Development 120, 2039-2050 (1994)
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β_{Heavy}-spectrin has a restricted tissue and subcellular distribution during *Drosophila* embryogenesis

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SUMMARY

The components of the membrane skeleton play an important role in maintaining membrane structure during the dynamic changes in cell shape that characterize development. β_{Heavy}-spectrin is a unique β-spectrin from *Drosophila melanogaster* that is closer in size (M_r=430×10^3) to dystrophin than to other β-spectrin members of the spectrin/α-actinin/dystrophin gene super-family. Here we establish that both the subcellular localization of the β_{Heavy}-spectrin protein and the tissue distribution of β_{Heavy}-spectrin transcript accumulation change dramatically during embryonic development.

Maternally loaded protein is uniformly distributed around the plasma membrane of the egg. During cellularization it is associated with the invaginating furrow canals and in a region of the lateral membranes at the apices of the forming cells (apicolateral). During gastrulation the apicolateral staining remains and is joined by a new apical cap, or plate, of β_{Heavy}-spectrin in areas where morphogenetic movements occur. These locations include the ventral and cephalic furrows and the posterior midgut invagination. Thus, dynamic rearrangement of the subcellular distribution of the protein is precisely coordinated with changes in cell shape.

Zygotic message and protein accumulate after the germ band is fully extended, in the musculature, epidermis, hindgut, and trachea of the developing embryo. β_{Heavy}-spectrin in the epidermis, hindgut, and trachea is apically localized, while the protein in the somatic and visceral musculature is not obviously polarized.

The distribution of β_{Heavy}-spectrin suggests roles in establishing an apicolateral membrane domain that is known to be rich in intercellular junctions and in establishing a unique membrane domain associated with contractile processes.

Key Words: spectrin, membrane skeleton, cell polarity, *Drosophila*, morphogenesis, development

INTRODUCTION

The precise mechanism(s) by which the membrane skeleton, a ubiquitous cellular structure that is closely juxtaposed to the inner surface of the plasma membrane, functions to maintain the integrity of the plasma membrane and contributes to cell adhesion, signal transduction and cell polarity (see Luna and Hitt, 1992 for review) have yet to be elucidated. This membrane skeleton was first recognized as a distinct part of the cytoskeleton in erythrocytes (Marchesi and Steers, 1968) where it is now known to give stability to the plasma membrane and shape to the erythrocyte itself (Elgasaeter et al., 1986). Mutations in any of several components of the membrane skeleton lead to a loss of normal erythrocyte shape and, due to fragility, haemolytic anaemia in both humans and mice (Bodine IV et al., 1984; Palek and Sahr, 1992).

An essential component of the erythrocyte membrane skeleton is a meshwork of spectrin molecules that interconnect short actin filaments at the membrane (see Luna and Hitt, 1992). Spectrins are long, heterodimeric molecules that consist of α and β subunits composed largely of a repetitive α-helical rod motif of 106 amino acids. α/β dimers associate head to head, and these tetramers form the basic actin crosslinking unit, with an actin binding domain on the β subunit at each end. Spectrin is part of a larger superfamily of actin crosslinking proteins that are found in a wide variety of non-erythroid tissues. These include α-actinins, dystrophins, actin binding proteins (ABP280, or filamin and ABP120), fimbrin and a M_r=300×10^3 muscle specific protein from *Drosophila* (Hartwig and Kwiatkowski, 1991; Volk, 1992). In addition, many other components of the membrane skeleton have isoforms in most non-erythroid tissues examined (Bennett, 1990).

Structural, and probably functional, diversity in spectrins comes from variation in both the α and β subunits. Sequences from several genes in several species show that this variation is generated by having multiple genes and by alternatively splicing mRNA from a single gene (McMahon et al., 1987; Moon and McMahon, 1990). Three protein isoforms of vertebrate β-spectrin have been described, suggesting that variability in the function of the membrane skeleton in diverse tissues is due, at least in part, to the particular β-spectrin

isoform that is expressed. There are two β-spectrin genes: one encodes the erythroid β-spectrin isoform (Winkelman et al., 1990a; Birkenmeier et al., 1988) and both encode non-erythroid isoforms (Winkelman et al., 1990b; Hu et al., 1992; Ma et al., 1993). In cells where the two non-erythroid isoforms co-exist they have differing subcellular distributions suggestive of differing functions (Lazarides et al., 1984). Vertebrates also have a β isoform that is not associated with α-spectrin and localizes to clusters of acetylcholine receptors at neuromuscular junctions (Bloch and Morrow, 1989), and a larger isoform that is found in the terminal web of chicken intestinal epithelial cells (TW260/240; Glenny et al., 1982). Finally there is a very large β isoform (M_r=430×10^3) in *Drosophila* termed β_{Heavy}-spectrin (β_H; Dubreuil et al., 1990) that may also be found in vertebrate brain (Vann Bennett personal communication).

The functions of spectrins go beyond the general maintenance of cell shape and integrity. They may establish membrane subdomains, participate in exocytosis, contribute to lymphocyte capping, and function in the polarization of epithelial cells (reviewed by Bennett, 1990; Morrow et al., 1991; Luna and Hitt, 1992). In addition, spectrin genes are developmentally regulated in many organisms (see Barakat-Walter and Riederer, 1991; Zimmer et al., 1992; Wessel and Chen, 1993). Clearly a complete understanding of how any spectrin isoform contributes to membrane skeleton structure and carries out these proposed functions will require a detailed knowledge of the protein's structure, regulation and distribution.

β_H in *Drosophila* is the largest β-spectrin isoform found to date and is comparable in size to dystrophin (M_r=430x10^3; Dubreuil et al., 1990). A partial cDNA sequence revealed that the N terminus has a typical actin binding domain followed by the 106 amino acid repeating sequence characteristic of spectrins. *Drosophila* β_H dimerizes with the same α-subunit as the conventional β isoform in flies, but has a longer rope-like, tetrameric spectrin morphology (250 nm versus 180 nm). This tetramer binds by its termini to filamentous actin (Dubreuil et al., 1990).

Here we demonstrate a dynamic, developmental stage-specific distribution of the β_H transcript and protein during embryonic development and characterize the subcellular distribution of β_H protein. Our analysis shows that β_H is maternally loaded into the egg and is redistributed on the membrane at specific locations and times prior to the presence of any β_H transcript. We also report on the subsequent accumulation of zygotic β_H transcript and protein, which occurs in a tissue specific manner. The developmental expression and localization of the β_H transcript and protein during embryogenesis indicates a complex pattern of transcriptional and post-transcriptional regulation, which points to roles in cellularization and early muscle development as well as epidermal and tracheal structure. The subcellular localization of this protein suggests that β_H is important in generating and/or maintaining cell polarity and that it is involved in contractile processes that lead to changes in the shape of cell sheets.

MATERIALS AND METHODS

Cell lines and *Drosophila* stocks

Kc_0 cells were grown in D22 medium without serum (Sang, 1981). *Drosophila* embryos for immunoblots and immunofluorescence were obtained from an Oregon-R wild-type strain. Live embryos for protein extraction were staged according to Campos-Ortega and Hartenstein (1985).

Antibodies

The β_H-specific antiserum no. 243 was produced by immunizing a rabbit with bacterially expressed β_H-fusion proteins. In the following descriptions numbering of base pairs is as set out in Dubreuil et al. (1990; EMBL/GenBank/DDBJ accession X53992). An *Eco*RI restriction fragment from pBH5 (1-3761 bp) was subcloned into the pUCX-2 vector and transformed into *Escherichia coli* (*E. coli*) strain DH5α. Induced β_H-β-galactosidase fusion protein was purified as inclusion bodies (Nagai and Thøgersen, 1987), solubilized in SDS and purified by SDS-polyacrylamide gel electrophoresis (SDS-PAGE; Laemmli, 1971; Higgins and Dahmus, 1979). The excised band was dried and ground to a fine powder, rehydrated in a minimal volume of water and used to immunize rabbits following standard protocols (Kiehart and Feghali, 1986). After boosting there was only a weak response. Two alternative fusion proteins were made by inserting a 0.76 kb *Bcl*I to *Bam*HI restriction fragment (153-912 bp) and a 2.85 kb *Bam*HI to *Eco*RI restriction fragment (912-3761 bp) of pBH5 into the vector pGEX-3X (Smith and Johnson, 1988) and transforming into the *E. coli* strain DH20. Together these two fusion proteins include 95% of the fragment of β_H encoded by the first fusion protein, coupled to glutathione-S-transferase (GST) instead of β-galactosidase. These proteins were also purified as inclusion bodies as described above, and were used to boost further the same rabbit in order to produce immune serum no. 243. This mixture of fusion proteins was also used to immunize mice for the generation of monoclonal antibodies (Cocalico Biologicals Inc., Reamstown, PA) and one of the whole immune sera (DUM2) is used in this paper.

