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Neurogenic and proneural genes control cell fate specification in the *Drosophila* endoderm

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SUMMARY

The *Drosophila* endoderm segregates into three non-neural cell types, the principle midgut epithelial cells, the adult midgut precursors, and the interstitial cell precursors, early in development. We show that this process occurs in the absence of mesoderm and requires proneural and neurogenic genes. In neurogenic mutants the principle midgut epithelial cells are missing and the other two cell types develop in great excess. Consequently, the midgut epithelium does not form. In *achaete-scute* complex and *daughterless* mutants the interstitial cell precursors do not develop and the number of adult midgut precursors is strongly reduced. Development of the principle midgut epithelial cells and formation of the midgut epithelium is restored in neurogenic proneural double mutants. The neurogenic/proneural genes are, in contrast to the neuroectoderm, not expressed in small clusters of cells but initially homogeneously in the endoderm suggesting that no prepattern exists which determines the position of the segregating cells. Hence, the segregation pattern solely depends on neurogenic/proneural gene interaction. Proneural genes are required but not sufficient to determine specific cell fates because they are required for cell type specification in both ectoderm and endoderm. Our data also suggest that the neurogenic/proneural genes are involved in the choice between epithelial versus mesenchymal cell morphologies.

Key words: *Drosophila*, endoderm, *achaete-scute* complex, *daughterless*, neurogenic genes, cell type specification

INTRODUCTION

The segregation of the epidermal and neural precursor cells in the *Drosophila* ectoderm has become a paradigm for the mechanism of cell type specification that involves transcriptional regulators that belong to the basic helix-loop-helix (bHLH) family. The *Enhancer of split* gene complex (E(SPL)-C) that comprises seven bHLH genes is required for the development of epidermal cells, and the *achaete-scute* gene complex (AS-C) that comprises four bHLH genes is necessary for the formation of neural cells (for review see Campuzano and Modolell, 1992; Ghysen et al., 1993; Campos-Ortega, 1993). The E(SPL)-C, together with *Notch* (*N*), *Delta* (*Dl*), *neuralized* (*neu*) and several other genes, belongs to a group of genes that has been collectively called neurogenic genes, because in each of the corresponding loss of function mutants an increased number of neural precursors develops (reviewed by Campos-Ortega, 1993). However, in the *achaete* (*ac*), *scute* (*sc*), *lethal of scute* (*l'sc*) and *asense* (*ase*) mutants of the AS-C, as well as in mutants for *atonal* (*ato*) and *daughterless* (*da*) neural precursor cells fail to develop (Garcia-Bellido, 1979; Brand and Campos-Ortega, 1988; Caudy et al, 1988; Jiménez and Campos-Ortega, 1990; Brand et al., 1993; Domínguez and Campuzano, 1993; Jarman et al., 1993a, 1994). These genes therefore have been designated as proneural genes (Ghysen and Dambly-Chaudière, 1989; Romani et al., 1989). More recently it has been shown that the neurogenic mutants also affect the development of many non-epidermal cell types (Cagan and Ready, 1989; Corbin et al., 1991; Ruohola et al., 1991; Hartenstein et al., 1992). That some of the proneural genes also must be involved in more than one developmental process is demonstrated by the requirement of *da* and *sc* for sex determination (reviewed by Jan and Jan, 1993a). However, our understanding of the function of the proneural genes in cell type specification has so far been limited to their participation in the development of the nervous system. We show here that both the neurogenic and proneural genes play a key role in cell type specification of the *Drosophila* endoderm.

The endoderm of *Drosophila*, as in most other animals, gives rise to part of the epithelial lining of the digestive tract. The endodermally derived gut epithelium is composed of different cell types. A serial subdivision of the gut tube into specialized regions along the anterior-posterior axis can be observed. Each region contains a main (or principle) cell type with distinct structural and physiological properties. In addition there are scattered specialized cells that are intermingled with the principle cells of the epithelium. Examples include the epithelial stem cells of the mammalian intestine and the adult midgut precursors in the *Drosophila* larval midgut (e.g. Leblond, 1981; Skaer, 1993). Although the structure and physiology of the gut has been studied extensively, little is known about the embryonic origin and specification mechanisms of endodermally derived cell types. We describe here that the *Drosophila* endoderm segregates into

three cell types, the principle midgut epithelial cells (PMECs), the interstitial cell precursors (ICPs) and the adult midgut precursors (AMPs) early in development. The PMECs form the larval midgut epithelium (Tepass and Hartenstein, 1994b), whereas the ICPs and AMPs initially retain their mesenchymal morphology and enter the midgut epithelium later in development (Reuter et al., 1990; Hartenstein and Jan, 1992). The latter two cell types form scattered populations that are mixed with the PMECs (Filshie et al., 1971, Skear, 1993). Here we show that the specification of PMECs, ICPs and AMPs takes place in the absence of mesoderm, and that it requires the activity of the neurogenic and proneural genes. Our observations suggest that proneural genes together with the neurogenic genes form a gene cassette (Jan and Jan, 1933c) that fulfils a similar function in the ectoderm and in the endoderm.

MATERIALS AND METHODS

Fly stocks and egg collections

The following mutations, which are described in Lindsley and Zimm (1992) if not otherwise indicated, were used in this study. A chromosome mutant for both *twist*^{IH} and *snail*$^{4.26}$. Mutations of neurogenic genes include *neu*^{IF65}, *Dl*^{9P39}, and *Dl*^{5}, and the deletions *Df(1)N*^{81K1}, *Df(3R)E(spl)*^{8D06} and *Df(3R)E(spl)*^{R1}. Mutations of proneural genes studied are *Df(1)sc*^{B57} and *Df(2L)da*^{KX136}. As wild-type stock we used Oregon R.

Germ line clones for a chromosome carrying *Df(1)N*^{81K1} and an *ase* promoter-*lacZ* construct (F:2.0; Jarman et al., 1993b) were generated as described (Tepass and Knust, 1993). The *Df(1)sc*^{B57} *Dl*^{9P39} double mutants marked with the enhancer-trap insertion B11-2-2 (Bier et al., 1989) were generated by crossing *Df(1)sc*^{B57}/+; +/+ ; *Dl*^{9P39}/+ X +/Y; B11-2-2/+ ; *Dl*^{9P39}/+. Among the offspring of this cross 6.25% of the embryos labeled with B11-2-2 have the genotype *Df(1)sc*^{B57}/Y; B11-2-2/+ ; *Dl*^{9P39}/*Dl*^{9P39}. These embryos can be recognized by their ameliorated neurogenic phenotype (Brand and Campos-Ortega, 1988). Flies were grown under standard conditions and crosses were performed at room temperature or at 25°C. Egg collections were done on yeasted apple juice agar plates. Embryonic stages are according to Campos-Ortega and Hartenstein (1985).

