase across the D-V axis (Martinez Arias, 1993). These cell shape changes and local cell rearrangements begin in the thoracic region and spread posteriorly (Martinez Arias, 1993).

Expression of six genes is required zygotically for germ-band retraction: the *Drosophila* homolog of the mammalian EGF receptor (variously called *top*, *flb* and DER and referred to here as *Egfr*) (Clifford and Schüpbach, 1989; Raz et al., 1991), the *Drosophila* homolog of the mammalian insulin receptor (encoded by the *inr* gene) (Fernandez et al., 1995), *hindsight* (*hnt*) (Wieschaus et al., 1984; Strecker et al., 1991, 1992), *tailup* (*tup*) (Nüsslein-Volhard et al., 1984), *u-shaped* (*ush*) (Nüsslein-Volhard et al., 1984) and *serpent* (*srp*) (Jürgens et al., 1984; Reuter, 1994). Four of these genes, the *Egfr* (Clifford and Schüpbach, 1992), *hnt*, *ush* and *srp* (Frank and Rushlow, 1996) are required for the maintenance of the differentiated amnioserosa.

In this report, we present our analysis of the *hnt* mutant phenotype and report the molecular cloning and analysis of the expression of the *hnt* gene. Our results indicate that *hnt* does not affect pattern formation or tissue specification prior to germ-band retraction. The sequence of the *hnt* cDNA and localization of HNT protein in nuclei suggest that it functions as a zinc-finger-containing transcriptional regulator. Strikingly, *hnt* is not expressed in the epidermal ectoderm that undergoes the cell shape changes and movements that drive germ-band retraction. Rather, *hnt* is expressed in the endoderm and amnioserosa prior to, during and after retraction. Based on analysis of single and double mutants that eliminate expression of *hnt* in the regions that normally form midgut and/or amnioserosa, we argue that *hnt* expression in the amnioserosa is crucial for germ-band retraction. Two models are presented: a 'physical' model in which *hindsight* functions to maintain the differentiated amnioserosa which then controls retraction through direct physical interaction with cells of the germ band; and a 'chemical' model in which *hindsight* functions to maintain the amnioserosa, which then produces or activates a signal that is received by the germ band and coordinates germ-band retraction.

MATERIALS AND METHODS

Drosophila genetics and culture conditions

Flies were raised on standard medium at 25°C unless otherwise specified. The original *hnt* alleles, hnt^{XE81} and hnt^{X001}, were isolated by Wieschaus et al. (1984). Two additional alleles, hnt^{EH587} and hnt^{EH704a}, were obtained in a subsequent EMS mutagenesis screen (Eberl and Hilliker, 1988). We report here, that a putative fifth allele, *l(1)EH275a* (Eberl and Hilliker, 1988) also referred to as hnt^{EH275a} (Ray, 1993), is in fact *not* allelic to *hnt*. Most other mutations used in this study are described in detail in Lindsley and Zimm (1992); alleles used were: $cact^{A2}$, $cact^{HE9}$, cta^{WU31}, $Egfr^{f1}$, fog^{S4}, hkb^{2}, peb^{1}, pll^{078}, pll^{385}, sax^{HB18}, sax^{WO18}, srp^{9L}, tld^{9}, tll^{1}, tor^{PM51}, tor^{splc} and zen^{f62}. $tld^{68\text{-}62}$ is described in Shimell et al. (1991). All zygotic lethal mutations were maintained over appropriate 'blue balancer' chromosomes harboring a P-element transgene that expresses β-galactosidase under the control of the *ftz* promoter: *FM7Z* (Kania et al., 1990), *CyOZ* (Raz and Shilo, 1993), *TM3Z* (S. Smolick-Utlaut and E. B. Lewis, personal communication). Flies carrying the temperature-sensitive rough-eyed mutation, *pebbled*, were raised at 28±1°C, the restrictive temperature.

Analysis of embryonic cuticles and quantitative analysis of germ-band retraction

Embryos were collected for 24 hours and allowed to age at least an additional 24 hours. In most cases, cuticles were prepared by clearing dechorionated embryos in mounting medium as described in Ashburner (1989), except that embryos were not fixed before mounting. In cases where the vitelline membrane was removed for analysis, unhatched embryos were dechorionated with 50% bleach for 2 minutes, transferred to methanol:heptane (1:1) and vortexed to remove vitelline membranes. Embryonic cuticles were then fixed, mounted and analyzed as previously described (Lamka et al., 1992). For quantitative analysis of the germ-band retraction defects, hatched versus unhatched embryos were counted and the latter class was then processed for cuticle analysis. The extent of germ-band retraction was measured by noting which abdominal segment was located at the posterior tip of the embryo; half-segment measures were used to increase resolution (see Results; Figs 1, 2). Complete retraction positions the telson at the tip.

Time-lapse videomicroscopy

Embryos were collected for 0.5 hour intervals from balanced heterozygous *hnt* females. The embryos were submerged in halocarbon oil and were filmed under bright-field illumination at 21±1°C for more than 12 hours, usually in groups of three to four at a time. Filming was done using a Dage-MTI CCD72 videocamera attached to a Nikon Diaphot-TMD inverted microscope and recorded with a Hitachi/GYYR time-lapse VCR.

Histology and immunohistochemistry

Antibody staining of embryos was according to established procedures (Macdonald and Struhl, 1986; Patel et al., 1989). Primary antibodies were mouse anti-ABD-B monoclonal antibody (mAb) (1:2 dilution) (Celniker et al., 1989); rat anti-CUT (1:300) (Blochlinger et al., 1990); mouse anti-FKH (1:33) (Y. M. Kuo and S. K. Beckendorf, personal communication); rabbit anti-KR (1:200) (Gaul et al., 1987; M. Levine, personal communication); rabbit anti-LAB (1:75) (Diederich et al., 1991); $mAbD_3$ (1:15) (68G5D3) (Giniger et al., 1993); mAb 22C10 (1:2) (Fujita et al., 1982); mouse anti-β-galac-

tosidase (1:200 to 1:500) (Promega, Inc.); mouse monoclonal anti-HNT antibody 27B8 1G9 (1:10 to 1:20) (see below). Secondary antibodies were alkaline phosphatase-conjugated goat anti-mouse, goat anti-rabbit or donkey anti-rat antisera (Jackson Immunoresearch) used at 1:500-1:2000 dilution or goat anti-mouse or goat anti-rabbit antisera conjugated to horse radish peroxidase (HRP) (Jackson Immunoresearch) used at a dilution of 1:300. Since all zygotic lethal mutations were maintained over appropriate 'blue balancer' chromosomes (above) mutant embryos were identified by simultaneously staining batches of embryos with both anti-β-galactosidase and the particular antibody of interest.

Nucleic acid manipulations and analysis

Standard protocols were as described (Sambrook et al., 1989). Overlapping phage genomic DNA clones covering about 70 kb proximal to the proximal deficiency breakpoint of *Df(1)rb*^{46} (Pflugfelder et al., 1990) were hybridized to ^{32}P-labeled cDNA probes synthesized from 0-3 hour embryonic poly(A) RNA. A 4.5 kb *Bam*HI fragment from phage clone X-59 (which substantially overlaps clone X-32) and located at +130 kb on the chromosomal walk (Pflugfelder et al., 1990) was identified as hybridizing to the cDNA and was then used to screen a cDNA library (Poole et al., 1985). We isolated a cDNA clone, designated E20, with a 2 kb insert and, using this as a probe, we screened a second embryonic cDNA library to isolate longer cDNA clones (Brown and Kafatos, 1988). Iterative screening using probes derived from the 5′-most portions of progressively longer cDNAs resulted in the isolation of long cDNA clones encompassing the entire open reading frame (e.g. NB701). Sequencing of cDNA inserts was done using the Sequenase kit (US Biochemical Corp.) or the Cycle Sequencing Kit (Applied Biosystems, Inc.).

Molecular analysis of mutant alleles

Genomic DNA was isolated from *hnt* mutant embryos using at least 100 embryos for each preparation. The polymerase chain reaction (PCR) was used to amplify fragments of the *hnt* coding region from this genomic DNA; for each amplified fragment, triplicate PCR reactions were set up and processed simultaneously. Primers were: forward 5′-GCCAGTCTCTCGGAATCGGG-3′ derived from nucleotides 719-738 of the *hnt* cDNA sequence; reverse 5′-TCGCAGGCGGGACAACTTAG-3′ derived from nucleotides 1806-1787 of the *hnt* cDNA sequence. After PCR amplification, the triplicate reactions were combined and the amplifed fragment was purified using QIAquick PCR Purification Kit (Qiagen). Amplified DNA was then sequenced. The primers relevant to the point mutation in *hnt*^{X001} were: forward 5′-TGCTATCCTCGGCTTCATCC-3′ derived from nucleotides 1110-1129 of the *hnt* cDNA sequence; reverse 5′-CTGCGACTGTGACTATGTCCAC-3′ derived from nucleotides 1473-1452 of the *hnt* cDNA sequence.