β_H-specific antibodies were affinity purified for immunofluorescent staining of embryos using the small proportion of soluble GST-fusion proteins that could be obtained following expression in the alternative host strain DH5α. These were purified using glutathione agarose beads (Sigma, St. Louis, MO; Smith and Johnson, 1988) and an equimolar mixture of the two proteins was coupled to CNBr-activated Sepharose 6MB (Pharmacia, Piscataway, NJ) in 50 mM sodium phosphate, pH 8.0. Unreacted sites on the matrix were inactivated by incubation in 50 mM Tris-HCl pH 8.0/200 mM glycine. The column was rinsed in elution buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl/3.5M KI) and equilibrated in wash buffer (50 mM Tris-HCl, pH 7.5/150 mM NaCl). The column was incubated in serum/0.05% NaN_3 at 4°C, rinsed with wash buffer and wash buffer plus 1 M KCl before eluting in elution buffer. Eluted fractions were immediately and extensively dialyzed against wash buffer supplemented with 0.05% NaN_3. Antibodies purified in this way were not concentrated further and were used for staining at a dilution of 1:25.

Drosophila α-spectrin was detected with antiserum no. 905 (Byers et al., 1987). *Drosophila* β-spectrin was detected with antiserum no. 89 (a gift from Dr Dan Branton; Byers et al., 1989). *Drosophila* nonmuscle (cytoplasmic) myosin was detected using antiserum no. 656 (Kiehart and Feghali, 1986).

Immunoblots

To effectively resolve β_H protein by SDS-PAGE we used 5.94% acrylamide: 0.06% bis-acrylamide gels with a 3.9% acrylamide: 0.1% bis-acrylamide stacking gel using the Laemmli buffer system (Laemmli, 1971) with the addition of 10 mM β-mercaptoethanol to the upper buffer chamber (Fritz et al., 1989).

Approximately 3×10^6 Kc_0 cells were pelleted briefly in a microfuge. 150 μl of boiling 2× loading buffer (8 M urea, 2 M thiourea, 3% SDS, 75 mM DTT, 25 mM Tris-HCl pH 6.8, 0.05% bromophenol blue; Fritz et al., 1989) was added and the sample was briefly disrupted using a motor-driven pestle. The sample was boiled for 10 minutes, insoluble material was removed by pelleting in a

microfuge and the supernatant resolved by SDS-PAGE as described above.

The gel was soaked and electroblotted to nitrocellulose (Schleicher and Schuell, Inc., Keene, NH) in 20 mM Tris/0.3 M glycine/0.01% SDS/20% methanol plus 10 mM β-mercaptoethanol (Fritz et al., 1989). Membranes were blocked in 10% normal goat serum (NGS; Sigma, St.Louis, MO) in 50 mM Tris-HCl, pH 7.7/150 mM NaCl (TBS) overnight at 4°C, briefly incubated in 10% NGS/TBS/0.1% Tween and then incubated in the primary antiserum diluted in 5% NGS/TBS/0.1% Tween for 1 hour at 37°C. Filters were washed in TBS/0.1% Tween and incubated in alkaline phosphatase-conjugated goat anti-rabbit antibody (Boehringer Mannheim, Indianapolis, IN) diluted 1:2000 in 5% NGS/TBS/0.1% Tween for 1 hour at 37°C. After washing in TBS/0.1% Tween, filters were rinsed in 100 mM Tris-Cl/pH 9.5/100 mM NaCl/50 mM $MgCl_2$ and reacted with a stabilized nitroblue tetrazolium/5-bromo-4-chloro-3-indolyl phosphate solution (Gibco BRL, Gaithersburg, MD) to detect alkaline phosphatase on the filter.

In situ hybridization to embryos

The probe used for the detection of β_H mRNA in whole embryos was the complete insert of pBH5 (1-4936) that was excised with *Apa*I and *Xba*I, and gel purified using Gene Clean (Bio101, La Jolla, CA). The DNA was labeled with digoxigenin using the Genius DNA labeling and detection kit (Boehringer Mannheim, Indianapolis, IN), hybridized to fixed embryos and detected according to the method of Tautz and Pfiefle (1989) with the modifications described in Michelson et al. (1990).

Immunofluorescence

Embryos were dechorionated in 50% bleach (The Clorox Company, Oakland, CA) for 2 minutes and rinsed extensively in deionised H_2O (dH_2O). Fixation was performed by gently tumbling the embryos in a 1:1 mixture of heptane, that had been previously saturated against a 0.25% aqueous solution of glutaraldehyde, and methanol for 30 minutes at room temperature. This treatment with glutaraldehyde does not produce a significant autofluorescence problem. The embryos that sank to the bottom of the methanol layer were recovered, rinsed once in methanol and rehydrated through a 70:30, 50:50, 30:70 series of methanol/PBT (342 mM NaCl, 6.7 mM KCl, 3.7mM KH_2PO_4, 10.4 mM NaH_2PO_4, 0.5% Triton X-100, pH 7.0) for 10 minutes each followed by two washes in PBT, also for 10 minutes each.

The fixed embryos were blocked and extracted in 25% NGS/PBT5% (PBT but with 5% Triton) for 2 hours at room temperature. The 5% Triton treatment at this stage helps antibody penetration in the later stage embryos. Following a brief rinse in 5% NGS/PBT the embryos were placed into the primary antibody diluted to the appropriate concentration in 5% NGS/PBT/0.05% NaN_3 and incubated overnight at room temperature with gentle agitation. The embryos were washed once briefly and 4 times for 10 minutes each in 5% NGS/PBT and were incubated in the appropriate secondary antibody. A 1:250 dilution of a FITC-conjugated goat anti-rabbit secondary antibody (Zymed, San Fransisco, CA) in 5% NGS/PBT/0.05% NaN_3 was used to detect staining by the rabbit sera no. 243 and no. 656. A 1:100 dilution of a TRITC-conjugated goat anti-mouse secondary antibody (Southern Biotechnology Associates, Inc., Birmingham, AL) was used to detect staining by the mouse antiserum DUM2. Secondary antibodies were incubated with the embryos overnight at room temperature and washed as before, but with one extra wash and a final more than 30 minute equilibration in Tris-HCl, pH 8.6, 50% glycerol, 2% propyl gallate as a mounting medium for fluorescence microscopy. Scanning laser confocal microscopy was performed using a Biorad MRC 600 microscope (Biorad, Hercules, CA). Images were level adjusted and combined into figure panels using Adobe Photoshop (Adobe Systems Inc., Mountain View, CA). False colour composites that mimicked the colours in the original sample were generated in Photoshop with the blend command. Images processed in Photoshop were exported to Canvas (Deneba Software, Miami, FL) where they were montaged, annotated, saved to disk for electronic submission for publication or outputted to a Techtronix Phaser IISDX dye sublimation printer for hard copy.

RESULTS

β_H is a spectrin and not a dystrophin

The published sequence of β_{Heavy}-spectrin (Dubreuil et al., 1990) is partial and represents about 5 kb of a mature 13 kb mRNA. The conceptual translation of the open reading frame in this sequence predicts a peptide with a M_r=189×10^3 which was shown to be part of a larger protein with a M_r=430×10^3. The sequence of this clone represents an N-terminal fragment of a member of the spectrin/α-actinin/dystrophin gene superfamily of actin cross-linking proteins in that it contains a consensus actin binding domain followed by twelve and a half copies of a typical 106 amino acid repetitive domain (Speicher and Marchesi, 1984).

Based on the size of the protein and the features itemized in the Discussion of that paper, it was speculated that β_H might be a *Drosophila* homologue of dystrophin (Dubreuil et al., 1990). The sequences of the C-terminal region of dystrophins are extraordinarily conserved and could, in principle, be used to determine the precise relationship of β_H and dystrophin (Lemaire et al., 1988; Love et al., 1989). We have recovered another partial cDNA that represents the final approx. 600 amino acids of the β_H protein, a detailed analysis of which will be presented elsewhere (G. H. Thomas and D. P. Kiehart, unpublished data[1]). There is no detectable similarity to dystrophin in this C-terminal clone beyond that expected for a member of the α-actinin/spectrin/dystrophin superfamily.