Markers, immunohistochemistry and histology

As cell type specific markers we used the enhancer-trap line B11-2-2 (Bier et al., 1989) and A490.2M3 (Bellen et al., 1989) and promoter-*lacZ* constructs of the *ase* gene (F:2.0; Jarman et al., 1993b) and of the *lab* gene (HZ550 and $C_27.31$; Tremml and Bienz, 1992). These enhancer-trap lines and promoter constructs express β-galactosidase which was detected with a polyclonal anti-β-galactosidase antibody (Cappel; dilution 1:2000). A polyclonal anti-*ase* antibody (Brand et al., 1993) was diluted 1:5000. Antibody stainings and sections of stained embryos were done as described previously (Tepass and Knust, 1993).

In situ hybridization

Digoxigenin-labeled DNA probes were prepared following manufacture instructions (Genius kit; Boehringer) using full length cDNAs of the AS-C genes *ac*, *sc*, *l'sc* (Cabrera et al., 1987) and the E(SPL)-C genes mβ, mγ, mδ, m3, m5 and m7, and a genomic *Hin*dIII-*Eco*RI fragment that contains the m8 coding region (Klämbt et al., 1989; Knust et al., 1992). In situ hybridizations to whole-mount embryos were prepared according to the protocol of Tautz and Pfeifle (1989). Embryos were dehydrated and embedded in a 1:3 mixture of methyl salicylate and Canada balsam.

RESULTS

The *Drosophila* endoderm segregates into three different cell types early in development

At the extended germband stage (stages 10 and 11) the endoderm splits up into three cell types, the PMECs, the ICPs, and the AMPs (Fig. 1). This process can be visualized by

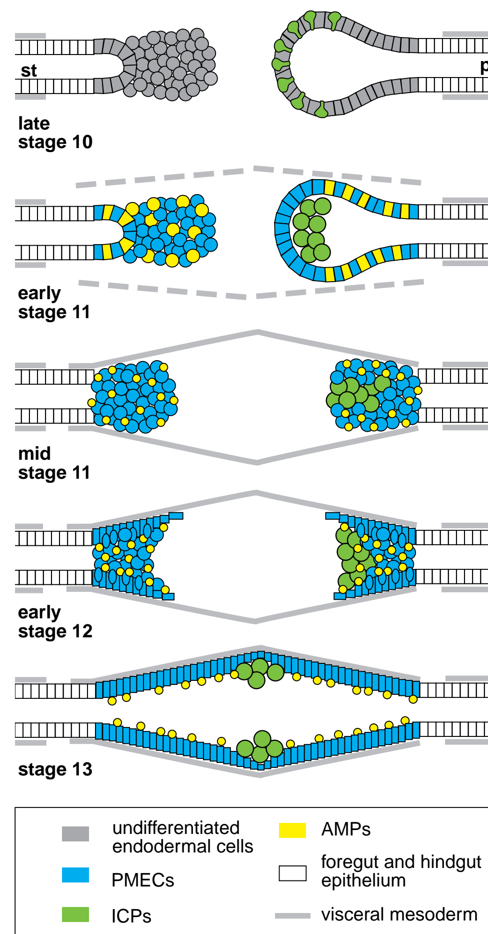


Fig. 1. Synopsis of endoderm development in *Drosophila*. At the extended germband stage the endoderm comprises an anterior and posterior part that are attached to the primordia of the foregut (stomodeum; st) and the hindgut (proctodeum; pr), respectively. Late stage 10: the ICPs segregate from the distal posterior endoderm that forms an epithelial pocket. Note the bottle shaped morphology of the ICPs before segregation. Early stage 11: the ICPs have delaminated into the lumen of the posterior endoderm. The AMPs have segregated from the proximal posterior endoderm and the anterior endoderm. The remaining endoderm cells are the PMECs. The visceral mesoderm has emerged in segmented clusters (Azpiazu and Frasch, 1993). Mid stage 11: anterior and posterior endoderm form a mesenchymal cell mass. The ICPs are located as a coherent cluster in the center of the posterior endoderm and the AMPs are scattered over the entire endoderm. Early stage 12: PEMCs have established contact with the visceral mesoderm that now forms a continuous band. The PMECs migrate along the visceral mesoderm towards the middle of the embryo, thereby reorganizing into an epithelial sheet. Stage 13: the PMECs have completed the formation of the midgut epithelium. The ICPs and AMPs remain as mesenchymal cells at the apical surface of the epithelium. They enter the midgut epithelium later in development.

following the expression of the *ase* gene (Fig. 2). ICPs are formed exclusively in the distal part of the posterior endoderm; AMPs derive, at a slightly later stage, from the proximal posterior endoderm and the entire anterior endoderm. Both cell populations are clearly distinct from a group of *ase*-expressing cells that are associated with the foregut and comprise the stomatogastric nervous system (Hartenstein et al., 1994) and cells at the tip of the Malpigian tubules (Hoch et al., 1994; own observations).

During the time at which ICP segregation takes place, the posterior endoderm forms a blind ending sac of epithelial cells. The ICPs assume a bottle shaped morphology with a constricted apical (luminal) surface and a rounded basal cell portion. Curiously, unlike most other delaminating cell populations, the ICPs delaminate towards the apical side of the epithelium, so that they come to lie in the lumen of the posterior endoderm. AMPs and PMECs form at a stage when all endodermal cells lose their epithelial morphology and form solid clusters of apolar, mesenchymal cells. Shortly thereafter, PMECs reorganize into an epithelium which will become the