Germ-line transformation and phenotypic rescue

A 6.3 kb *Ssp*I-*Not*I fragment containing the entire NB701 cDNA was cloned into the *Hpa*I-*Not*I sites of the *pCaSpeR-hs* vector (Thummel and Pirrotta, 1991) and transformed into the germ line (Rubin and Spradling, 1982; Spradling and Rubin, 1982). Two transgenic lines were obtained: *hs-hnt:E* (X chromosome) and *hs-hnt:M* (2nd chromosome). For rescue experiments, virgin females from each of the four *y hnt* mutant stocks were mated to males from the *hs-hnt:M* transgenic line. The experimental crosses were: *y hnt/FM7Z* × *w*^{1118}*/Y; hs-hnt:M/hs-hnt:M*; control crosses were: *y hnt/FM7Z* × *w*^{1118}*/Y*. All progeny from the experimental crosses carry one copy of the *hs-hnt* transgene and were collected on yeasted grape juice-agar plates at 2 to 2.5 hour intervals. 3.5 or 4 hours after collection, embryos were heat shocked twice at 36.5°C for 0.5 hour per treatment with an interval of 0.5 hour at 25°C between heat shocks. Embryos were then allowed to develop at 25°C before cuticles were mounted for analysis. *hnt* mutant embryos were identified on the basis of the *y* marker.

Whole-mount RNA tissue in situ hybridization

Whole-mount RNA tissue in situ hybridization was based on previously published protocols (Tautz and Pfeifle, 1989; Ding et al., 1993). Digoxigenin (DIG)-labeled *hnt* DNA probe was synthesized from a 2 kb *Eco*RI fragment containing the 3′ end of *hnt*. DIG-labeled *hnt* antisense RNA probe was synthesized by in vitro transcription off the T7 promoter using cDNA NB701 as template. Both DNA and RNA probes gave identical results.

Expression of HNT fusion protein and production of anti-HNT antibodies

A 907 bp *Bgl*II-*Bam*HI fragment from cDNA NB701 (nucleotides 2710 to 3617; codons 824 to 1125) encoding a 302 amino acid HNT polypeptide was cloned into the *Bam*HI site of *pGEX1* (Smith and Johnson, 1988). The GST-HNT fusion protein was expressed and purified from *E. coli* (Ausubel et al., 1987). To generate anti-HNT antibodies, 50 μg of purified GST-HNT fusion protein suspended in RIBI adjuvant (RIBI Biochem) was injected into three Balb/c mice (Simenson). Antisera from the mice were tested by ELISA against the fusion protein and on fixed *Drosophila* embryos. The blood from the mouse that produced the strongest response was saved as anti-HNT polyclonal antiserum. This mouse was then killed for monoclonal antibody production that followed standard procedures (Harlow and Lane, 1988) and used HL-1 myeloma cells (Hycor). Monoclonal supernatants were tested on fixed embryos. The monoclonal anti-HNT antibody used in most of our experiments is designated 27B8 1G9 and was used at a 1:10 to 1:20 dilution of supernatant.

RESULTS

hindsight function is required for the morphogenetic movements that drive germ-band retraction

Four embryonic lethal alleles of the X-linked gene *hindsight* (*hnt*) have been reported (Wieschaus et al., 1984; Eberl and Hilliker, 1988; Lindsley and Zimm, 1992). A fifth potential allele, *l(1)EH275a* (Eberl and Hilliker, 1988), is not allelic to *hnt* (see below). We demonstrate here and in a separate study that *pebbled* (*peb*) mutations are viable alleles of *hnt* (M. L. R. Y., Q. Sun, M. L. L. and H.D. L., unpublished data). The four embryonic lethal alleles *hnt*^{XE81}, *hnt*^{X001}, *hnt*^{EH704a} and *hnt*^{EH587} display qualitatively similar phenotypes: hemizygous (*hnt/Y*) embryos fail to retract their germ bands (Figs 1, 2). All such embryos have the correct number of thoracic and abdominal segments which are patterned normally. As a consequence of failed germ-band retraction, the embryos are U-shaped with their posterior region folded onto the dorsal side (Fig. 1B-D). Additionally, mutant embryos show defects in head involution and often have a severely disrupted cephalopharyngeal skeleton (Fig. 1B-D) (Ray, 1993; Yip, 1995; Frank and Rushlow, 1996).

l(1)EH275a (Eberl and Hilliker, 1988) has previously been classified as a semi-lethal *hnt* allele and referred to as *hnt*^{275a} (Ray, 1993). Germ-band retraction occurs in most *l(1)EH275a* embryos, but these usually die with head defects and occasional dorsal holes. Normal looking *l(1)EH275a* escaper adult males can emerge but they are often sterile, sometimes exhibiting partially unrotated external genitalia. Thus it has not previously been possible to determine whether *l(1)EH275a* is indeed allelic to *hnt* (Eberl and Hilliker, 1988). We have recently used an autosomal duplication of the wild-type *hnt* locus to carry out inter se complementation tests among all putative *hnt* alleles (B. Reed and H. D. L., unpublished obser-

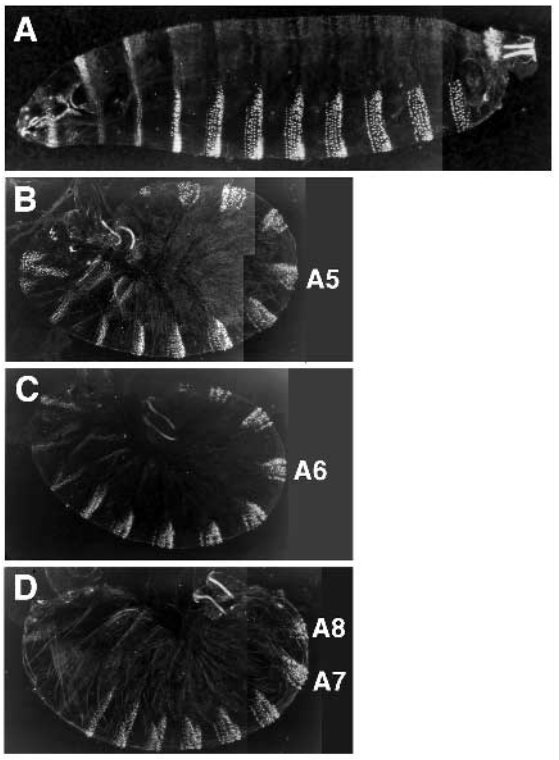

Fig. 1. *hindsight* mutant cuticular phenotypes. (A) Wild-type stage 17 embryonic cuticle; (B-D) cuticles of stage 17 hemizygous hnt^{X001} mutant embryos. (B) The 'strong' unretracted germ-band phenotype. Cuticle of a hemizygous hnt^{X001} mutant embryo with abdominal segment 5 (A5) at the posterior pole. Note the normal thoracic and abdominal segments and the abnormal head skeleton. (C) An 'intermediate' germ-band retraction phenotype with A6 at the posterior pole. (D) A 'weak' germ-band retraction phenotype with A7.5 at the posterior pole. Embryos are oriented with anterior to the left and dorsal toward the top of the page.

vations). *l(1)EH275a* complements embryonic lethal *hnt* alleles (hnt^{XE81}, hnt^{X001}, hnt^{EH704a}) (B. Reed and H. D. L., unpublished observations). *l(1)EH275a* also complements a chromosomal deletion, *Df(1)ovoG6* (Pflugfelder et al., 1990), that removes most of the *hnt* locus (B. Reed and H. D. L., unpublished observations). In addition, *l(1)EH275a* complements the viable *hnt* allele, hnt^{peb} (M. L. R. Y. and H. D. L., data not shown; see below). We thus conclude that *l(1)EH275a* is not allelic to *hnt*.

We have identified an additional, viable *hnt* allele that was originally named *pebbled* (*peb*) because of its rough eye phenotype (Lindsley and Zimm, 1992). None of the four embryonic lethal alleles complements the *pebbled* rough eye phenotype (Table 1), suggesting that *hindsight* and *pebbled* are allelic. Detailed analyses of *hnt* functions during eye development will be reported separately (M. L. R. Y., Q. Sun, M. L. L. and H.D. L., unpublished data). We shall refer to *peb* henceforth as hnt^{peb}. Table 1 presents a summary of *hnt* mutant phenotypes.