Distribution of β_H zygotic transcript accumulation

β_H transcript accumulation during embryogenesis was examined by hybridizing the β_H 5 kb cDNA to embryos. Despite protein sequence similarity between β_H and other spectrins, this probe has only limited similarity to them at the DNA level and has not shown any cross-hybridization in numerous probings of DNA and RNA blots (data not shown).

Our data suggest that β_H transcript is not maternally loaded. The β_H gene shows no detectable transcript accumulation until stage 10 (fully extended germband) of embryogenesis, after which transcript accumulates throughout embryogenesis. β_H transcripts are always confined to a specific subset of cells and tissues (Fig. 1). The first transcripts accumulate at stage 10 in the primordia of the foregut and hindgut (Fig. 1A,B); and then in numerous mesodermal cells that appear in a segmentally repeated pattern (Fig. 1C,D). These labeled mesodermal cells subsequently increase in number (Fig. 1F,G) and are probably somatic muscle precursor cells. Concomitantly β_H transcript accumulates deeper in the embryo, in the splanchnopleura, which gives rise to the visceral mesoderm (Fig. 1E,H). β_H is thus expressed in much or all of the muscular tissue of the

[1]The sequence will be fully analysed in a future publication, when we have completed the cloning and sequencing of the entire β_H cDNA. It is reported here insofar as it is important in establishing that β_H is a spectrin and not a dystrophin. The complete DNA sequence of this new cDNA (pBH12) is available under Genbank accession no. U07629.

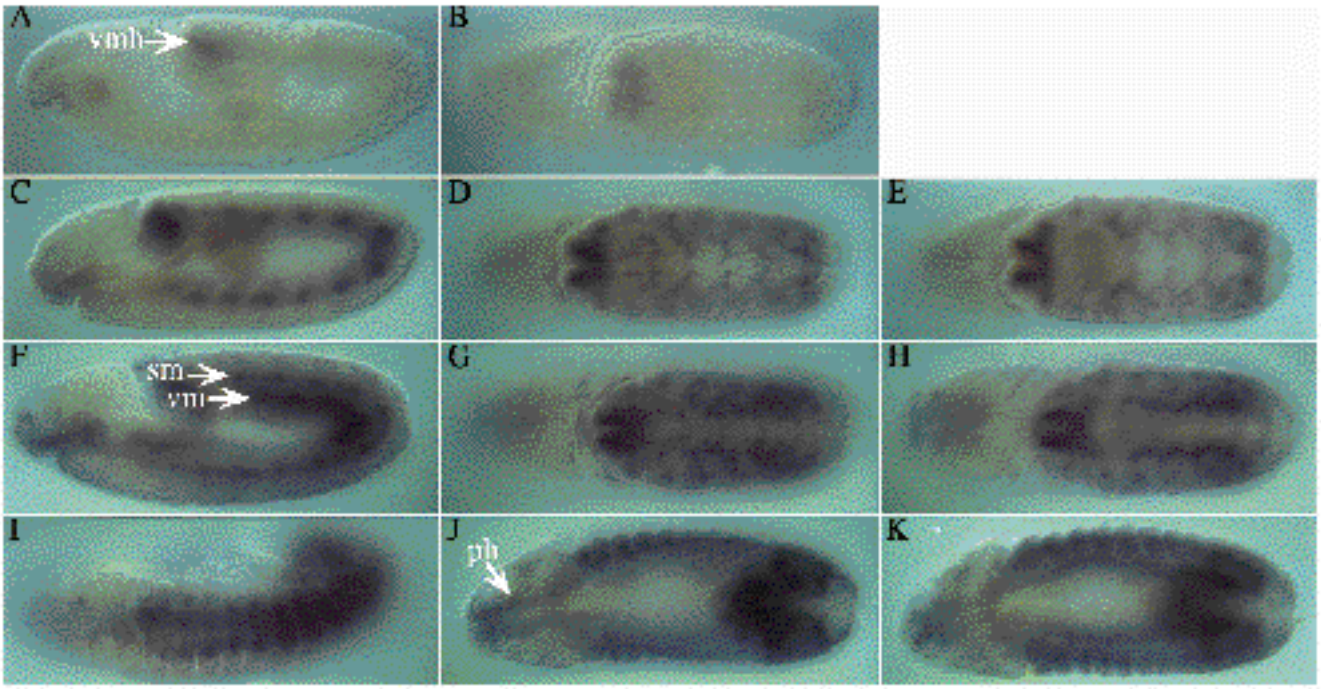

Fig. 1. β_H transcript accumulates in a restricted tissue set during embryogenesis. The insert of pBH5 (see Materials and Methods) was used as a probe for β_H transcripts in whole *Drosophila* embryos. Four embryos are shown, one on each row in two or three different views. The embryos are at stages 10 (A, B), 11 (C-E), late 11 (F-H) and 12 (I-K). In all panels anterior is to the left. A, C, F and I are lateral views (dorsal is up). B, D, G and J are dorsal views. E, H and K are dorsal views at a deeper focal plane. sm, somatic muscle precursors; vm, visceral muscle precursors (splanchnopleura); vmh, precursors of the visceral mesoderm of the hindgut; ph, developing pharyngeal musculature. Thus β_H is expressed in both ectodermal and mesodermal tissue.

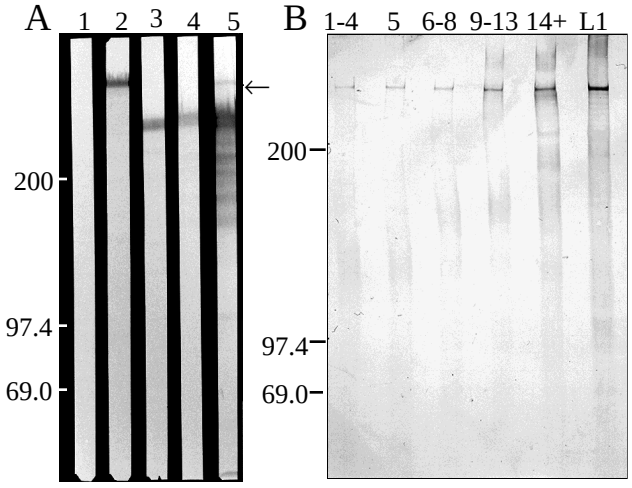

Fig. 2. Antiserum no. 243 reacts specifically with β_H and demonstrates that β_H is present throughout embryonic development. (A) Immunoblots demonstrating the specificity of the 243 antiserum for β_H. Kc_0 cell protein was resolved by SDS-PAGE in a curtain gel configuration, then blotted to nitrocellulose. Neighbouring strips from the blot were probed as follows: Lane 1, preimmune serum no. 243 (1:2000). Lane 2, β_H specific immune serum no. 243 (1:2000). Lane 3, serum no. 89 directed against *Drosophila* β-spectrin (1:2000). Lane 4 and 5, serum no. 905 directed against *Drosophila* α-spectrin (1:10,000 and 1:2000 respectively). The migration of molecular size standards ($M_r \times 10^{-3}$) are indicated to the left of the panel. The position of β_H is indicated by the arrow. (B) An immunoblot showing the expression levels of β_H protein during embryonic development. Protein samples were resolved by SDS-PAGE, blotted to nitrocellulose and probed with antiserum no. 243 at a dilution of 1:1333. The migration of molecular size standards ($M_r \times 10^{-3}$) are indicated to the left of the panel. The numbers at the top of the lanes refer to the stage of development of the embryos that contributed to each sample (Campos-Ortega and Hartenstein, 1985) and L1 indicates that first instar larvae were the source of the sample. For each lane 20 embryos or larvae were used, so the loadings are equivalent (except for the first instar larvae which had hatched and had started to feed). β_H can also be detected in unfertilized eggs at levels similar to stage 1-4 embryos indicating that it must be maternally loaded (data not shown).

developing embryo at a time comparable to the transcription factors *nautilus* (Michelson et al., 1990) and S59 (Dohrmann et al., 1990) which are the earliest known markers of muscle tissue. As the germband starts to retract expression is also seen in the abdominal and thoracic epidermis (Fig. 1I-K) and, after dorsal closure, in the trachea (data not shown).