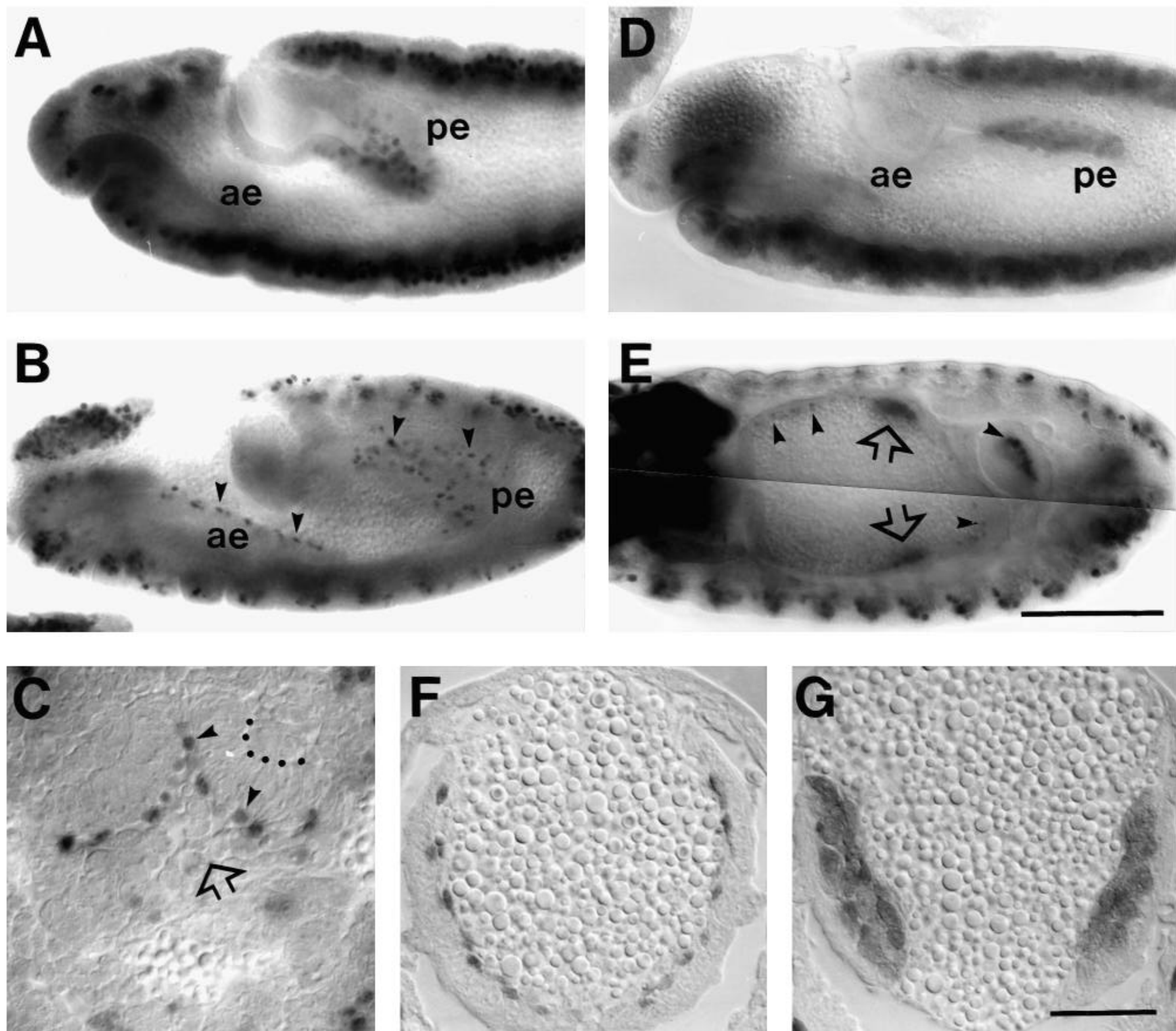


Fig. 2. Development of the ICPs and AMPs in wild type monitored by *ase* expression. (A-C) *ase* antibody staining (Brand et al., 1993). (D-G) Expression of *ase* promoter-*lacZ* construct (F:2.0; Jarman et al., 1993b). (A) At late stage 10 *ase* protein is first seen in the ICPs in the lumen of the posterior endoderm (pe) shortly after they have delaminated. (B) At stage 12 *ase* protein is expressed in the small AMPs (arrowheads) that are scattered over the anterior (ae) and posterior endoderm. (C) Cross section of the posterior endoderm at stage 12. Dotted line marks the boundary between the visceral mesoderm and the epithelial PMECs. *ase*-positive AMPs (arrowheads) are distributed over the surface of the epithelium; ICPs (open arrow) have lost *ase* expression and are located more interiorly. (D) Expression of β-galactosidase is delayed compared to the *ase* protein. Expression is first observed in the ICPs in a mid stage 11 embryo. (E) Due to the perdurance of β-galactosidase *ase* expression can be followed into late embryonic stages. At stage 14 ICPs (open arrows) form two clusters in the center of the developing midgut; AMPs (arrowheads) are scattered over the entire midgut. (F) Section of the anterior midgut at stage 14, showing AMPs at the apical surface of the midgut epithelium. (G) Section of the middle midgut at stage 14, showing clustered ICPs. Anterior is to the left in A,B,D and E. Scale bars: A,B,D,E 70 μm; C,F,G 30 μm.

larval midgut epithelium. Both ICPs and AMPs initially remain as mesenchymal cells in the lumen of the forming midgut. The ICPs form a coherent cluster of large cells located towards the center of the midgut and the AMPs are small cells distributed evenly over the entire midgut.

Early cell type specification in the endoderm occurs in the absence of the mesoderm

Recent studies have shown that several aspects of endoderm development require interactions between mesoderm and endoderm (Reuter et al., 1993; Tepass and Hartenstein, 1994b; Bienz, 1994). In order to determine whether the specification of PMECs, ICPs and AMPs is also influenced by the mesoderm, we assayed for the development of these cells in *twist snail* double mutant embryos, which entirely lack the mesoderm (Grau et al, 1984). In this mutant a regular midgut epithelium does not form; instead, the endoderm cells remain as solid mesenchymal clusters (Tepass and Hartenstein, 1994b). However, the segregation into PMECs, ICPs and AMPs still occurs. Similar to what has been shown above for wild-type embryos, in *twist snail* double mutants a fraction of endodermal cells express *ase* (Fig. 3). Some of the *ase*-positive cells form a cluster of large cells (the ICPs), whereas other, small *ase*-positive cells are scattered over the entire endoderm (the AMPs).

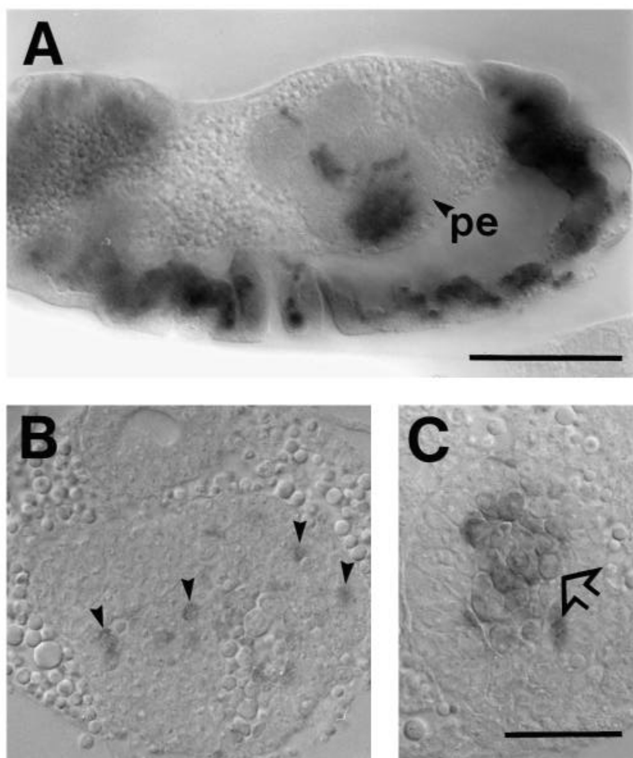


Fig. 3. Endoderm development in embryos that lack mesoderm. A-C show *twist snail* double mutants labeled with an *ase* promoter-*lacZ* construct. (A) Lateral view of a stage 14 embryo; only a fraction of the cells of the posterior endoderm (pe) express *ase*. (B,C) Cross sections of the posterior endoderm showing scattered AMPs (arrowheads in B) and a cluster of ICPs (open arrow in C). Anterior is to the left in (A). Scale bars: A 70 μm; B,C 30 μm.