While previous studies have carried out a survey of cuticular and/or amnioserosal defects, to date there has been no quantitative analysis of germ-band retraction in *hnt* mutant embryos (Eberl and Hilliker, 1988; Ray, 1993; Frank and Rushlow, 1996). We quantified germ-band retraction by examining populations

Table 1. *hindsight* mutant phenotypes

Alleles	Embryonic lethality	Germ-band retraction	Complementation of *pebbled* rough eye phenotype
hnt^{XE81}	lethal	– – –	no
hnt^{EH587}	lethal	– –	no
hnt^{EH704a}	lethal	– –	no
hnt^{X001}	lethal	–	no
hnt^{peb}	viable	+	no

– – –, strong failure of retraction (mode of retraction distribution at abdominal segment 5.0, see Fig. 2).
– –, intermediate failure of retraction (mode at 6.0, see Fig. 2).
–, weak failure of retraction (mode at 7.0, see Fig. 2).
+, retraction complete.

of mutant embryos and plotting the distribution of abdominal segments lying at the posterior tip (Fig. 2; see Materials and Methods). The embryonic-lethal *hnt* alleles represent a phenotypic series with respect to failure of germ-band retraction (Figs 1, 2). When hemizygous, a deletion of the *hnt* locus, $Df(1)bi^{D3}$ (Banga et al., 1986; Oliver et al., 1988), results in 100% of the embryos with abdominal segment 4.5 to 6.0 at the posterior tip (Fig. 2A). The strongest *hnt* allele studied, hnt^{XE81}, is similar to the deletion in that the mode of the distribution lies at abdominal segment 5.0. However, this distribution is broader than that produced by the deletion; 12% of the embryos have abdominal segment 6.5 or greater at the posterior tip (Fig. 2B), a situation not seen with $Df(1)bi^{D3}$. Weaker still are hnt^{EH704a} and hnt^{X001}; respectively, 36% and 68% of the embryos have segment 6.5 or greater at the posterior tip (Fig. 2C,D). hnt^{EH587} is very similar in distribution to hnt^{EH704a} (data not shown).

hindsight mutants do not exhibit defects in pattern or tissue specification prior to germ-band retraction

We wished to determine whether the morphogenetic process of germ-band retraction failed in *hnt* mutants without significant defects in tissue specification or pattern formation. With the exception of the head, the cuticle of *hnt* mutant embryos does not show any pattern defects (Fig. 1) (Yip, 1995). In order to study the internal tissues, we examined embryos carrying the strongest mutant allele hnt^{XE81} with a panel of antibodies that recognize proteins/epitopes with different temporal and spatial expression patterns and functions (Table 2). With one exception, all of these markers exhibited no obvious differences between hnt^{XE81} mutant embryos and wild-type embryos prior to germ-band retraction. The exception, KRÜPPEL, which accumulates in wild-type embryos in the nuclei of amnioserosal cells, is absent from most but not all of these cells in stage 11 *hnt* mutant embryos (Fig. 3) (Ray, 1993).

hindsight mutants undergo normal germ-band extension

Our examination of the external and internal tissues of *hnt* mutants revealed neither pattern abnormalities nor defects in tissue specification prior to germ-band retraction. However, it remained possible that, while cell position and tissue specification were normal, earlier morphogenetic events were abnormal and thus that failure of germ-band retraction was a secondary consequence of such defects. For example, if *hnt* mutants were

defective in the timing or the spatial aspects of germ-band extension, germ-band retraction might fail as a consequence of inappropriate positioning of tissues to receive a 'retraction signal'. In order to avoid the ambiguities inherent in analyses of fixed material, we used time-lapse video microscopy of living *hnt*^{EH704a}*/Y* mutant embryos to determine whether any morphogenetic defects were visible in the mutants at these earlier stages. Videotaped *hnt* mutant embryos were identified on the basis of their failure to retract their germ bands by 13 hour post-fertilization and these embryos were then studied retrospectively for earlier morphogenetic events. +/+, *hnt* /+, +*/Y* and *hnt/Y* mutant embryos extend their germ bands at indistinguishable rates and with similar spatial relationships. The first difference appears after germ-band extension is complete: three quarters of the embryos (presumably *hnt*/+, +/+ or +/*Y*) initiate germ-band retraction normally while the remaining quarter of them (presumably *hnt/Y*) either did not initiate retraction or initiated premature retraction-like movements (4 hours after the beginning of germ-band extension in *hnt/Y* versus 7.5 hours in *hnt*/+, +/+ or +/*Y* siblings at 21 ± 1°C) but failed to complete the process. The proportion of the embryos that underwent retraction-like movements correlated with the strength of the *hnt*^{EH704a} allele determined on the basis of cuticle analysis: 7/11 videotaped mutant embryos (57%) underwent some retraction-like movements, consistent with the fact that 59% of the *hnt*^{EH704a} cuticles exhibited abdominal segment 6.0 or greater at the posterior tip (Fig. 2C). We conclude that the *hnt* gene is not required for early embryonic pattern specification, tissue specification or earlier morphogenetic events, such as germ-band extension.

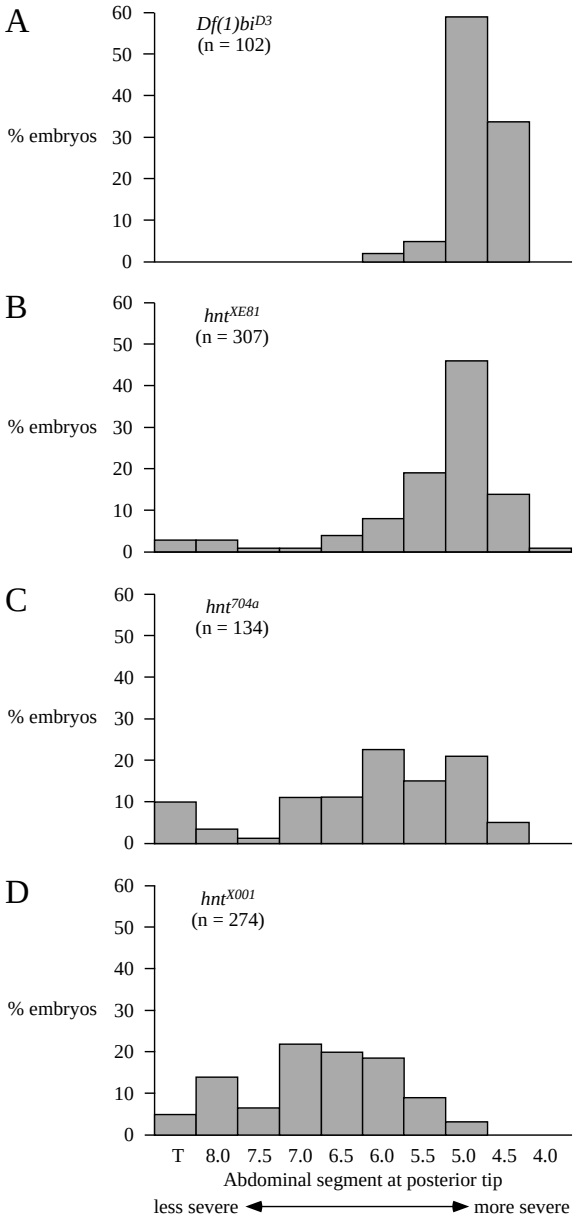

Fig. 2. Quantitative analysis of germ-band retraction in embryonic lethal *hindsight* mutants. Populations of unhatched embryos (*n*, number of unhatched embryos) were scored for the abdominal segment at the posterior tip (abscissa) (see Materials and Methods and Results for details). The percentage (%) of the unhatched embryos with a particular abdominal segment at the posterior is given on the ordinate. (A) *Df(1)bi*^{D3}. Deletion of the *hnt* gene results in 98% of the embryos with abdominal segment 5.0±0.5 at the posterior tip. (B) *hnt*^{XE81}. This strongest allele of *hnt* results in 79% of the embryos with abdominal segment 5.0±0.5 at the posterior tip. (C) *hnt*^{704a}. This intermediate *hnt* allele results in only 41% of the embryos with abdominal segment 5.0±0.5 at the posterior tip. (D) *hnt*^{X001}. This weak *hnt* allele results in only 12% of the embryos with abdominal segment 5.0±0.5 at the posterior tip.

The *hindsight* gene encodes a putative zinc-finger transcription factor

Previous studies mapped *hnt* within polytene chromosome region 4C5/6 (Oliver et al., 1988; Lindsley and Zimm, 1992). In contrast to published data (Oliver et al., 1988), we found that the deficiency chromosome, *Df(1)rb*^{46}, complements the rough eye phenotype of *hnt*^{peb}, placing *hnt* proximal to the proximal breakpoint of *Df(1)rb*^{46}. A chromosomal walk that spanned the proximal breakpoint of *Df(1)rb*^{46} had been conducted in this region (Pflugfelder et al., 1990). Using over-

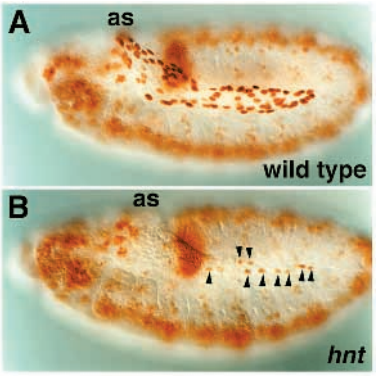

Fig. 3. KRÜPPEL expression is lost from most cells of the amnioserosa (as) of *hnt* embryos. (A) Germ-band-extended Oregon-R (wild-type) stage 11 embryo stained for KRÜPPEL protein. Note the presence of KRÜPPEL in the amnioserosa. (B) Germ-band-extended *hnt*^{XE81}/Y mutant stage 11 embryo stained for KRÜPPEL protein. Note the absence of KRÜPPEL from most but not all cells of the amnioserosa (arrowheads point to amnioserosal nuclei that contain KRÜPPEL). Other aspects of KRÜPPEL expression are normal. Embryos are oriented with anterior to the left and dorsal toward the top of the page.