Developmental expression of β_H protein

The β_H-specific antiserum (no. 243, see Materials and Methods) reacts specifically with the single protein band of the expected M_r=430×10^3 on immunoblots of *Drosophila* cells solubilized in SDS, and does not react with either α- or β-spectrin (Fig. 2A; the no. 905 antiserum directed against α-spectrin is less specific and, at high concentration, reacts weakly with β_H). Protein extracts made from equal numbers of developmentally staged embryos were analyzed for the presence of β_H protein on immunoblots using this antiserum and the results indicate that β_H protein is present throughout embryonic development

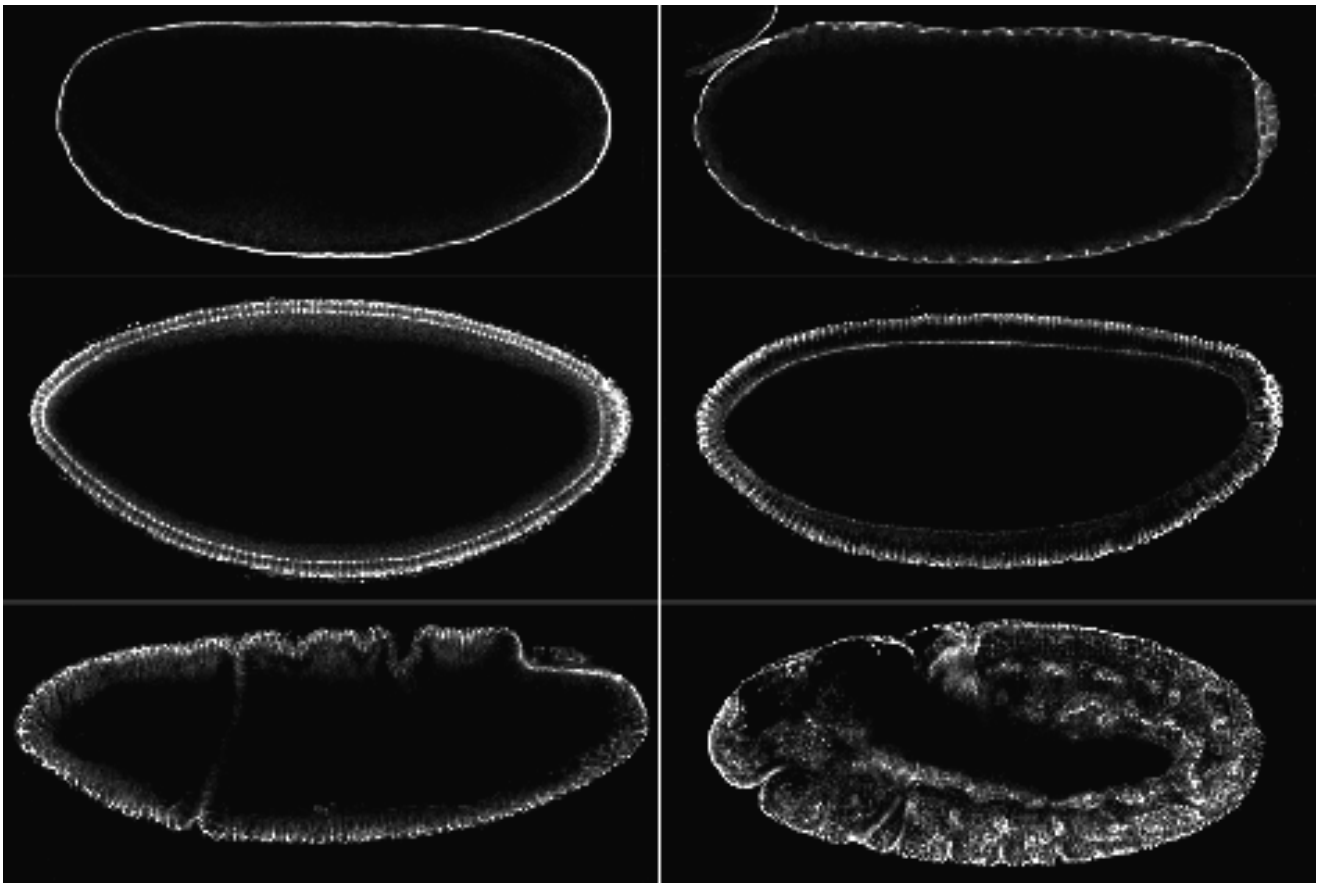

Fig. 3. Maternally supplied β_H protein is associated with specific membrane subdomains. Fixed embryos were stained with affinity purified β_H antibodies from antiserum no. 243 and are shown in sagittal or parasagittal confocal section. (A) In cleavage stage embryos (stage 1-2) β_H is uniformly associated with the plasma membrane. An identical distribution is seen when unfertilized eggs are stained (data not shown). (B) In embryos showing the transient mitotic furrows during syncytial blastoderm (stage 3) β_H is seen to be associated with the invaginating membranes. (C) In the early (slow) phase of cellularization (stage 5) β_H is seen apicolaterally and in a region near the furrow canals. (D) In the late (fast) phase of cellularization. β_H is similarlly distributed as in (C), but as the invaginations reach their fullest extent β_H is lost from the region of the furrow canal. In addition, an apical cap, or plate, of β_H is now evident (arrow) in those cells that are going to form the posterior midgut invagination. (E) In early gastrulation (stage 7) only apicolateral β_H remains, plus the apical cap, or plate, of staining at the posterior midgut invagination (arrow). (F) During late germband extension (stage 11-12) when zygotic expression of β_H begins, the protein is evident in the somatic and visceral muscle precursors (sm and vm respectively).

(Fig. 2B). β_H protein is also present in unfertilized eggs (data not shown) indicating that the protein is maternally contributed. There is a slight increase in the amount of protein after stage 9-11 whereupon it remains relatively constant until hatching.

Distribution of β_H protein in the developing embryo

During the cleavage stage of *Drosophila* embryogenesis, the egg remains a syncytium and the nuclei migrate out to the surface of the egg to lie just below the plasma membrane after 10 nuclear cycles. The next three divisions are also syncytial but the plasma membrane transiently invaginates between each nucleus during mitosis. After nuclear cycle 13, cellularization begins and the plasma membrane invaginates around each nucleus to form individual cells as gastrulation begins (reviewed by Schejcter and Weischaus, 1993).

Maternal β_H protein distribution

Maternal β_H protein is closely associated with the membrane of the egg during the syncytial stages of embryogenesis (Fig. 3A), and shows its first dynamic involvement with the developmental process when it becomes associated with the transient mitotic furrows during nuclear cycles 11, 12 and 13 (Figs 3B, 4B,C). During cellularization, β_H protein is seen in two locations. Some β_H remains apically localized in the cell as a circumferential band of staining on the lateral membrane (apicolateral; Fig. 4D,F,G), but staining is also seen to descend with the invaginating cell membranes (basal), and is concentrated in a region adjacent to the acto-myosin ring (Figs 3C,D, 4F-J and see below; Young et al., 1991). The basal staining disappears during the latter part of cellularization (towards the end of the fast phase; Mahowald, 1963b) and prior to gastrulation, suggesting that it plays a specific role during the cellularization process itself. The basal distribution of β_H appears to be lost first from the ventral cells that invaginate 5-10 minutes ahead of the dorsal cells (Mahowald, 1963a; Fig. 3D).