Neurogenic genes are required for the development of PMECs

In embryos with reduced or no function of the neurogenic loci E(SPL)-C, *N*, *Dl* and *neu*, midgut development is severely disturbed (Hartenstein et al., 1992; Reuter et al., 1993), and in *N* mutants the number of AMPs is increased (Hartenstein et al., 1992). Further analysis revealed that in mutants carrying null alleles of *Dl* and in embryos that lack maternal and zygotic *N* expression, all endodermal cells express *ase* (Fig. 4B,D). Expression of *lab*, which serves as a marker for a subset of PMECs (Reuter et al., 1990; Fig. 4G), is absent in these mutants (data not shown). These findings indicate that PMECs do not develop. Correspondingly, in sections of such mutant embryos, no trace of a midgut epithelium can be found (Fig. 4B,D). Approximately 30-50% of the *ase*-positive cells in the mutant embryos form a coherent cluster of large cells, while the remaining *ase*-positive endodermal cells form a solid mass of small cells, suggesting that both ICPs and AMPs develop in excess numbers (not shown).

In embryos mutant for a strong but not amorphic *neu* allele (not shown) and in embryos that lack only zygotic *N* function, a small fraction of PMECs develops (Fig. 4C). Embryos carrying deletions removing the entire E(SPL)-C which show a consistent extreme neuralization of the ectoderm display a variable endodermal phenotype (Fig. 4E,F). In some embryos, the large majority of endodermal cells turn into *ase*-positive AMPs and ICPs, whereas in others a substantial fraction of PMECs appears, which are able to form small epithelial islands (Fig. 4E,I). In conclusion, lack of neurogenic gene function leads to an increase of the number of AMPs and ICPs at the expense of PMECs. Even though they become attached to the visceral mesoderm, AMPs and ICPs are incapable of forming an epithelium.

Proneural gene function is required for the development of ICPs and AMPs

Midgut development was studied in embryos carrying *Df(1)sc*^{B57}, which removes all four genes of the AS-C, and *Df(2L)da*^{KX136}, which deletes the *da* locus. Because the endoderm expression of *ase* depends on AS-C and *da* function (Brand et al., 1993), the enhancer-trap lines B11-2-2 or A490.2M3 were used as general endodermal markers and PMECs, ICPs, and AMPs distinguished on the basis of their size, shape and position (Tepass and Hartenstein, 1994b; Fig. 5). In AS-C mutants, ICPs are absent and the number of AMPs is strongly reduced. Since no cell death could be seen in the developing midgut (not shown) it is likely that the cells that would have developed as ICPs and AMPs in wild type, develop as PMECs instead. The PMECs of AS-C mutants form a structurally normal midgut epithelium. A loss/reduction of ICPs and AMPs also occurs in *da* mutant embryos. However, in this mutant, cell death is apparent in the developing midgut in both A490.2M3-labeled (Fig. 5F) and toluidine blue stained sections (not shown). This suggests that in *da*, the development of ICPs and AMPs may be initiated, but is terminated shortly thereafter by apoptosis.

The developmental fate of the ICPs, which had previously been called large basophilic cells because of their staining properties (Campos-Ortega and Hartenstein, 1985), has to date been unknown. The finding that the ICPs are missing in AS-C

and *da* mutants allowed us to address the issue of ICP fate. Using *lab* as a marker for the copper cells (Hoppler and Bienz, 1994), we can show that this cell type is present in normal amounts in AS-C and *da* mutants (Fig. 5G-I), but that they are not mixed with unlabled cells as in wild type (not shown) indicating that the ICPs represent the precursors of the interstitial cells and that the copper cells develop as a subpopulation of the PMECs.

The midgut epithelium forms in AS-C *Dl* double mutants

In double mutant combinations between neurogenic and proneural genes a prevalance of the proneural and a suppression of the neurogenic phenotype was observed in the neuroectoderm (Brand and Campos-Ortega, 1988; Heitzler and Simpson, 1991). Our analysis of endoderm development in *Dl* AS-C double mutants yields similar results (Fig. 6). An appar-

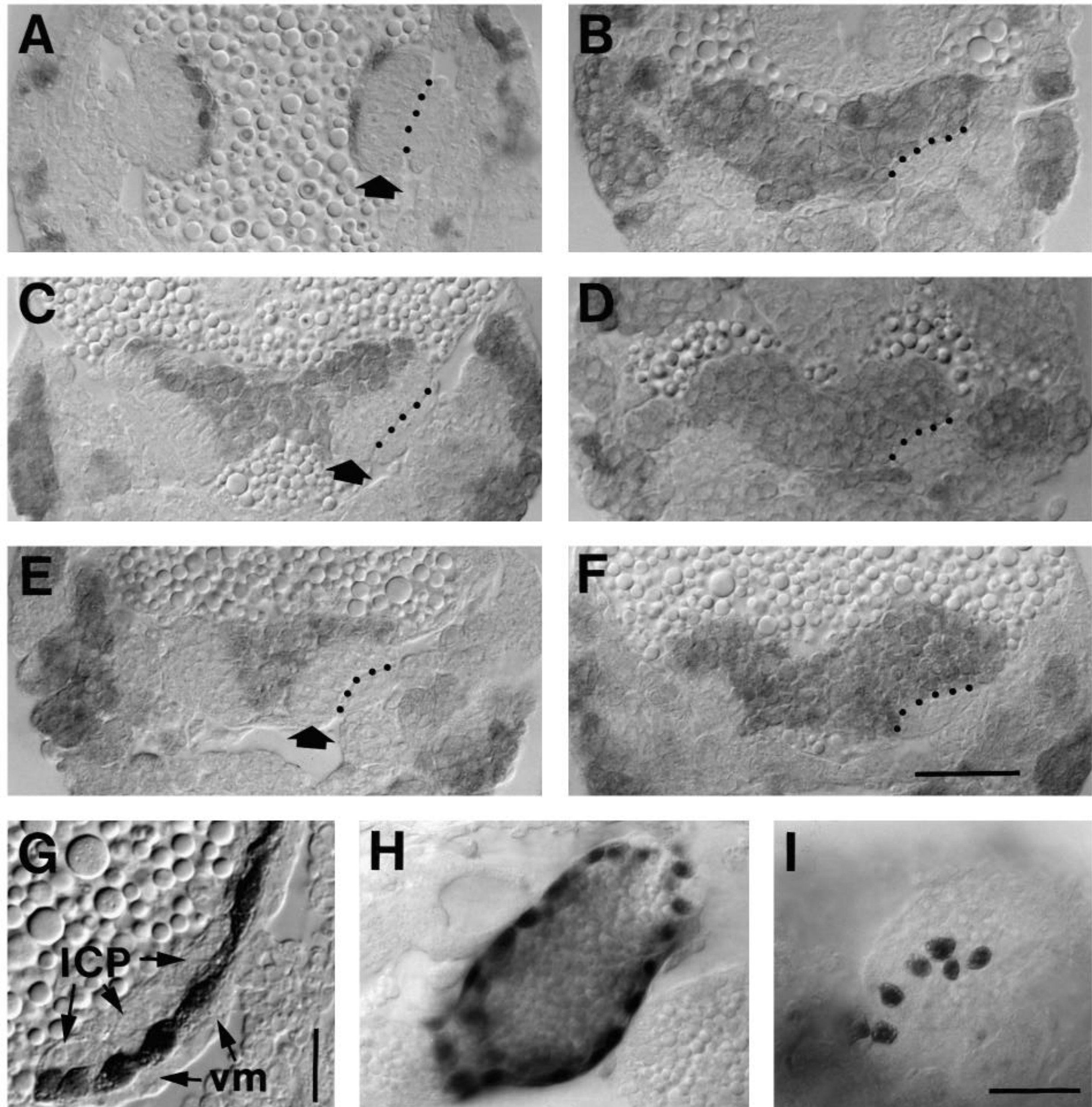
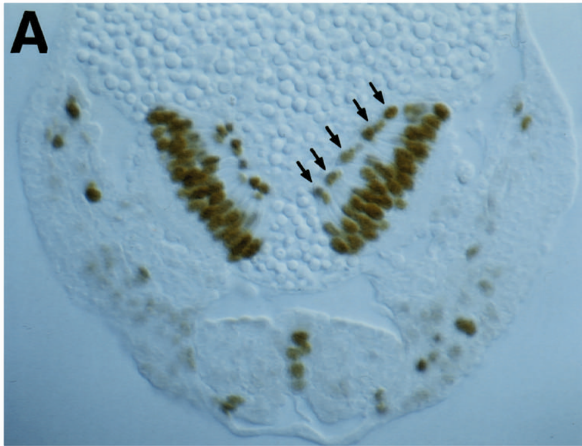