Table 2. Molecular markers used to examine *hnt* mutant embryos

Molecular marker	Relevant tissue marked	Expression in *hnt* mutant embryos
α-Abdominal B	Hindgut, posterior spiracles	Normal
α-cut	Malphigian tubules, peripheral nervous system	Normal
α-forkhead	Invaginating foregut, midgut, hindgut, salivary glands	Normal
α-Krüppel	Amnioserosa, central nervous system, muscle precursor cells	Loss of most amnioserosal expression; otherwise normal
α-labial	Endodermal cells around second midgut constriction	Normal
mAb D_3	Developing tracheal system	Normal
mAb 22C10	All neuronal cells	Normal

lapping phage clones proximal to the breakpoint (kindly provided by Dr G. Pflugfelder), we initiated a search for early embryonic transcripts encoded by the region. One *Bam*HI fragment at +130 kb on their walk was identified and used to screen two embryonic cDNA libraries (see Materials and Methods). Two cDNAs, denoted E20 (the first to be identified) and NB701 (one of the longest) were analyzed in detail.

Sequence analysis of NB701 and E20 revealed that E20 was a partial cDNA and that both clones were otherwise identical in sequence. NB701 contained a single large open reading frame (ORF) of 1920 codons with 241 bp of 5′- and 163 bp of 3′-untranslated regions. Conceptual translation of the ORF is shown in Fig. 4A. It encodes a protein with several characteristics of transcription factors [analyses were conducted using the BLAST algorithm (Altschul et al., 1990)]: 14 C_2H_2 type zinc fingers (Fig. 4B) in widely spaced clusters, multiple glutamine-rich domains, proline-rich domains, serine/theronine-rich domains and acidic/charged domains (Fig. 4C).

We confirmed that this cDNA is encoded by the *hnt* gene in three ways. First, we made an *hsp70* promoter-*hnt* cDNA transgene using the NB701 clone and produced transgenic flies using P-element-mediated transformation (Rubin and Spradling, 1982; Spradling and Rubin, 1982). This transgene rescues germ-band retraction in embryos mutant for any of the four embryonic lethal alleles (Table 3). On average, 50% of the heat-shocked genotypically *y hnt* embryos carrying one copy of the transgene fully retracted their germ bands (‘T’ at posterior tip). In contrast, <5% of control hnt^{XE81} and hnt^{X001} embryos complete germ-band retraction (Table 3 cf. Fig. 2). The heat-shock regimen used did not rescue the *hnt* mutant head defects, nor did it induce any dominant pattern or morphogenetic defects. Second, we identified the molecular lesion

Table 3. Rescue of germ-band retraction in *hindsight* mutant embryos using a *hs-hnt* transgene

Embryonic genotype	Age at first heat shock	Age at second heat shock	% of mutant embryos rescued (*n*)
y hnt^{XE81}/Y	4-6 hours	5-7 hours	<5 (43)
y hnt^{X001}/Y	4-6 hours	5-7 hours	<5 (21)
y hnt^{XE81}/Y; *hs-hnt:M*	4-6 hours	5-7 hours	44 (70)
y hnt^{XE81}/Y; *hs-hnt:M*	3.5-6 hours	4.5-7 hours	46 (82)
y hnt^{X001}/Y; *hs-hnt:M*	4-6 hours	5-7 hours	63 (27)
y hnt^{X001}/Y; *hs-hnt:M*	3.5-6 hours	4.5-7 hours	52 (62)
y hnt^{EH587}/Y; *hs-hnt:M*	4-6 hours	5-7 hours	51 (83)
y hnt^{EH587}/Y; *hs-hnt:M*	3.5-6 hours	4.5-7 hours	73 (56)
y hnt^{EH704a}/Y; *hs-hnt:M*	4-6 hours	5-7 hours	65 (46)
y hnt^{EH704a}/Y; *hs-hnt:M*	3.5-6 hours	4.5-7 hours	84 (67)

n, total number of *y hnt* embryos scored.

caused by the EMS-induced allele hnt^{X001}. Sequence analysis of fragments PCR-amplified from hnt^{X001} genomic DNA revealed a C to T transition that introduced a premature stop codon at amino acid residue 348 in place of a glutamine (CAG to TAG) (Fig. 4A,C). The truncated protein is predicted to contain only 3 of the 14 zinc fingers. Third, none of the four lethal *hnt* alleles shows detectable immunostaining when examined with our anti-HNT antibodies (data not shown). We conclude that we have cloned the *hnt* gene and that it encodes a putative zinc-finger transcription factor.

hindsight is not expressed in the epidermal ectodermal cells that undergo shape change and movement during germ-band retraction, but is expressed in the amnioserosa and midgut

The embryonic expression of *hnt* was determined using whole-mount tissue in situ hybridization to visualize *hnt* RNA (data not shown) and anti-HNT antibodies to visualize HNT protein (Fig. 5). HNT protein is localized to nuclei as expected of a transcription factor. No *hnt* mRNA or protein is detectable before stage 5 of embryogenesis, consistent with the fact that analysis of embryos produced by homozygous clones of *hnt* cells in the germ line revealed no requirement for *hnt* expression or function during oogenesis (Wieschaus and Noell, 1986). *hnt* mRNA accumulation begins in the cellular blastoderm (stage 5) in a posterior-terminal domain corresponding to the posterior midgut primordium and dorsally in the presumptive amnioserosa (stages are according to Campos-Ortega and Hartenstein, 1985). HNT protein appears in these cells slightly later, at stage 6 (Fig. 5A). During stage 7, dorsal expression expands to cover the entire presumptive amnioserosa from the cephalic furrow to the posterior midgut primordium (Fig. 5B). Anteroventral staining, corresponding to the anterior midgut primordium, is first detected at stage 8 (Fig. 5C). Accumulation in these tissues continues as gastrulation proceeds (Fig. 5D). Commencing at stage 11, accumulation is also detectable in the cells of the emerging larval peripheral nervous system and the tracheal system (Fig. 5E-G). Expression also occurs in peripheral and CNS glial cells (M. L. L. and H. D. L., unpublished data). The complex expression pattern of *hnt* suggests that it may have multiple functions during embryogenesis. Functions during the development of the embryonic nervous system (M. L. L. and H. D. L., unpublished data) and during adult eye development (M. L. R. Y., Q. Sun, M. L. L. and H. D. L., unpublished data) will be reported elsewhere. Here we focus on *hnt* functions in germ-band retraction since the first detectable morphogenetic defect in *hnt* mutants is failure of this process. Strikingly, *hnt* is absent from the epidermal ectodermal cells that undergo the cell shape changes and movements that drive germ-band retraction (Martinez Arias, 1993). Since *hnt*