Redistribution of β_H to an apical cap or plate in the cell antic-

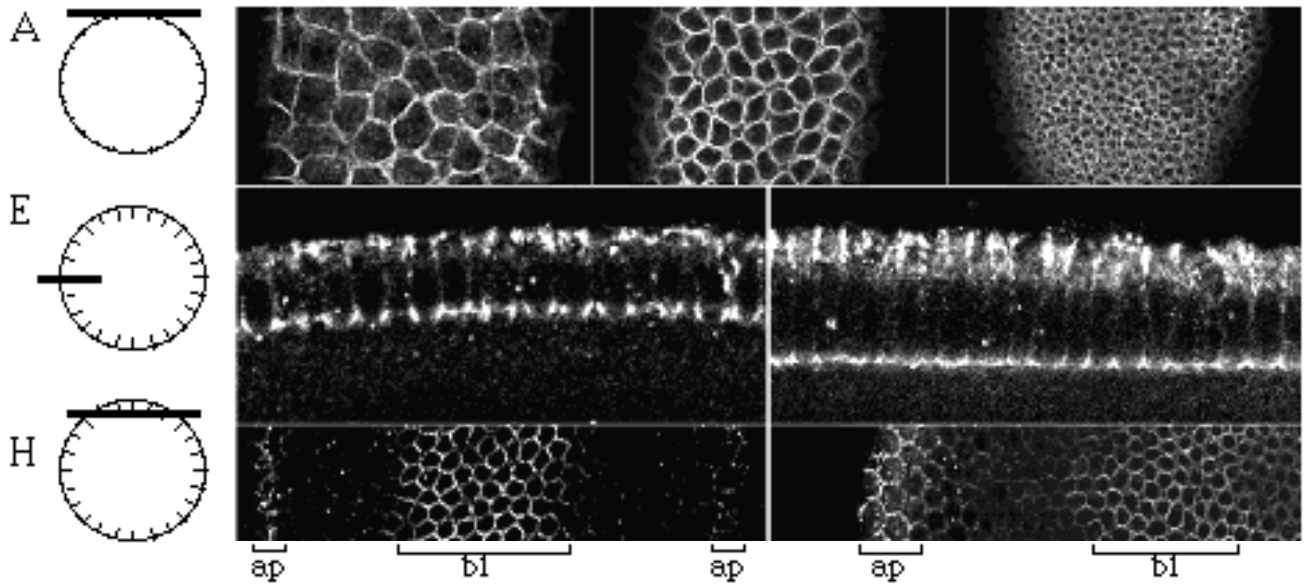

Fig. 4. Maternally supplied β_H is associated with transient mitotic furrows prior to cellularization and is localized to specific membrane subdomains during cellularization. Confocal sections showing the distribution of β_H in embryos stained as in Fig. 3. (A,E,H) Schematic cross sections of embryos indicating the plane of sectioning (bar) in the accompanying rows of pictures. B and C show the association of β_H with the transient mitotic furrows during two mitoses prior to cellularization. (D) β_H distribution at the onset of cellularization. F and G show in detail the two membrane associated regions of β_H protein during mid (F) and late (G) cellularization. The outer surface of the embryo is up in these panels. (I,J) β_H distribution in the same embryos as F and G, but in glancing section. The apical and basal domains are indicated by brackets labeled 'ap' and 'bl' respectively.

ipates cell sheet morphogenesis. Whereas most of the β_H at the cellular blastoderm stage is seen to be apicolaterally localized in all cells except the pole cells (Fig. 3C,D), certain regions that are undergoing contractile processes to produce morphogenetic movements (ventral and cephalic furrows, and the posterior midgut invagination), have an additional cap of β_H across the apical ends of the cells (Figs 3E, 6A,B). In the case of posterior midgut invagination, for example, the redistribution of β_H to a cap, or plate, at the apical surface of the cell clearly anticipates any gross visible movement of the cell sheet (Fig. 3D). Dynamic redistribution of β_H throughout these early developmental stages is therefore correlated with changes in cell shape that are all probably occurring due to contractile forces generated by nonmuscle myosin. The source of the β_H for the new apical domain could either be from a cytoplasmic pool (that is substantial but not well fixed using this methodology) or from a pre-existing membrane domain.

Zygotic β_H protein distribution

Anti-β_H staining in embryos at later stages, after zygotic transcription has initiated (stage 10), recapitulates the transcript accumulation seen with the whole embryo hybridizations. There is, in addition, visible but diminishing staining in all cells that we attribute to the perdurance of the maternally loaded protein.

(i) β_H is closely associated with the plasma membrane of the somatic and visceral musculature: in stage 10-12 embryos we see bright β_H staining in the somatic and visceral muscle precursors (Figs 3F, 5A,B). The somatic muscle precursors are visible in the ventral mesoderm just above the developing central nervous system (CNS), and appear in a segmentally repeated pattern. As the germband retracts these cells move to more lateral positions, and staining can ultimately be seen in the maturing somatic musculature (Fig. 5D,G,H).

The visceral musculature originates in segmentally repeated clusters of cells (Azpiazu and Frasch, 1993; Bodmer, 1993) that join together along the anterior-posterior axis and form the characteristic palisade cell structure of the splanchnopleura (Campos-Ortega and Hartenstein, 1985). Concomitant with the appearance of β_H in the somatic muscle precursors, the protein is also seen in the developing splanchnopleura (Figs 3F, 5B,D-F), and can eventually be seen staining the mature visceral musculature around the midgut epithelium (Fig. 5E,F).

The earliest sites of β_H transcription are associated with the morphogenesis of both the foregut and the hindgut at the stomodeal and proctodeal invaginations respectively (Fig. 1A,C). The former ultimately results in the staining of both the pharyngeal musculature and oesophagus for β_H protein (Fig. 5E,F). The latter site of early transcription is in a layer of cells on the inner surface of the proctodeum (Fig. 1D,E) which go on to give rise to the visceral mesoderm of the hindgut. These cells stain with β_H antibodies and form a layer that stretches along most of the dorsoventral extent of this invagination (Fig. 5A,B). These cells are still evident at later stages of development (e.g., Fig. 5C) and move in an anterior direction along the hindgut. In contrast to the other tissues described below there is no obvious polarization of the cortex in muscle tissue that is reflected in the β_H distribution.

(ii) β_H is apically localized in the trachea: as with other invaginations during embryogenesis each of the 20 bilaterally symmetric tracheal placodes reorganize their membrane skeleton such that β_H is seen across the apices of the cells as they move into the embryo during stage 10 (Fig. 6C, arrows). At this stage β_H transcription is not detectable in the epidermis, so we assume that this is still maternal protein that is being