Fig. 4. Endoderm defects in neurogenic mutants. (A-F) Cross sections of the anterior endoderm of stage 13 embryos labeled with the *ase* promoter *lacZ* construct. Dotted lines mark the boundary between visceral mesoderm and endoderm. (A) Wild-type embryo. Some *ase*-expressing AMPs are located at the apical side of the epithelial PEMCs (arrow). (B) *Dl* mutant. All endodermal cells express *ase* and form a multilayered cluster. (C) Embryo lacking zygotic *N* expression. AMPs are increased in number, but some PMECs have developed (arrow) which form an epithelium attached to the visceral mesoderm. (D) Embryo that lacks maternal and zygotic *N* expression. Same phenotype as in (B). (E) Embryo deficient for the E(SPL)-C. Same phenotype as in C. (F) Embryo deficient for the E(SPL)-C. Same phenotype as in B and D. (G-I) Embryos labeled with a *lab* promoter *lacZ* construct. (G) Cross section of a stage 14 wild-type embryo. The *lab*-expressing cells are a subpopulation of PMECs that form a monolayer separating the visceral mesoderm (vm) and the ICPs (ICP). The number of *lab*-expressing cells is strongly reduced in E(SPL)-C mutants (I) compared to wild type (H). Scale bars: A-F 30 μm; G 10 μm; H, I 30 μm.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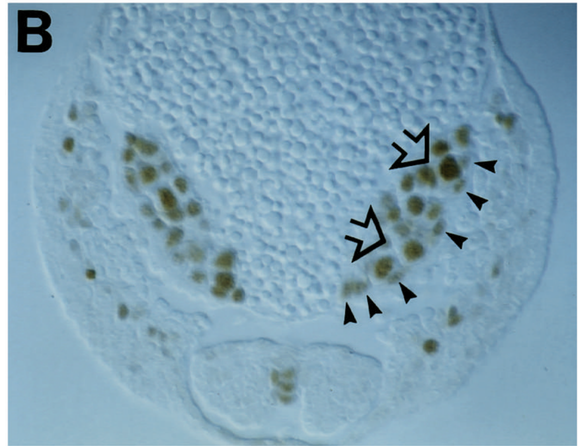
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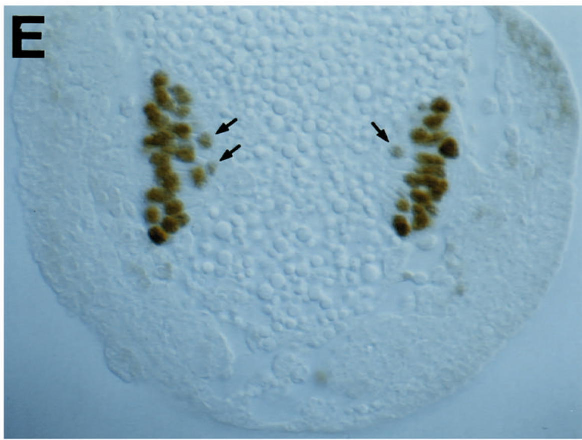
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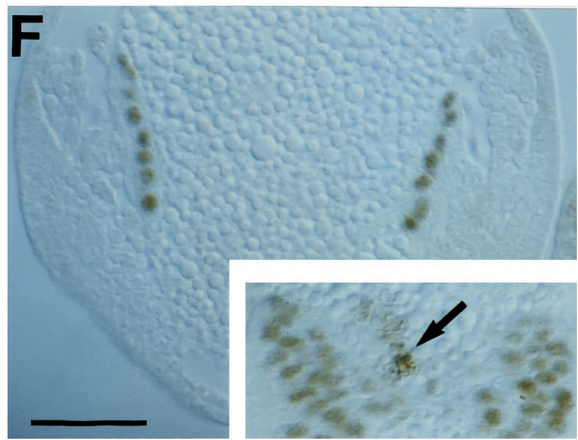
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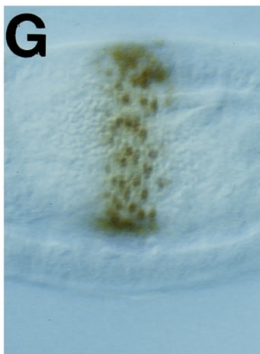
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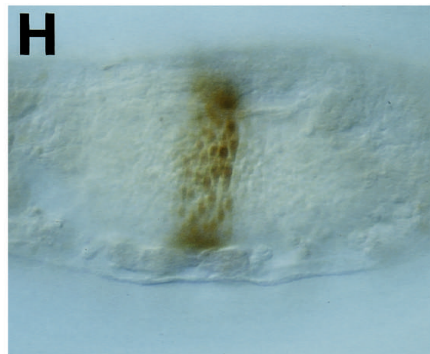
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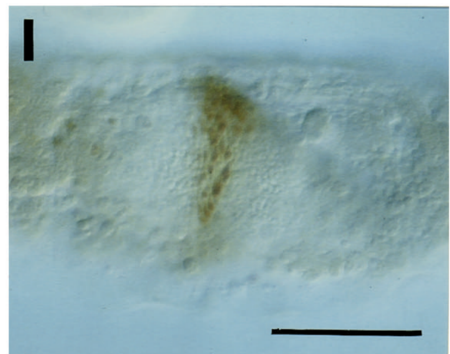
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ently normal midgut epithelium forms in these double mutants suggesting that the PMECs develop and differentiate normally. As in AS-C mutants we find that the ICPs are missing in the double mutants (Fig. 6) and that the number of AMPs is reduced (not shown). Taken together, these observations suggest that the proneural phenotype is epistatic to the neurogenic phenotype in the endoderm, implying that the neurogenic genes are not required for the reorganization of the PMECs into an epithelium once the PMECs are specified.