A

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1    MLAAQQQHNNSTVVLEMERQRRDSTTSESSLEHLDLGRTPKKLGGNSGSTQTTSTPHELA
61   TVTSSRKRKIRHLQLNHHQQQQHHQQSDLLSDEDVVEAEAEEDEDDEDGDQVAALGSRNL
121  GRHKQRRSGGATTQASIVMDYSSGDASSLRKKFRLNRSAASLSESGFVDASSTTGHSGYL
181  GNSSSATNTTATSGIGASAVAPSPVGGAAINAASSSSGSSSGGSGGSSPQGQCLSSGESG
241  IGAGDEHMKYLCPICEVVSATPHEFTNHIRCHNYANGDTENFTCRICSKVLSSASSLDRH
301  VLVHTGERPFNCRYCHLTFTTNGNMHRHMRTHKQHQVAQSQSQSQQQQSLQQQQQSQQQR
361  RQQQQHQPSQQQQNPAQQQLMGNTLSAGAESYESDASCSTDVSSGHSHSRSSSSLNNNNN
421  NSHKANNNLKDLEELEVSTEDQDTENKQRRLKTTINNNIIESEQQEDMDDEEADDADVAM
481  LTSTPDVATLLAGASASGAASRSPTPSPSASPALLLSCPACGASDFETLPALCVHLDAMH
541  SDIPAKCRDCEVIFATHRQLQSHCCRLPNALAGGLPPLLGASSSPLHNEEPEDEEHGDDE
601  DLEQKERLASQSEDFFHQLYLKHKTANGCGAISHPPSPIKHEPADTKDLADIQSILNMTS
661  SSSSFLRNFEQSVNTPNSSQYSLDGRDQEEEAQDAFTSEFRRMKLRGEFPCKLCTAVFPN
721  LRALKGHNRVHLGAVGPAGPFRCNMCPYAVCDKAALVRHMRTHNGDRPYECAVCNYAFTT
781  KANCERHLRNRHGKTSREEVKRAIVYHPAEDAGCEDSKSRLGEDLADTSFRSISPTPPPP
841  PVNESKSQLKHMLLGENHLAPVNQQPPLKIQVKSLDQLVDKKPSAPAPQQQQQQQQEKS
901  GSALDFSMDVLDLSKKPTGGASLTPAVTRTPTPAAVAPVTPGGVGTPDLAAAIEQQQLLL
961  AQQQLFGAGGEYMQQLFRSLMFQSQTSGFPFFPFMAPPPPQANPEKPPMVSPPNRINPMP
1021 VGVGVGVPVPPGGPVKMVIKNGVLMPKQKQRRYRTERPFACEHCSARFTLRSNMERHVKQ
1081 QHPQFYAQRERSAHHVMRGRGASNVAAAAAAAAAAAAPTVMAGGPGSSGFGSNHHHGHGH
1141 GSHGHAPISEQVKCAILAQQLKAHKNTDLLQQALAHGSSSVAGNPLLHFGYPLTNPSPMH
1201 NGSSQGNGQATAMDDDEPKLIIDEDENEHDHEVEAEDVDDFEEDEDEEEMDEPEDEPELI
1261 LDEQPAEKEAEEEQELPKPLEQLGTKEAAQKMAETILEQAIKAGKPLSPPPTKENASPAN
1321 PTVATTMQEPAITAPSTNPSSLKTMIAQAEYVGKSLKEVASSPFKDESQDLVPVAKLVDN
1381 ATSQNMGFNSYFRPSDVANHMEQSDEEGLVASGSASESNNSGTEDVTSSSSSSEPKKKSA
1441 YSLAPNRVSCPYCQRMFPWSSSLRRHILTHTGQKPFKCSHCPLLFTTKSNCDRHLLRKHG
1501 NVESAMSVYVPTEDVSEPIPVPKSVEEIELEEQRRRQEAEREKELELERERELERERELE
1561 RERQLEKEKERERQQLIQKLAAQMNAAATAAAVVAAASAVNGGASGGPHGPIADALAGGD
1621 LPYKCHLCEGSFAERLQCLEHIKQAHAHEYALLLAKGAIETESLEANPHQQPSQQAVHSD
1681 DEAPNGGGNRGKYPDYSNRKVICAFCLRRFWSTEDLRRHMRTHSGERPFQCDICLRKFTL
1741 KHSMLRHMKKHSGRAHNGDTPGSDCSDDEQVSSPPSTPHPTQPTSANNNNSCHNNNNNAN
1801 NNNNNNNNNNNNSSSKLGLKLHDLLDKASEWRASRLGEHKENMGEATPSGATVAGSDLIG
1861 NLLGISDQGILNKLLSSRTRRPNFWVWTTSERNSSDNRATPRAINTGVAAVLHRLTYTKA
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B

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C2H2 Zn-finger Consensus    E[K/R]P[F/Y]xCxxCxxxFxxx[X/S]xLxxHxxxHTG

Finger  1    247    HMKYLCPICEVVSATPHEFANHIRCHNY
Finger  2    279    TENFTCRICSKVLSSASSLDRHVLVHTG
Finger  3    307    ERPFNCRYCHLTFTTNGNMHRHMRTHKQ
Finger  4    513    ALLLSCPACGASDFETLPALCVHLDAMHS
Finger  5    542    DIPAKCRDCEVIFATHRQLQSHCCRLPN
Finger  6    706    RGEFPCKLCTAVFPNLRALKGHNRVHLG
Finger  7    738    AGPFRCNMCPYAVCDKAALVRHMRTHNG
Finger  8    766    DRPYECAVCNYAFTTKANCERHLRNRHGK
Finger  9   1056    ERPFACEHCSARFTLRSNMERHVKQQHPQ
Finger 10   1445    PNRVSCPYCQRMFPWSSSLRRHILTHTG
Finger 11   1473    QKPFKCSHCPLLFTTKSNCDRHLLRKHGN
Finger 12   1620    DLPYKCHLCEGSFAERLQCLEHIKQAHAH
Finger 13   1698    NRKVICAFCLRRFWSTEDLRRHMRTHSG
Finger 14   1726    ERPFQCDICLRKFTLKHSMLRHMKKHSG
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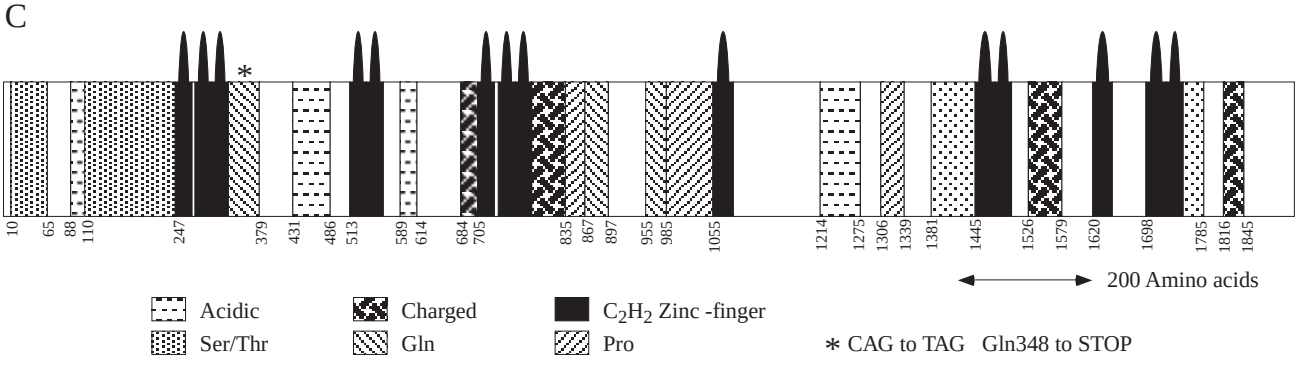
RNA and HNT protein accumulate in the endoderm and amnioserosa prior to germ-band retraction, it seemed likely that *hnt* expression in one or both of these tissues plays a role in the control of this morphogenetic process (see below).

hindsight expression is regulated by the terminal and dorsal-ventral patterning pathways

We carried out a survey of the genetic control of *hnt* expression in order to enable us to begin to dissect which aspects of *hnt* expression are important for germ-band retraction. *hnt* expression in the midgut is controlled by the maternal and zygotic members of the *torso*-mediated 'terminal' pathway as suggested by our previous genetic studies (Strecker et al., 1989, 1991, 1992; Strecker and Lipshitz, 1990). Embryos produced by homozygous *torso* loss-of-function mutant females lack HNT expression in the posterior midgut which lies within the domain of *tor* function. Instead of extending their germ bands dorsoanteriorly, most such embryos form spiralled germ bands (Fig. 6A) (Schüpbach and Wieschaus, 1986, 1989). Reciprocally, embryos from homozygous $torso^{splc}$ gain-of-function mothers lack dorsal expression (i.e. in the presumptive amnioserosa) consistent with conversion of central cell fates to more terminal ones (Schüpbach and Wieschaus, 1989; Strecker et al., 1989, 1991, 1992; Strecker and Lipshitz, 1990). These embryos also show expanded expression of HNT in the enlarged posterior midgut primordium and twisted gastrulation (Fig. 6B) (Yip and Lipshitz, 1996). Two genes, *tailless* (*tll*) and *huckebein* (*hkb*), have been identified as key zygotically expressed components of the *torso*-mediated terminal pathway (Klingler et al., 1988; Strecker et al., 1989; Weigel et al., 1990). *tll*

Fig. 4. Sequence of HINDSIGHT. (A) Predicted amino acid sequence of the HNT protein based on cDNA sequence analysis (nucleotide sequence not shown but entered in the GenBank database: accession number U86010). The cysteine and histidine residues in the zinc fingers are underlined and the residue mutated from a glutamine to a stop codon (#348) in hnt^{X001} is in bold. (B) Alignment of the 14 zinc fingers. (C) Schematic representation of the HNT protein sequence indicating the zinc fingers and domains rich in glutamine, proline, serine/threonine, acidic or charged residues.

C



mutant embryos have an abnormal acron anteriorly, and lack abdominal segments 8-10, hindgut and Malpighian tubules posteriorly (Strecker et al., 1986, 1988; Pignoni et al., 1990). *hkb* is required for the formation of endodermal midgut and stomodeum (Weigel et al., 1990; Brönner et al., 1994). *tll* mutations have little effect on HNT expression (Fig. 6C); from analysis of *hkb tll* double mutant embryos (Fig. 6E), it is clear that the only loss of HNT expression in *tll* mutants occurs in the region from which the Malpighian tubule primordia originate, consistent with the reported role for *tll* and *hnt* in the development of these structures (Strecker et al., 1986, 1988; Harbecke and Lengyel, 1995). *hkb* mutant embryos lack HNT expression in the regions from which the anterior and posterior midgut normally arise; expression remains only in the presumptive ureter of the Malpighian tubules (Fig. 6D). In *hkb tll* double mutant embryos, HNT is not expressed at all in the domains that would form anterior and posterior midgut and Malpighian tubule primordia; expression does, however, occur in the amnioserosa (Fig. 6E). Germ-band retraction occurs in *tll* or *hkb* single mutants (see Fig. 7B) as well as in *hkb tll* double mutants, suggesting that midgut expression of HNT is not necessary for germ-band retraction.