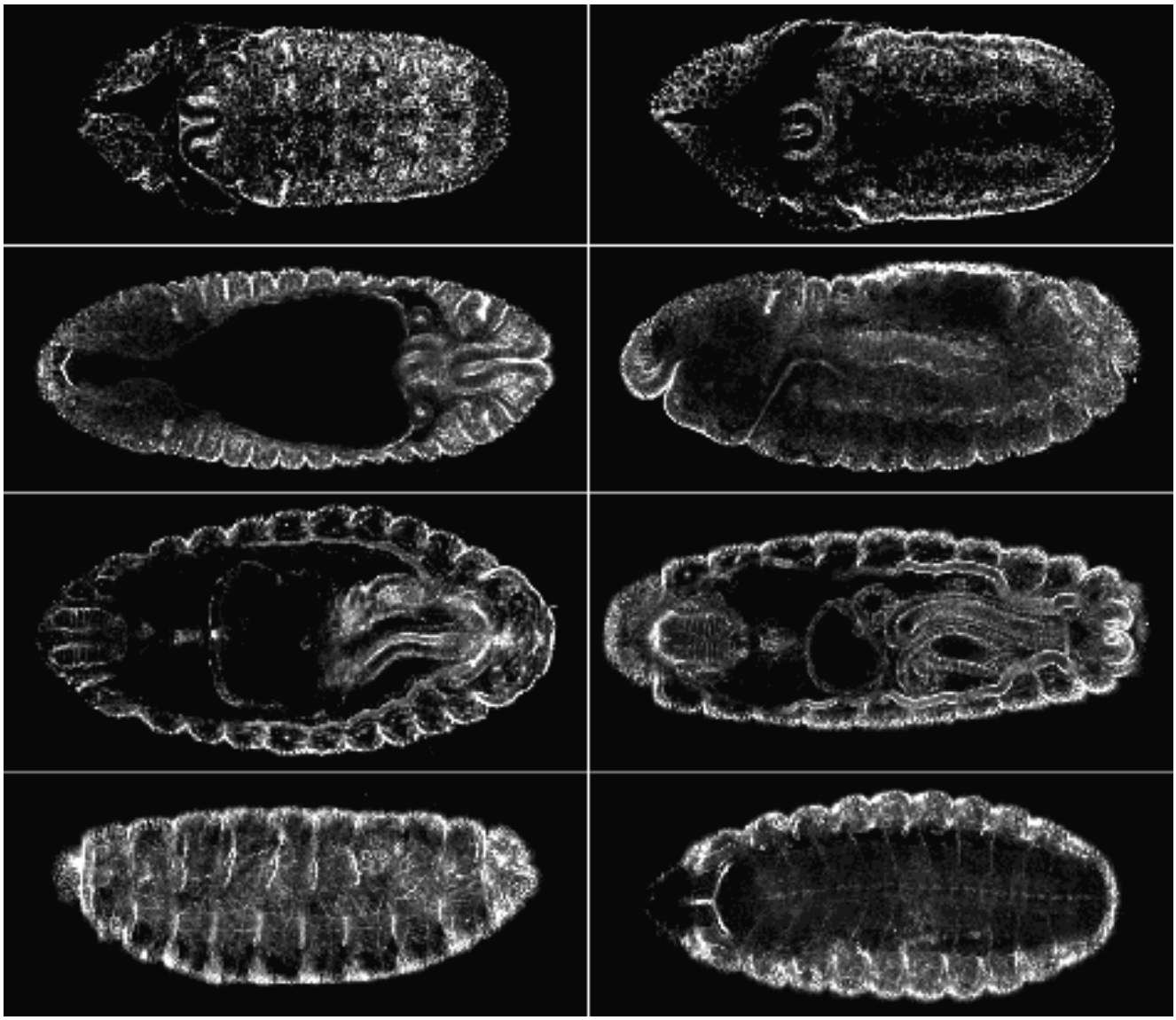

Fig. 5. Zygotic β_H protein has a specific tissue and subcellular distribution. Embryos were stained as in Fig. 3 and are shown in a variety of orientations as confocal sections. In all panels the cells of the epidermis are conspicuously stained around the apical end of their lateral membrane. In some of the pictures this signal is saturating in order to see fainter internal staining. (A) An optical section in the ventral mesoderm below the dorsally presented ventral epidermis shows the segmental repeat of somatic muscle precursors that have elevated levels of β_H (Stage 11-12). (B) The same embryo as in A, but optically sectioned deeper to view the primordia for the visceral mesoderm of the hindgut (vmh) and midgut (vm). (C) An optical section of a stage 13 embryo shows intense staining of the spiracles (sp), hindgut (hg), visceral mesoderm of the hindgut (vmh) and cells at the entrance to the foregut (asterisk-see also D) as well as the epidermis. (D) The same embryo as that in C but shown in parasagittal optical section to illustrate staining of the visceral mesoderm of the midgut (vm), the lumen of the salivary glands (sg) and the optic lobe invagination (ol), as well as one row of somatic muscles (sm, running from asterisk to asterisk). (E) An optical section of a stage 15 embryo shows the prominent staining of the pharyngeal musculature (ph), hindgut (hg), trachea (tr) and epidermis. (F) An optical section of a stage 16 embryo with a well developed gut shows similar features to that in E. (G) An optical section just below the epidermis of a stage 16 embryo highlights the somatic musculature and tracheal branches (arrows indicate three examples of the latter). (H) An optical section through the central nervous system of a stage 16 embryo shows the lumenal staining of the ventral branches of the trachea that penetrate this region (arrows indicate three examples). Note also the bright staining in the lumen of the salivary glands and duct (asterisk) and the somatic musculature just inside the epidermis.

redistributed. As the trachea mature, the sections of the longitudinal trunk connect, the transverse and ventral branches grow out, and transcription starts in this tissue (data not shown). Although the staining is greatest at the abdominal spiracles, the protein can be detected at the lumenal surface of all the cells in the network (Fig. 5E-H) including the branches that penetrate the CNS (Fig. 5H).

(iii) β_H is a prominent component of the cells in the epidermis and amnioserosa: β_H transcript accumulation begins in the epidermis as the germband retracts (Fig. 1I), and this tissue stains intensely for β_H protein (Fig. 5). With the

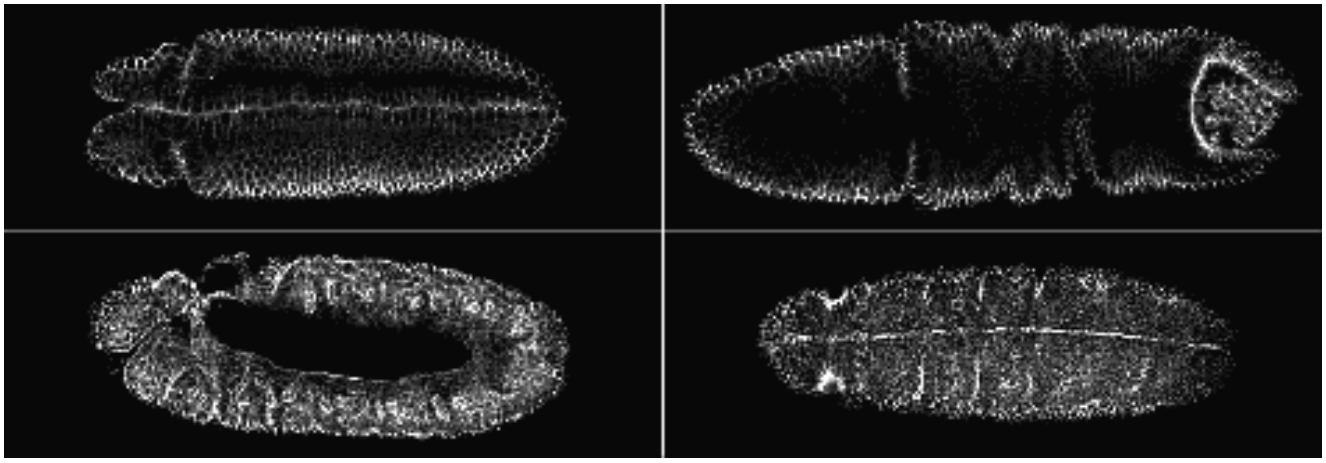

Fig. 6. β_H is redistributed to an apical cap or plate at regions of cell sheet morphogenesis. (A) A ventral optical section of a stage 7 embryo shows the apical localization of β_H in the cephalic (asterisks) and ventral (arrows) furrows. (B) A dorsal view of the same embryo as (A) shows the apical localization of β_H in the posterior midgut invagination (asterisk). A and B are views of the same embryo as in Fig. 3E so as to convey the three dimensional distribution of β_H in the embryo. (C) A lateral view of a stage 11-12 embryo showing apical localization of β_H in the cells invaginating to form the trachea (arrowheads indicate four examples). (D) Ventral view of a stage 12 embryo showing apical distribution of β_H in the cells invaginating to form the salivary glands (asterisks) and also the staining along the ventral midline between the two rows of mesectodermal cells (arrows).

exception of sites of invagination the protein is seen exclusively in an apicolateral distribution that was originally established with maternal β_H protein.

The amnioserosa also stains very intensely for β_H after but not before germband retraction (Fig. 5D, mid-dorsal margin) and we have never detected transcript accumulation here using the digoxigenin-labeled whole-embryo hybridization methods. Two possibilities most easily account for the increase in protein staining in the apparent absence of transcription. Either β_H is assembled into the membrane skeleton from a cytoplasmic β_H pool that is not visualized, or transcription is occurring but is not detected. The amnioserosa is a particularly delicate and exposed structure and our failure to detect transcription may be due to excessive damage to this tissue during the proteolysis step of the hybridization procedure. It is unlikely that this is due to cross reaction with another protein since the serum sees only one band on immunoblots (Fig. 2A) and the stainings shown were performed using affinity purified antibodies (Figs 3-6).

(iv) β_H is also localized in an apical cap or plate at later sites of invagination: staining at the apical ends of the cells at the salivary gland placodes is evident as soon as the organ invaginates (e.g., Fig. 6D), and continues to be present at a high level (Fig. 5D,H) on the lumenal surface of the cells. It is not known exactly when transcription of the β_H gene starts in the salivary gland as whole-mount hybridization is less efficacious after dorsal closure and the start of cuticle deposition, and prior to that the staining intensity of the epidermal layer is very high, making weak internal signals hard to visualize.

The bright line of β_H staining along the ventral midline, which forms as the ventral furrow invaginates and cells on either side come into contact, persists for some time after the ventral furrow disappears (Fig. 6D) and accumulation of β_H transcript starts in the ectoderm. This line of staining lies between the two rows of mesectodermal cells which will later become part of the CNS at stage 12-13 (Campos-Ortega and Hartenstein, 1985), approximately when the β_H staining disappears from the midline. Since this line appears prior to detectable transcript accumulation in the ectoderm it is likely to be established with maternally supplied protein.

Comparison of the distributions of β_H and cytoplasmic myosin

The presence of β_H staining at sites where contraction is generated by cytoplasmic myosin raises the possibility that these two proteins colocalize in the cell. The basal staining for β_H during cellularization is very close to the region that contains the actomyosin contractile ring at the base of the forming cells, and cytoplasmic myosin has also been observed at the apices of the cells that form the midgut invagination (Young et al., 1991). Double staining for both proteins simultaneously reveals that they are in distinct domains at cellularization but colocalize (at this resolution) in the posterior midgut invagination (Fig. 7). Thus, while β_H could be involved in the contractile process at the latter location it is unlikely that this is the case at cellularization.

In summary, the data indicate that the lateral cell membranes are divided into at least four distinct subdomains during cellularization. These are summarized in Fig. 8A.