Expression of E(SPL)-C and AS-C genes in the endoderm correlates with the segregation of ICPs, AMPs and PMECs

The expression of the E(SPL)-C and AS-C genes in the neuroectoderm is tightly regulated temporally and spatially and correlates with the segregation of neural and epidermal precursors (Cabrera et al., 1987; Romani et al., 1987, 1989; Cubas et al., 1991; Martín-Bermudo et al., 1991; Skeath and Carroll, 1991, 1992; Knust et al., 1987, 1992). In the endoderm the bHLH genes of the E(SPL)-C (mβ, mγ, mδ, m3, m5, m7, and m8, which corresponds to the *E(spl)* gene) are expressed in very similar patterns, with the exception of m5 for which no expression was detected. Expression is first seen in late stage 9/early stage 10 embryos in the distal posterior endoderm, rapidly followed by a uniform expression in the entire anterior and posterior endoderm. During late stage 10/early stage 11, E(SPL)-C expression disappears, first from the distal posterior endoderm, then from the remaining endoderm (Fig. 7A-C). Weak expression of some of the E(SPL)-C transcripts was seen in the ICPs at stage 11.

The AS-C genes are expressed in distinctly different patterns in the endoderm. We were unable to detect *ac* transcript in the endoderm, although the same embryos showed intense labeling of proneural clusters in the neuroectoderm. *l'sc* expression (Fig. 7D-G) appears in the distal posterior endoderm at the end of stage 8. During stage 9 uniform expression is seen in the entire endoderm. *l'sc* mRNA disappears from the endoderm during stage 10, initially from the distal posterior endoderm, shortly thereafter from the remaining parts of the endoderm. Weak *l'sc* signal remains in the ICPs at stage 11. The pattern of *sc* expression follows that of *l'sc* with a temporal offset (Fig. 7H-N). *sc* expression, which is initially (early stage 10) homogeneous, increases in the presumptive ICPs before they segregate from the distal posterior endoderm. Slightly later, the level of *sc* in the presumptive AMPs is also increased. The pattern of sc expression reveals that the presumptive ICPs and AMPs are evenly spaced before their segregation. They are separated by a single cell diameter (Fig. 7I). Expression remains strong in both ICP and AMP cells during and after their segregation until mid stage 11, while the PMECs lose *sc* transcript. *ase*, as described above, is expressed exclusively in the ICPs (late stage 10 to mid stage 11) and AMPs (early stage 11 to late stage 12) during and after their segregation.

Fig. 5. Endoderm defects in AS-C and *da* mutants. (A-F) Cross sections of the anterior midgut (A,C,E) and the middle midgut (B,D,F) of stage 13 wild-type (A,B), AS-C deficient (C,D) and *da* deficient (E,F) embryos. Embryos are labeled with the enhancer trap lines B11-2-2 (A-D) or A490.2M3 (E,F) that are expressed in all endodermal cells. (A) In wild type, numerous AMPs (small arrows) are scattered over the midgut epithelium formed by the PMECs. The number of AMPs is strongly reduced in AS-C (C) and *da* (E) mutants. (B) ICPs (open arrow) form a cluster of large cells in wild type. They are separated from the visceral mesoderm by a monolayer of PMECs (arrowheads; compare to Fig. 4G). The PMECs in this region are flat to cuboidal, in contrast to the PMECs in the anterior and posterior midgut which are columnar (compare to A). ICPs are missing in AS-C (D) and *da* (F) mutants. Inset in F shows labeled cellular debris (arrow) in the posterior midgut of a *da* mutant embryo presumably representing degenerated ICPs. (G-I) Wildtype (G), AS-C (H) and *da* (I) mutant labeled with a *lab* promoter-*lacZ* construct. *lab* is expressed in a subpopulation of PMECs that are located between the ICPs and the visceral mesoderm (Reuter et al., 1990; see Fig. 4G). In both AS-C and *da* deficient embryos, these cells develop normally. In (G-I) anterior is to the left. Scale bars: A-F 30 μm; G-I 70 μm.

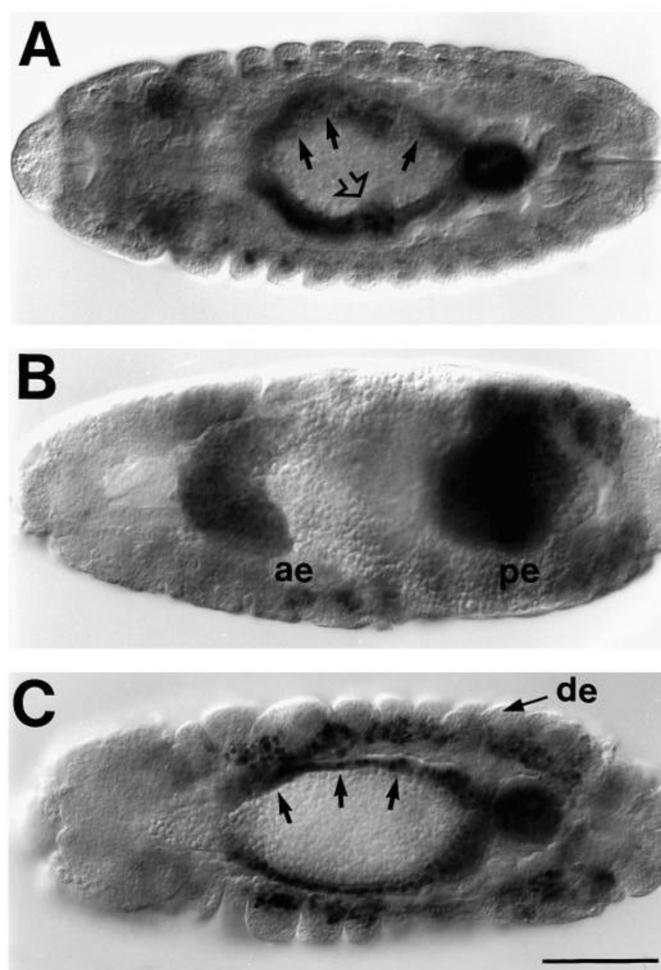

Fig. 6. Endoderm defects in *Dl* AS-C double mutants. (A) Wildtype, (B) *Dl* mutant, and (C) *Dl* AS-C double mutant at stage 14 labeled with the general endodermal marker B11-2-2. (A) The PMECs have formed the midgut epithelium (arrows). The ICPs form a centrally located cluster (open arrow). (B) No midgut epithelium has formed in *Dl* mutants and the anterior (ae) and posterior (pe) endoderm remain as large mesenchymal clusters that consist only of AMPs and ICPs (see Fig. 4). (C) In the *Dl* AS-C double mutant formation of the midgut epithelium is restored (arrows) but the ICPs are missing. Note also the substantial rescue of dorsal epidermis (de) that has been reported previously (Brand and Campos-Ortega, 1988). Anterior is to the left. Scale bar for A-C: 60 μm.