HNT expression was assayed in four mutants that are defective in germ-band retraction: *serpent* (*srp*), *u-shaped* (*ush*), *tailup* (*tup*) and the EGF receptor (*Egfr*) (Nüsslein-Volhard et al., 1984; Clifford and Schüpbach, 1989). HNT expression is not affected in *ush*, *tup* and *Egfr* mutants (Fig. 6G shows an *Egfr* mutant; data not shown for *ush* and *tup*). These results suggest that *hnt* either resides upstream of these three genes in the same hierarchy or one or more of these genes functions in a parallel pathway involved in germ-band retraction. In contrast, endodermal expression of HNT is missing in *srp* mutant embryos (Fig. 6F). This last result is consistent with the fact that *srp* is required to establish the identity of the endodermal midgut; loss-of-function mutations in *srp* result in transformation of the endoderm into ectoderm (Reuter, 1994).

HNT expression in the amnioserosa is regulated by the dorsoventral pathway. Dorsal HNT expression is reduced in genetically ventralized embryos such as those produced by *saxophone* (*sax*) or *cactus* (*cact*) females (Fig. 6H shows an embryo from a *cact* female; data not shown for *sax*) or homozyous for *zen* or *tolloid* (*tld*) (Fig. 7C for *tld*; data not shown for *zen*). Reciprocally, dorsal HNT expression expands ventrally in dorsalized embryos (Fig. 6I shows an embryo from a *pelle* (*pll*) female). Anterior midgut expression of HNT is affected by the dorsoventral pathway (e.g. Fig. 6I) since anterior midgut development requires inputs from both terminal and dorsoventral pathways (Reuter and Leptin, 1994; Yip and Lipshitz, 1996). Since dorsoventral mutants fail to undergo germ-band extension, the role of amnioserosal expression of HNT in germ-band retraction could not be assayed in embryos singly mutant for any these genes (but see below).

The *concertina* (*cta*) and *folded gastrulation* (*fog*) genes are required for proper ventral furrow formation and posterior midgut invagination (Zusman and Wieschaus, 1985; Parks and Wieschaus, 1991; Sweeton et al., 1991; Costa et al., 1994). HNT expression is unaffected by either mutation (Fig. 6J shows an embryo from a *cta* female; data not shown for *fog*).

The amnioserosa but not the midgut is required for germ-band retraction

hkb mutants eliminate midgut cell fates and expression of HNT in the presumptive midgut regions at the embryonic termini (Figs 6D, 7C). However *hkb* mutant embryos undergo normal germ-band extension and retraction (Fig. 7C,D) (Reuter et al., 1993). Thus, neither the midgut per se nor HNT expression in

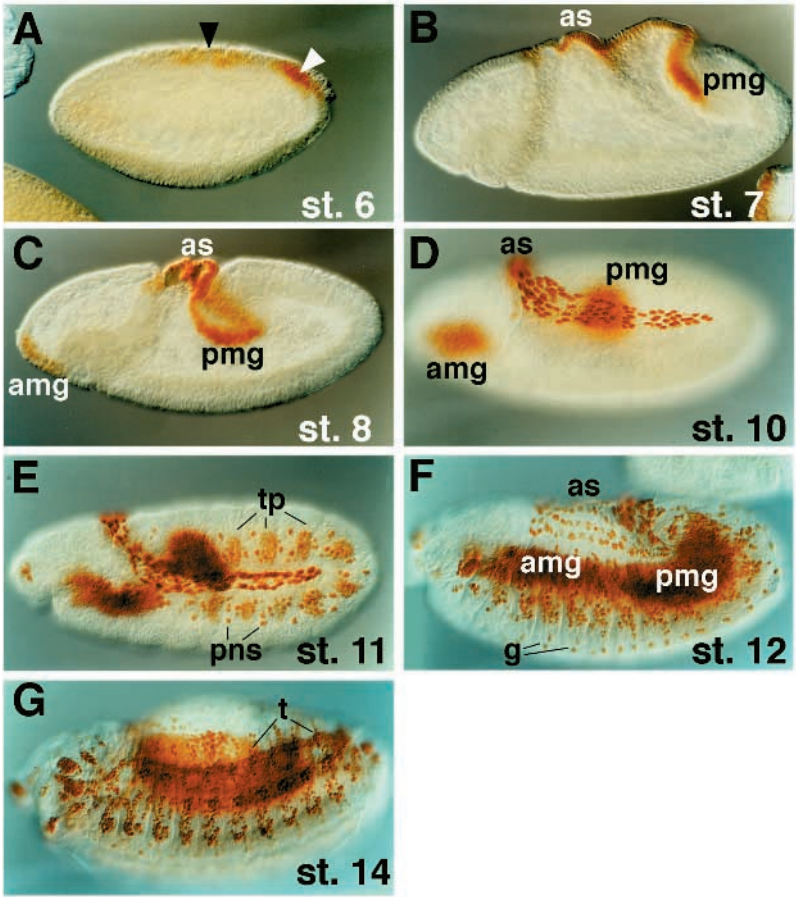


Fig. 5. HNT protein expression. (A) HNT protein starts to accumulate in stage 6 embryos in the posterior midgut primordium (white arrowhead) and dorsally in the presumptive amnioserosa (black arrowhead). (B) In stage 7 embryos, HNT protein can be seen in the posterior midgut (pmg) and amnioserosa (as). (C) At stage 8, HNT protein accumulates in the anterior midgut (amg) primordium as well as the posterior midgut and amnioserosa. (D) A fully germ-band-extended embryo (stage 10) shows HNT protein in the midgut and in the large nuclei of amnioserosal cells. (E) HNT appears in the tracheal pits (tp), peripheral and CNS glial cells (not visible) and peripheral nervous system (pns) as they form in stage 11 embryos. In the embryo shown here, CNS expression has not yet begun. (F) During germ-band retraction, HNT can be seen in the CNS glial cells (g) of stage 12 embryos. (G) A germ-band-retracted embryo (stage 14) shows continued accumulation of HNT in the amnioserosa (out of focal plane), midgut, CNS (out of focal plane), pns and tracheae (t). Embryos are oriented with anterior to the left and dorsal toward the top of the page.