DISCUSSION

Dynamic changes in the expression pattern and subcellular distribution and redistribution of β_{Heavy}-spectrin (β_H) take place during the embryonic development of *Drosophila.* β_H is localized to specific membrane sub-domains and shows a restricted tissue distribution at specific times during embryonic development. Maternally provided protein is dynamically redistributed without detectable transcription of the β_H gene, presumably by a post-translational mechanism, and results in the assembly of new membrane associated domains of β_H. Subsequently, transcriptional regulation gives rise to tissue specific

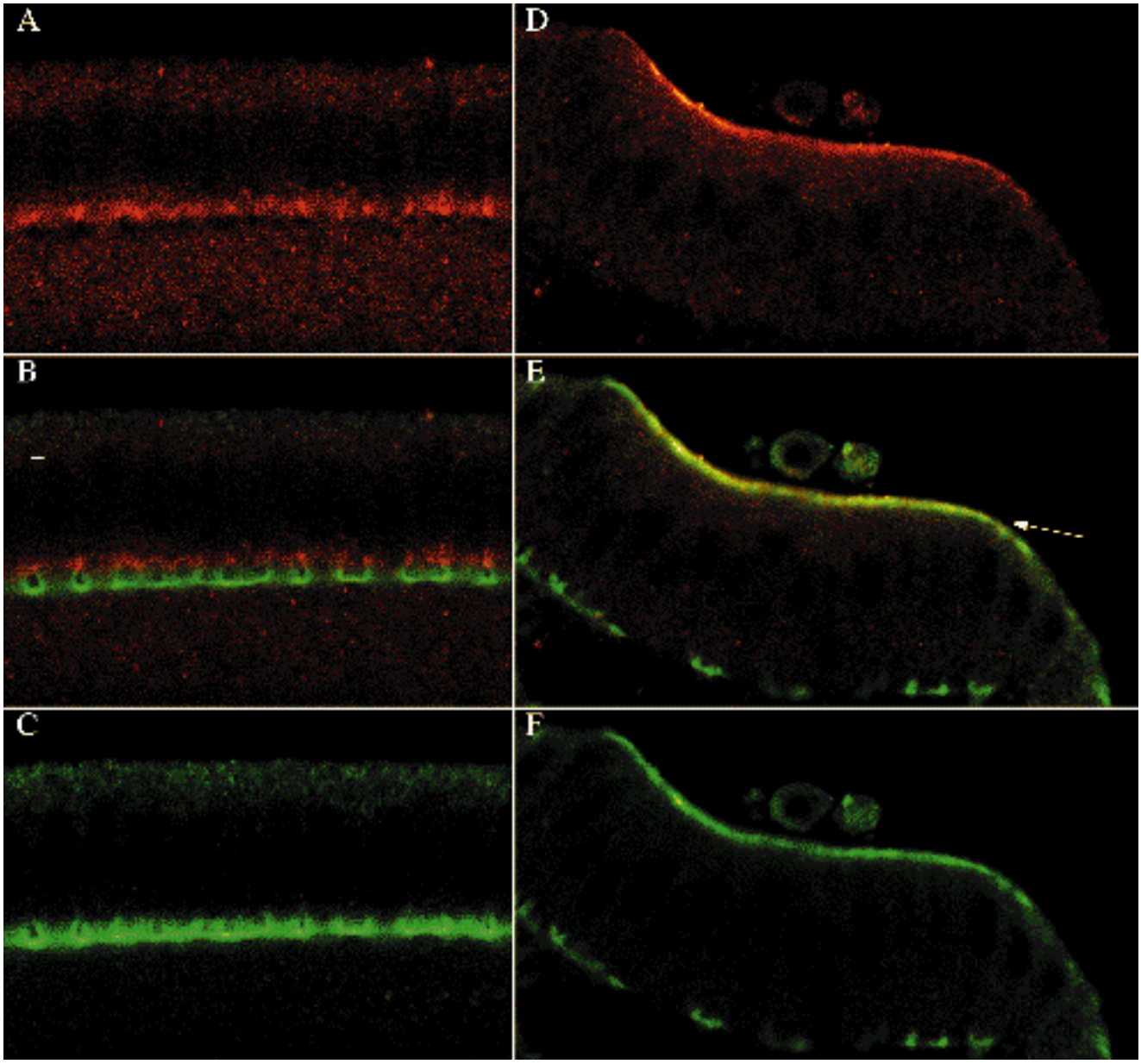

Fig. 7. β_H and cytoplasmic myosin are in distinct domains at cellularization but colocalize at the posterior midgut invagination. Dual labeling for β_H (with the mouse serum DUM2 diluted 1:250) and cytoplasmic myosin (with rabbit serum no. 656 diluted 1:1000) at sites of contraction is shown. (A-C) A sagittal optical section of part of an embryo in mid cellularization (stage 5). (D-F) A parasagittal optical section of the posterior midgut invagination at the beginning of gastrulation (stage 7). A and D are stained for β_H, C and F for cytoplasmic myosin. B and E are false colour composites where red is β_H and green is myosin. At cellularization the domain containing β_H is clearly distinct from that containing myosin (B). The bracket indicates the location of the nuclear layer. During gastrulation and the formation of the posterior midgut invagination β_H and myosin colocalize (E, arrow) at the apices of the contracting cells. Note that in order to covisualize these two proteins it was necessary to add a heat treatment step to the fixation protocol (Miller et al., 1989) prior to methanol step, and that this does not preserve the apicolateral domain of β_H.

β_H expression, although post-transcriptional pathways are likely to continue to direct the protein to specific sub-cellular locations. Together, our observations point to a role for this protein in the maintenance of membrane structure during the diverse cell shape changes that characterize early development.

β_H shows five basic subcellular distributions (a-e) during embryonic development (summarized in Fig. 8). Staining of the cell membranes may be (a) uniform, as in the case of the musculature. Alternatively it may be restricted to one of three distinct membrane subdomains. (b) β_H may be located apically at sites of morphogenesis and in the lumen of the trachea and hindgut; (c) it may be restricted to an apicolateral band around the cell during cellularization and in the epidermis; or it can occupy a basolateral domain (d), during cellularization at the top of the furrow canals. Finally, (e) there is a substantial cytoplasmic pool of β_H (data not shown) that

is not visible in the micrographs presented here due to the fixation technique used.

Comparison with α-spectrin

α-spectrin staining during cellularization is most prominent at or very near the basal domain of β_H protein, but is present at a lower level along most or all of the lateral membranes (Pesacreta et al., 1989). As with β_H, the basal α-spectrin disappears towards the end of cellularization, and it was suggested that this protein was mobilized to an apicolateral position (Pesacreta et al., 1989). Prior to this redistribution of some of the α-spectrin, β_H is simultaneously present in the apicolateral and basal domains, suggesting that either the additional apicolateral α-spectrin (i) is bound to β-spectrin (rather than β_H), (ii) is accompanied by additional β_H or (iii) it binds to pre-existing apicolateral β_H that was not previously bound to α-spectrin. Bloch and Morrow (1989) have reported the existence of a β-spectrin that is not associated with α-spectrin; however, we have no biochemical data at present that addresses this possibility for β_H.

During gastrulation α-spectrin is primarily apicolateral in its distribution (Pesacreta et al., 1989), closely resembling that of β_H; however, at later stages it completely outlines the cells of the epidermis, presumably in association with β-spectrin, while β_H remains apicolaterally distributed. An apical cap of α-spectrin is also seen at the cephalic furrow and the posterior midgut invagination, and is concentrated at the tracheal placodes (Pesacreta et al., 1989), consistent with α- and β_H-subunits operating as a 'conventional' heterodimeric spectrin. Wessel and Chen (1993) have reported a concentration of α-spectrin at sites of active morphogenesis in the sea urchin embryo, so the reorganization of the cortical cytoskeleton may be a general feature of morphogenetic movements.

Functions implied by the distribution of the β_H protein

Spectrins are involved in a number of cellular processes that occur at or close to the plasma membrane, with at least two distinct types of membrane association. In erythrocytes, spectrin is an integral part of the membrane skeleton where it is necessary for stabilization of the cell membrane since various human mutations in α- or β-spectrin lead to fragile erythrocytes (Palek and Sahr, 1992). In non-erythroid cells, spectrins have also been implicated in cell adhesion by their direct association with cell adhesion molecules (N-CAM$_{180}$, Pollerberg et al., 1987; E-cadherin, Nelson et al., 1990), suggesting that spectrin is again closely juxtaposed to the plasma membrane at junctional complexes. However, the spectrin isoform TW260/240 (Glenny et al., 1982) is not closely opposed to the plasma membrane but is found in the actin rich cortical cytoplasm where it crosslinks actin bundles as they emerge from microvilli in the terminal web (Hirokawa et al., 1983).

The localization of β_H during the development of the *Drosophila* embryo is consistent with a number of functions that have been ascribed to other spectrins, but since this study is essentially descriptive we will not dwell at length on these correlations.