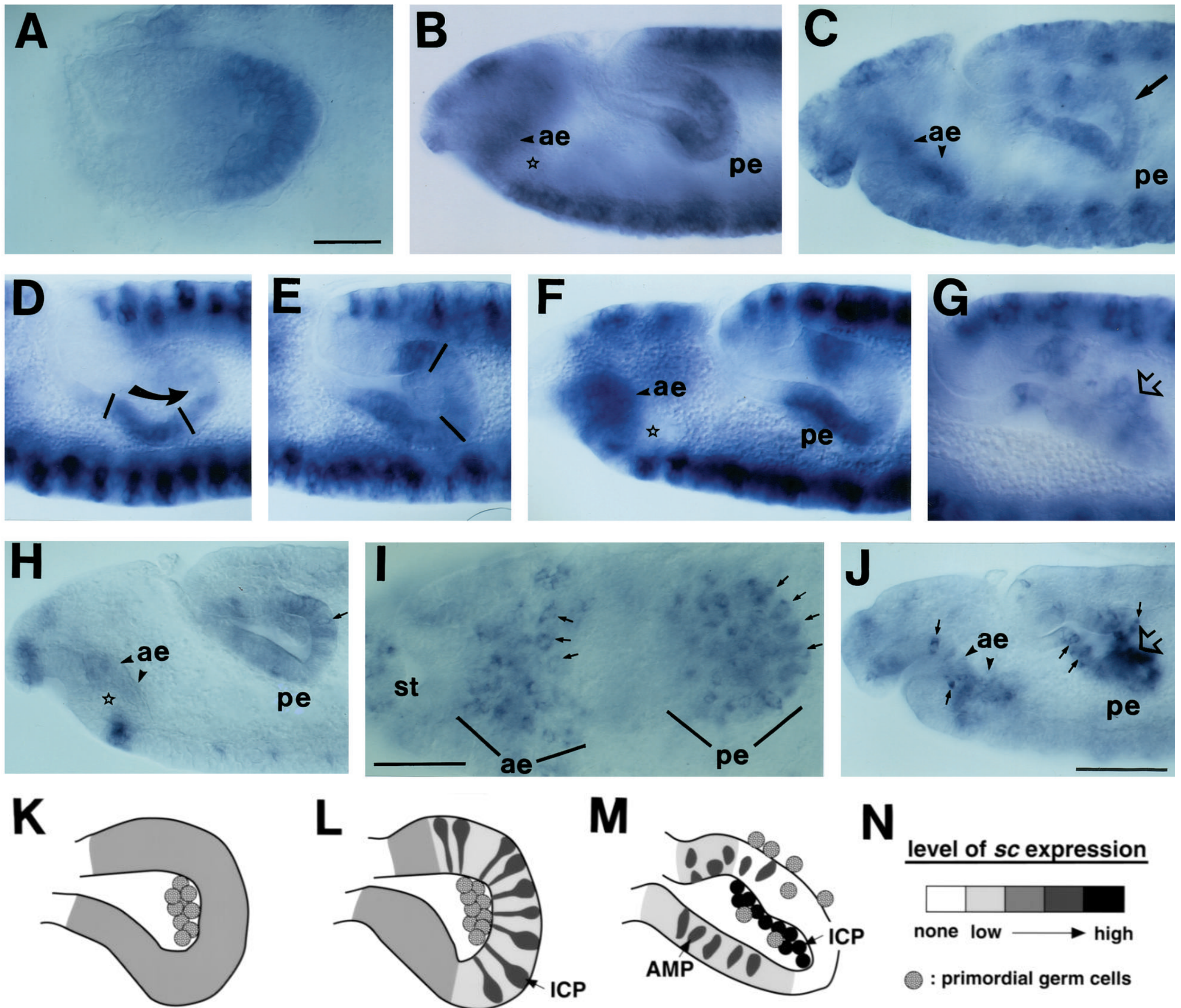

Fig. 7. Expression of E(SPL)-C and AS-C in the endoderm. (A-C) Endoderm expression of E(SPL)-C mγ. (A) Late stage 9; expression is first seen in the posterior endoderm. (B) Early stage 10; uniform expression in the anterior (ae) and posterior (pe) endoderm. Cells indicated by the star belong to the mesoderm. (C) Late stage 10; expression has decreased in the distal posterior endoderm (arrow) but is still present in the remaining endoderm. (D-G) Endoderm expression of *l'sc*. (D) Late stage 8; expression is seen in the distal posterior endoderm (between bars). During the completion of germband extension the posterior endoderm will turn about 90° in the direction indicated by the arrow (Campos-Ortega and Hartenstein, 1985). (E) Stage 9; ubiquitous expression in the posterior endoderm. Note that *l'sc* transcript level has already decreased in the distal part (between bars). (F). Early stage 10; *l'sc* expression is strong in the anterior and the proximal posterior endoderm, but not detectable in the distal posterior endoderm. Star indicates mesodermal cells. (G) Early stage 11; weak *l'sc* expression is seen in the ICPs (open arrow). (H-J) Endoderm expression of *sc*. (H) Mid stage 10; ubiquitous expression is observed in the anterior and posterior endoderm. Scattered cells in the distal posterior endoderm have started to increase *sc* expression (arrow). Star indicates mesodermal cells. (I) Ventral view of a late stage 10 embryo. Cells scattered over the entire endoderm have increased *sc* expression, while adjacent cells have decreased *sc* expression. Note the regular spacing of labeled cells (arrows) that are separated usually by a single cell diameter. (J) Early stage 11; ICPs that have delaminated into the lumen of the posterior endoderm show high levels of expression (open arrow). AMPs (arrows) are still intermingled with the remaining endodermal cells. (K-N) Camera lucida tracings of *sc* expression in the posterior endoderm in mid stage 10 (K), late stage 10 (L) and early stage 11(M). N provides a key for (K-M). *sc* expression increases first in the presumptive ICPs in the distal posterior endoderm (L). Note the bottle shaped morphology of the ICPs at this stage. (M) After ICPs have delaminated, the distal posterior endoderm is free of staining. At this stage, presumptive AMPs in the proximal posterior endoderm have accumulated *sc* transcript. The primordial germ cells penetrate the wall of the posterior endoderm at the time of ICP delamination. Anterior is to the left in all panels. Scale bars: A, 20 μm; B-H,J, 60 μm; I, 45 μm.

sc expression is elevated in neurogenic mutants

To study the interactions between neurogenic and proneural genes in midgut development we examined *sc* expression in neurogenic mutants (Fig. 8). In *Dl* mutant embryos the level of *sc* expression is uniform throughout the endoderm and substantially increased compared to presumptive ICPs and AMPs in wild-type embryos (Fig. 8A,B). These findings suggest that the normal activity of neurogenic genes negatively regulates proneural gene transcription in all endodermal cells, i.e. initially also in those cells that accumulate *sc* in wild type and develop as AMPs or ICPs.

Embryos carrying deletions of the E(SPL)-C show a variable increase in *sc* expression (Fig. 8C,D). Thus, in some E(SPL)-C mutant embryos high levels of *sc* expression occurred in the entire endoderm, while in other mutant embryos only scattered patches of endoderm cells showed elevated *sc* expression. This finding is consistent with our phenotypic observations, which show that in *Dl* mutants PMECs are missing altogether, whereas in E(SPL)-C mutants the number of PMECs is variably reduced. It seems likely that other, yet unidentified factors, possibly additional bHLH genes located outside of the E(SPL)-C, cooperate with the E(SPL)-C bHLH genes in order to suppress proneural gene activity in the endoderm.