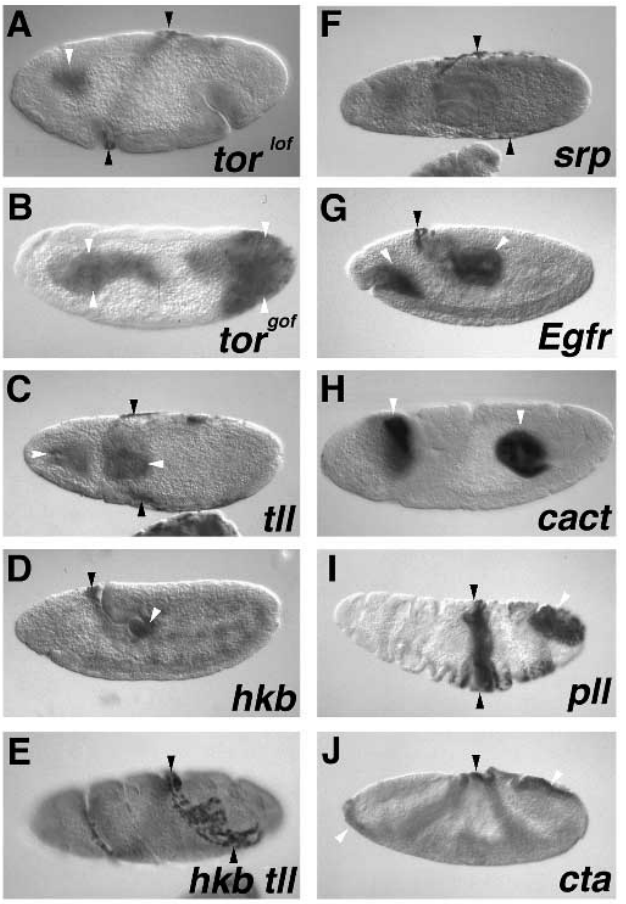


Fig. 6. Regulation of HNT expression. (A) Embryo from a homozygous *torso* loss-of-function (tor^{PM51}) mother lacks HNT in the posterodorsal region while accumulation still occurs in the presumptive amnioserosa dorsally (black arrowheads) and in the anterior midgut rudiment (white arrowhead). The twisted gastrulation phenotype exhibited by *tor* loss-of-function mutants is apparent. (B) Embryos from homozygous $torso^{splc}$ gain-of-function mothers lack dorsal HNT accumulation while the enlarged posterior and anterior midgut primordia (white arrowheads) exhibit concomitant expanded HNT accumulation. (C) The HNT accumulation pattern is largely unaffected in a tll^{1} mutant embryo (amnioserosa, black arrowheads; midgut, white arrowheads). (D) HNT accumulation in the anterior and posterior termini is lost in hkb^{2} mutant embryos (see also Fig. 7A). Posteriorly, only the Malpighian tubule primordium expresses HNT (white arrowhead). Amnioserosal expression is normal (black arrowhead). (E,F) Accumulation of HNT in the regions that normally give rise to endoderm and Malpighian tubules is lost while amnioserosal expression (black arrowheads) is unaffected in hkb^{2} tll^{1} double mutant embryos (E; gastrulation is twisted in these embryos) and in srp^{9L} mutant embryos (F). (G) HNT expression is normal in $Egfr^{f1}$ mutant embryos (amnioserosa, black arrowhead; midgut, white arrowheads). (H) Embryos derived from $cactus^{A2}/cactus^{HE9}$ mothers lose dorsal HNT expression while midgut expression remains (white arrowheads). (I) Embryos derived from $pelle^{078}/pelle^{385}$ mothers have an expanded amnioserosa and thus an expanded dorsal domain of HNT accumulation (black arrowheads), normal expression in the posterior midgut (white arrowhead) and loss of HNT from the region that would normally form the anterior midgut. (J) Embryos from $concertina^{WU31}$ females have normal HNT expression in the amnioserosa (black arrowhead) and in the midgut (white arrowheads). Embryos are oriented with anterior to the left. Dorsal is toward the top of the page in A, B, D, E, G, H, I and J, while the embryos in C and F are viewed from the dorsal side.

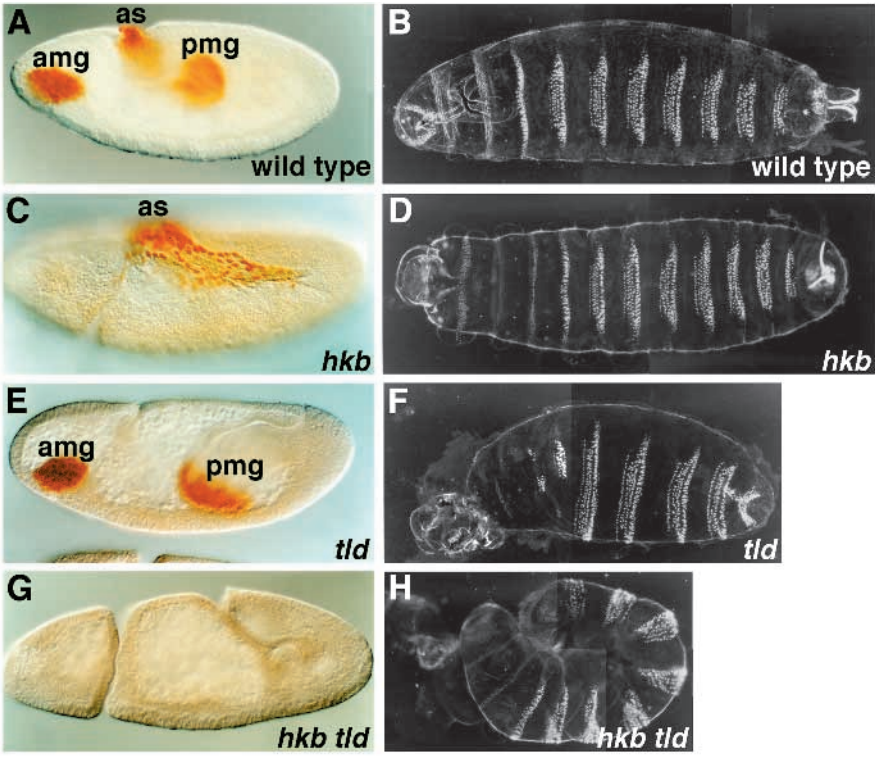


Fig. 7. HINDSIGHT expression and germ-band retraction in *hkb* and *tld* single and double mutants. (A,C,E,G) Embryos in which HNT protein was visualized using anti-HNT antibody 27B8 1G9 (see Materials and Methods). (B,D,F,H) Cuticle preparations. (A,B) Wild-type embryos. (C,D) hkb^{2} mutant embryos lack expression of HNT in the terminal domains that would normally form the midgut (C). However, germ-band extension (C) and retraction (D) occur normally. (E,F) $tld^{9}/tld^{68\text{-}62}$ mutant embryos lack dorsal expression of HNT in the region that would normally form the amnioserosa (E) and fail in germ-band extension (E,F; in F A8 is out of the focal plane). (G,H) hkb^{2} tld^{9} double mutant embryos lack HNT expression in both the termini and the dorsocentral regions (G). They undergo germ-band extension (G) but fail in retraction (H). In all cases, anterior is to the left and dorsal towards the top of the page. See legend to Fig. 5 for abbreviations.

the termini is necessary for germ-band retraction, while dorsal HNT expression in the amnioserosa is sufficient for retraction.

Strongly ventralizing dorsoventral pattern mutants such as *zen* and *tld* eliminate amnioserosa cell fates and dorsal

expression of HNT (Fig. 7E; $tld^{9}/tld^{68\text{-}62}$). However, assaying the function of the amnioserosa and dorsal HNT expression in germ-band retraction is not possible using these mutants because they fail to undergo germ-band extension (Fig. 7E,F).

hkb^{2} tld^{9} double mutant embryos lack HNT expression dorsally as well as in the termini (Fig. 7G) but undergo germ-band extension (Fig. 7G,H), thus allowing us to assay germ-band retraction in the absence of both midgut and amnioserosa. Strikingly, hkb^{2} tld^{9} double mutant embryos fail to undergo germ-band retraction (Fig. 7H). These results suggest that the amnioserosa per se — and likely HNT expression in this tissue — is necessary for germ-band retraction (see Discussion).

DISCUSSION

We have shown that *hnt* gene function is required for germ-band retraction and that *hnt* mutations do not cause defects in this morphogenetic process as an indirect consequence of mis-specification of cellular positional values or tissue identity. *hnt* encodes a large zinc-finger protein that accumulates in the nuclei of several tissues prior to germ-band retraction. Strikingly, *hnt* is not expressed in the epidermal ectoderm, the tissue that undergoes the morphogenetic alterations that drive retraction. Analyses of *hnt* expression and germ-band retraction in mutants that lack specific tissues in which HNT normally accumulates suggests that *hnt* expression in the extraembryonic amnioserosa is necessary and sufficient for retraction.

Two lines of evidence support the conclusion that failure of germ-band retraction is a primary effect of *hnt* mutations. First, *hnt* mutant embryos undergo normal pattern and tissue specification in the germ band prior to retraction. Second, the temporal and spatial aspects of germ-band extension are indistinguishable in wild-type and in *hnt* mutant embryos. Thus, there are no detectable defects either in tissue specification or in morphogenesis prior to germ-band retraction. Of eight molecular markers assayed, one – KRÜPPEL – was abnormal in *hnt* mutants prior to germ-band retraction. KRÜPPEL is absent from many but not all amnioserosal cells by stage 11 (similar observations have been reported by Ray, 1993). Absence of KRÜPPEL at this stage correlates with the recently reported premature apoptosis of the differentiated amnioserosa in *hnt* mutants (Frank and Rushlow, 1996). The amnioserosal cells of hnt^{XE81}mutant embryos display similar morphology to those of the wild type until apoptosis commences, leading us to suspect that the lack of KRÜPPEL in the amnioserosa of *hnt* mutants is an indirect consequence of premature apoptosis, rather than that the *Krüppel* gene is a regulatory target of HNT. The absence of any retraction defects in *Krüppel* mutants is consistent with this suggestion.

The *hnt* gene encodes a large protein with fourteen C_2H_2-type zinc fingers. This type of zinc finger is found in many transcription factors and has been shown to function as a DNA-binding domain (Pabo and Sauer, 1992). The arrangement of the HNT zinc fingers is unusual in that twelve of the fourteen fingers occur in widely spaced clusters, each of which includes two or three tandemly arranged zinc fingers. Only two of the fourteen zinc fingers — the ninth and twelfth — are unclustered. The fifth zinc finger does not have the second conserved histidine residue and therefore may be non-functional. If so, then four clusters of zinc fingers alternate with isolated functional zinc fingers throughout the length of the protein. In addition to the zinc fingers, the HNT protein contains several structural domains commonly found in transcriptional regulators, including multiple glutamine-rich, proline-rich, serine/theronine-rich and acidic/charged domains. Each of these types of domains has been shown to function in *trans* to mediate protein-protein interactions important for transcriptional control (Courey and Tjian, 1988; Mermod et al., 1989; Ptashne and Gann, 1990; Stringer et al., 1990; Tanaka and Herr, 1990; Dynlacht et al., 1991; Lin and Green, 1991; Lin et al., 1991; Madden et al., 1991; Han and Manley, 1993). These structural motifs, combined with the nuclear localization of HNT protein as revealed by antibody staining, make it highly likely that HNT protein functions as a transcription factor. The fact that the hnt^{X001} mutation results in a protein product truncated after the first three zinc fingers but is only a weak allele (Fig. 2D), suggests that partial function can be conferred by the first cluster of three zinc fingers.