β_H is present in an apicolateral domain in the outer epithe-

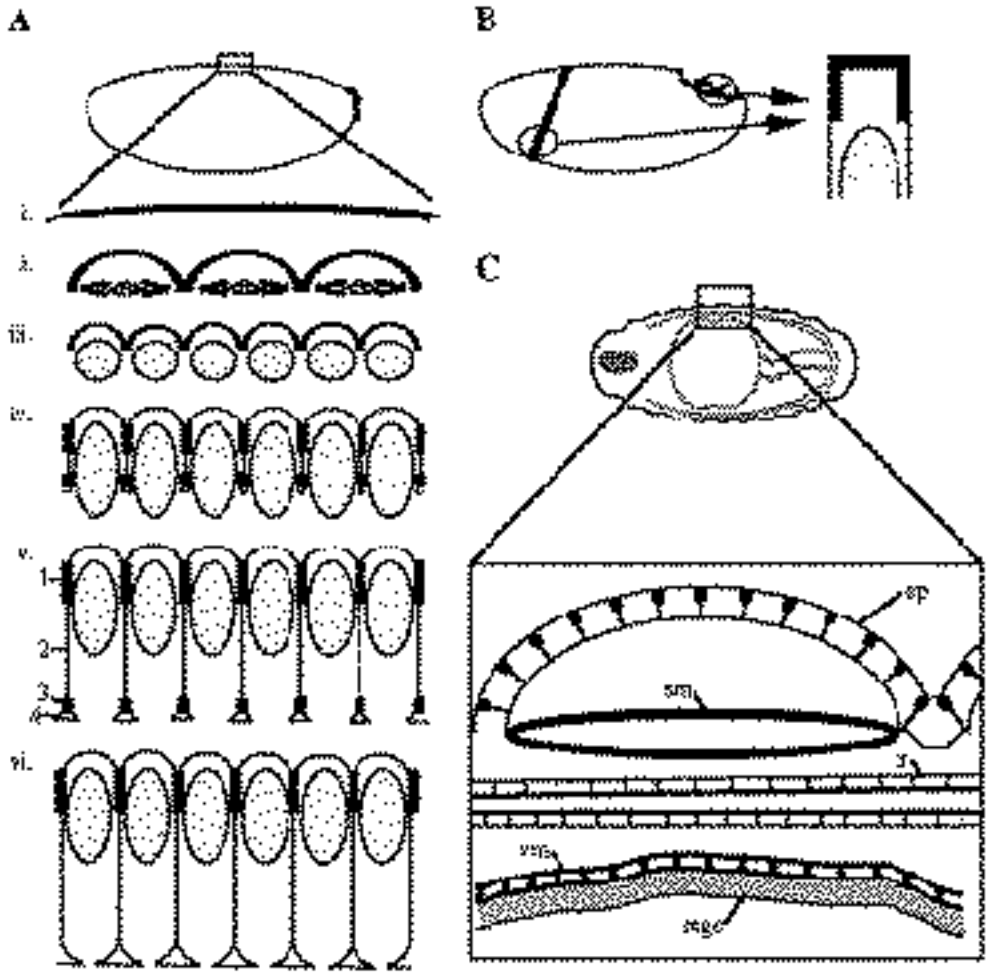

Fig. 8. Summary of the dynamic redistribution of membrane associated β_H during development in representative tissues. (A) Prior to cellularization β_H is present as a uniform layer (i) that invaginates with the transient mitotic furrows (ii), during cellularization (iii-vi) the β_H staining splits into two domains. Colabelling for cytoplasmic myosin reveals that the invaginating membranes at cellularization can be divided into four distinct domains. There is an apicolateral domain (1) containing β_H, a mediolateral domain (2) devoid of β_H, a basal domain (3) containing β_H, and finally a contractile domain (4) defined by the presence of cytoplasmic myosin and actin. (B) At regions of cell sheet morphogenesis (e.g. the cephalic furrow and posterior midgut invagination) an additional apical cap or plate of β_H is seen. (C) Later in development β_H is apicolaterally localized in the epidermis (ep), apically localized to the tracheal lumen (tr), but uniformly distributed around the somatic (sm) and visceral (vm) musculature. The midgut epithelium (mge) is devoid of β_H.

lium of the embryo from cellularization onwards in a region known to be rich in a variety of junctional complexes (Mahowald, 1963a; Eichenberger-Glinz, 1979). β_H may be associated with cell adhesion molecules in this region or could be involved in establishing and/or maintaining the membrane domain in which they reside. During cellularization adjacent cell membranes become loosely adherent above the furrow canals (Eichenberger-Glinz, 1979). A domain of β_H located on the inside of the cell at the base of this region may be associated with transmembrane cell adhesion molecules and may play a role in facilitating a "Velcro-like" joining of neighbouring cell membranes as they extend.

At regions of contraction that lead to morphogenetic movements, and in the lumen of the hindgut and trachea, β_H is also found in an apical cap. At these locations β_H may crosslink actin farther from the membrane, more akin to the TW260/240 molecule (in the terminal web; Hirokawa et al., 1983) and thus, for example, stablilize the F-actin meshwork upon which myosin generates the force for an apical contraction during cell sheet folding. Alternatively, β_H could be more closely juxtaposed to the membrane (comparable to spectrin in the erythrocyte) during contractile events to permit stabilization of the membrane during the blebbing associated with apical contractions (Leptin and Grunewald, 1990; Young et al., 1991), and during the more global changes in cell shape associated with musclular contractions. Regardless of its precise ultrastructural position, β_H could be playing a role in stabilizing the actin rich cortex and overlying membrane.

During muscle development, individual myoblasts fuse to form the final syncytial muscle (e.g., Bate, 1990). It is possible that β_H has a role in this process. Certainly β_H is one of the earliest markers of somatic muscle development where it is expressed at about the same time as the transcription factors nautilus (Michelson et al., 1990) and S59 (Dohrmann et al., 1990) prior to and concomitant with myoblast fusion.

Finally, the line of β_H staining between the two rows of mesectodermal cells (Fig. 5D) is similar to that of the *Toll* protein (Hashimoto et al., 1991). These mesectodermal cells will be moving into the embryo to contribute to the nervous system (Campos-Ortega and Hartenstein, 1985) and so β_H is continuing to mark a region of active morphogenesis; however, the presence of Toll also implies that this isa specialized region of cell-cell contact in which β_H might participate.

The source of the β_H that is associated with the contractile regions, particularly in the formation of the apical cap, is unknown, but it apparently moves to these locations without de novo transcription and translation. β_H could either remoblilize from a pre-existing membrane location or be taken from the cytoplasmic pool of the protein, which is substantial but not revealed with this fixation protocol (data not shown). In either case it seems likely that some kind of post-translational mechanism must operate to redirect the protein to its new domain.

PERSPECTIVE

β_H is positioned to contribute in a number of ways to membrane structure and dynamics during the development of the fly. The staining patterns presented in this paper establish that β_H is in the correct place at the right time to function as the intracellular anchor for a cell adhesion protein(s) that are required at specific subcellular locations, and that it may play some role in structures that perform muscle and nonmuscle contractile processes. One potentially unifying feature of the nonmuscle tissues that express β_H is that they traverse the entire length of the organism (epidermis and trachea) in structures that must withstand the contractile waves that are used for locomotion in the larvae. This is also consistent with β_H acting to reinforce the cells or their attachment to one another.

Most β-spectrins interact with several proteins along their length (Bennett, 1990) and the sequence of β_H so far indicates the presence of the usual spectrum of domains. β_H also contains a Src-homology 3 domain not found in other β-isoforms and probably interacts with other proteins some of which may be involved in signal transduction processes (Schlessinger, 1993). In addition to Toll, β_H colocalizes, at various times and places, with three other membrane associated proteins that are important for the correct development of the fly embryo. These are armadillo (Riggleman et al., 1990), crumbs (Tepass et al., 1990) and Notch (Kidd et al., 1989). This suggests that β_H may also have a role as an intracellular anchor in cell communication processes in addition to the structural roles discussed in this paper.

We would like to thank the following people for their help, advice or encouragement with various aspects of this project: Susan Abmayr, David Alcorta, Bill Gelbart, Alan Michelson, Kim Morrison, Norbert Perrimon, Esther Siegfried, David Strehlow, George Serbedzija, Todd Stewart, Nate Weymouth, Lori Worth and the members of the Kiehart, Goldstein, Branton and Gelbart laboraties. A special thanks goes to John Lee for invaluable advice on numerous occasions. This work was supported by grants to D. P. K. from The Muscular Dystrophy Association of America, The March of Dimes Research Foundation and by the National Institutes of Health (grant no. GM33830). This paper is dedicated to the memory of E. Anne Thomas.

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(Accepted 18 March 1994)