The *hs-hnt* transgene rescues the morphogenetic process of germ-band retraction without causing any dominant gain-of-function defects, strongly suggesting that the role of HNT is permissive rather than instructive; that is, that HNT is involved in the spatial and/or temporal coordination of germ-band retraction, rather than in the implementation of the morphogenetic cell shape changes and movements. The fact that HNT is a zinc-finger protein that is expressed in a distinct set of tissues from those that undergo the morphogenetic alterations, is consistent with this possibility.

Expression of *hnt* in the amnioserosa dorsally versus in the posterior midgut primordium is regulated, respectively, by the dorsoventral and the terminal gene hierarchies, while expression of *hnt* in the anterior midgut primordium receives input from both of these pathways (Yip and Lipshitz, 1996). *hnt* is positioned downstream of the genes that specify endodermal (midgut) identity, such as *hkb*. In contrast, mutations in the early morphogenetic control genes *cta* and *fog* do not affect *hnt* expression. This last result suggests that distinct pathways control the early morphogenetic movements that result in internalization of the mesoderm and endoderm (controlled by *cta* and *fog*) versus the later morphogenetic movements that drive germ-band retraction (controlled by the U-shaped class of genes that includes *hnt*).

HNT expression is normal in embryos mutant at any of four other loci required for germ-band retraction: *ush*, *tup*, *Egfr* (this study) and *inr* (M. L. L. and H. D. L., unpublished data). Furthermore, the *hs-hnt* transgene is unable to rescue *ush*, *tup* and *Egfr* mutants under experimental conditions that rescue *hnt* (M. L. R. Y. and H. D. L., unpublished data). These data are consistent with *ush*, *tup*, *inr* and *Egfr* residing either downstream of *hnt* or in a parallel pathway that regulates germ-band retraction. In contrast, endodermal expression of HNT is missing in a fifth mutant that affects germ-band retraction, *srp*. This last result is consistent with the fact that *srp* is required to establish the identity of the endodermal midgut and is expressed in both the anterior and posterior midgut primordia through stage 9 (Reuter, 1994; Rehorn et al., 1996). Not surprisingly, then, HNT expression is absent in *srp* mutants since the endoderm is absent. The fact that germ-band retraction is normal in *hkb* mutants that lack midgut but fails in *srp* mutants in which the midgut is converted into foregut and hindgut, suggests that loss of HNT from presumptive midgut per se does not result in

failure of germ-band retraction; rather the failure of retraction in *srp* mutants must have some other cause.

The most striking result of the present studies is the absence of *hnt* expression in the epidermal ectodermal cells that undergo the morphogenetic alterations that accomplish germ-band retraction, and the presence of *hnt* expression in several other tissue types — notably the endoderm and amnioserosa — prior to and during germ-band retraction. The question thus arises as to the role of these tissues in germ-band retraction. Definitive conclusions will require analyses of embryos genetically mosaic for *hnt* (M. L. L. and H. D. L., unpublished data). However, at this point, several tentative conclusions can be reached on the basis of genetic and phenotypic studies.

The tissue actually responsible for executing germ-band retraction is the epidermal ectoderm. As described in the Introduction, morphological and anatomical analyses of developing embryos show that epidermal ectodermal cells undergo extensive shape changes as well as local rearrangements during germ-band retraction (Martinez Arias, 1993). These processes initiate in the thoracic region and proceed posteriorly, driving germ-band movement around the posterior pole of the embryo (Martinez Arias, 1993). In contrast, the mesoderm is dispensable for germ-band retraction since embryos lacking mesoderm undergo normal germ-band retraction (Leptin et al., 1992). The fact that germ-band retraction is normal in *hkb* mutants that lack midgut, and thus lack HNT expression in the termini (Fig. 7C,D) suggests that neither the midgut per se nor HNT expression in the termini is essential for germ-band retraction. Further, embryos in which the migration and morphogenesis, but not specification, of the endoderm are disrupted can still undergo germ-band retraction (Reuter et al., 1993; Tepass and Hartenstein, 1994). Thus, neither endodermal nor mesodermal cells per se, nor the migration and morphogenesis of these tissues, are necessary to direct germ-band retraction.

In contrast to the endoderm and mesoderm, a crucial role for the extraembryonic, amnioserosal cells in programming germ-band retraction is likely. First, since the amnioserosa is present in *hkb* mutants, which successfully complete germ-band retraction, the amnioserosa is sufficient to program retraction in the absence of midgut. Second, reduction in the size of the amnioserosa (in embryos mutant for weakly ventralizing alleles of zygotically active dorsoventral pattern genes) results in a U-shaped phenotype (Arora and Nüsslein-Volhard, 1992). This suggests that retraction is sensitive to the size of the amnioserosa, consistent with the amnioserosa being necessary for retraction. Third, our double mutant analysis (Fig. 7G,H) confirms that the amnioserosa is necessary for germ-band retraction.

Consistent with an important role for HNT expression in the amnioserosa, and for the amnioserosa per se, it has recently been shown that *hnt*, *srp* and *ush* mutations all result in premature death of the differentiated amnioserosa (Frank and Rushlow, 1996). Indeed, the timing of initiation of the premature germ-band retraction-like movements that we observed in a subset of *hnt* mutant embryos, correlates well with the stage at which the amnioserosa cells have been reported to undergo premature apoptosis in these mutants (Frank and Rushlow, 1996). Why amnioserosal death should result in premature initiation of this morphogenetic process cannot yet be explained. However, it suggests that the amnioserosa plays both a negative and a positive role in coordinating germ-band retraction: it prevents premature retraction movements while promoting retraction at the appropriate stage of embryogenesis.

Two classes of models – not mutually exclusive – could explain the role of the amnioserosa in germ-band retraction. The first, or 'physical' model, proposes that physical contact between the amnioserosa and the epidermal ectoderm is important for retraction. For example, cell shape changes in the amnioserosa may be necessary to drive, or to allow, retraction. The second, or 'chemical' model, suggests that a (possibly diffusible) signal from the amnioserosa to the epidermal ectoderm directs or coordinates the ectodermal cell shape changes that drive germ-band retraction. These models differ in several respects. Most importantly, the former predicts that the amnioserosa must be present for retraction to occur, while the latter predicts that the amnioserosa per se may be dispensible as long as the appropriate signals to the ectoderm are provided.

Several lines of evidence make us supect that the amnioserosa produces or activates signal(s) that coordinate the morphogenetic alterations in the ectoderm during germ-band retraction. Among the loci required for germ-band retraction, *hnt*, *srp*, *inr* and *Egfr* are the only four for which information regarding the molecular nature of the gene products and expression patterns is available (this study; Raz and Shilo, 1993; Fernandez et al., 1995; Rehorn et al., 1996). Expression and function of the zinc-finger-containing GATA-like factor, SRP, has been considered above. Both the EGFR (M. L. L. and H.D.L, unpublished data) and the INR (Fernandez et al., 1995) are expressed throughout the embryo with the exception that the INR is never present in the amnioserosa and the EGFR is absent from the amnioserosa after stage 10 (M. L. L. and H. D. L., unpublished data). Based on these expression patterns and the fact that the products of these last two genes are transmembrane receptor tyrosine kinases, it is possible that coordinating signals from the amnioserosa are received in the ectoderm by the INR and/or EGFR and transduced into the shape changes and local cell rearrangements that drive germ-band retraction. The coordinating signal(s) produced by the amnioserosa may be the ligands for these receptor(s). Alternatively they could be an activity or activities that process or activate the ligand(s). Or they could function more indirectly through effects on the extracellular matrix. Future genetic and molecular analyses will focus on specific tests of these models.

Independent of whether the 'physical' or the 'chemical' model is correct, it will be important for future analyses to distinguish alternative mechanisms by which *hnt* functions in the amnioserosa to program the germ-band retraction process. It is possible that *hnt*'s only function in the amnioserosa is to prevent premature apoptosis (Frank and Rushlow, 1996). In *hnt* mutants physical contact between the amnioserosa and epidermal ectoderm, or chemical signaling from the amnioserosa to the epidermal ectoderm, would then be disrupted as a secondary effect of loss of the amnioserosa. Alternatively, *hnt* may play a dual role in the amnioserosa: on the one hand, *hnt* might function to promote survival of the amnioserosal cells (preventing, for example, premature retraction movements) while, on the other hand, *hnt* might play a direct role in regulating production or activation of amnioserosa-to-ectoderm signal(s) that coordinate germ-band retraction.

HNT is expressed in several tissues other than the amnioserosa in the developing embryo. These include the midgut, the ureter of the developing Malpighian tubules, the

developing tracheal system and glial cells in the nervous system (Fig. 5). Taken together with the fact that HNT is likely to be a transcription factor, it is possible that HNT controls additional morphogenetic events during embryonic and postembryonic development by transcriptionally regulating sets of genes that function to coordinate these processes. Future genetic and molecular analyses will address this possibility.

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