DISCUSSION

The diverse cell types found in the epithelial lining of the *Drosophila* larval midgut are specified by at least two different mechanims. During an early endoderm autonomous mechanism involving the activity of the neurogenic and proneural genes, three cell types, the AMPs, ICPs and PMECs, are generated. In neurogenic mutants the number of AMPs and ICPs is strongly increased at the expense of PMECs. In proneural mutants, however, ICPs are absent and the number of AMPs is strongly reduced. The AMPs and the ICPs alone, in contrast to the PMECs, are not capable of forming an epithelium as can be seen in neurogenic mutants. After the PMECs have formed an epithelium, they are regionally specified to form several structurally distinct subpopulations. Previous studies have shown that the specification of at least some of these different PMEC fates depends on interactions between the visceral mesoderm and the endoderm (reviewed by Bienz, 1994).

The apparently normal segregation of AMPs, ICPs and PMECs in embryos without mesoderm is a novel feature of endoderm differentiation in *Drosophila*. We have not formally proved that the segregation of AMPs and ICPs from the

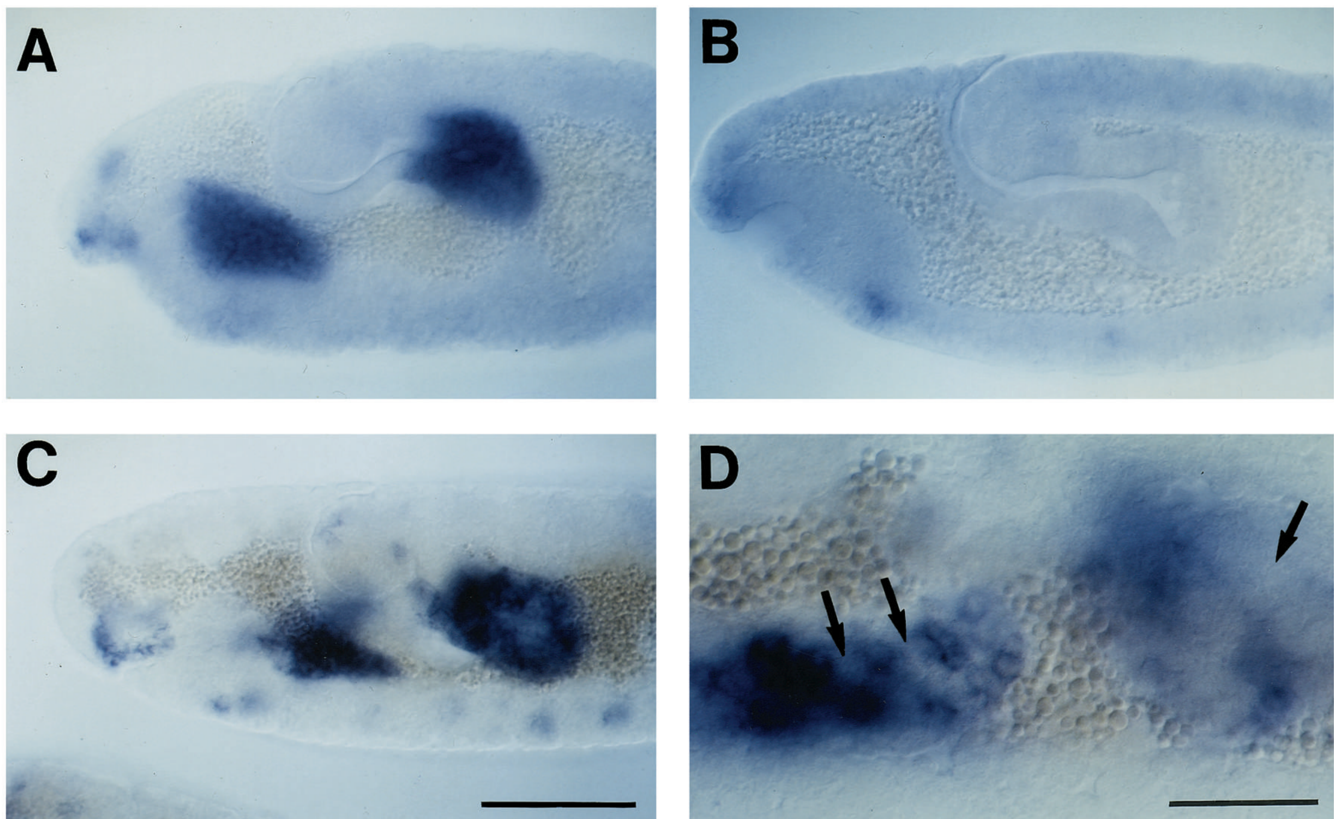

Fig. 8. *sc* expression in neurogenic mutants. (A) Late stage 10 *Dl* mutant showing uniformly high levels of *sc* expression. (B) Late stage 10 wild-type control embryo from the same staining batch as the embryo in A. The color reaction was stopped as signal just emerged in the wild-type embryos. Note that endoderm expression is hardly detectable, by contrast to the *Dl* mutant shown in A, indicating that level of sc expression is substantially elevated in *Dl* compared to wild type embryos. (C,D) *sc* expression in E(SPL)-C deficient embryos is increased compared to wild type. Some embryos show a rather uniform accumulation of *sc* transcript (C), while in others many cells do not increase *sc* expression (arrows in D). Anterior is to the left. Scale bars: A-C, 70 µm; D, 30 µm.

remaining endodermal cells, the PMECs, is a process that requires only endoderm autonomous functions. Two arguments, however, strongly support this notion. First, we found that this segregation process takes place in embryos that do not have mesoderm. Secondly, the neurogenic and proneural genes whose normal function is required for the segregation process are expressed in the endoderm at the time when the segregation takes place (Romani et al., 1987; Knust et al., 1987; Hartley et al., 1987; Kidd et al., 1989; Godt, 1990; Kooh et al., 1993; Brand et al., 1993; this work). The dynamics of the expression of the proneural genes and the E(SPL)-C in relationship to ICP/AMP segregation is similar to the dynamics observed for the expression of these genes during neuroblast segregation from the neuroectoderm, where an autonomous requirement for several of the neurogenic and proneural genes has been demonstrated by mosaic analysis (reviewed by Campos-Ortega, 1993; Simpson et al., 1993).

The endoderm is a homogeneous field of neurogenic and proneural gene activity

Several bHLH genes of the E(SPL)-C, *l'sc* and initially also *sc,* are expressed uniformly throughout the endoderm. This represents a fundamental difference to the expression pattern of these genes in the neuroectoderm. Here the proneural genes are activated in small groups of cells, called proneural clusters, by the anterior-posterior and dorsal-ventral patterning genes (Martìn-Bermudo et al., 1991; Skeath et al., 1992). Each proneural cluster forms an equivalence group from which a single neural precursor is selected while the remaining cells form epidermal precursors (Heitzler and Simpson, 1991; Ghysen et al., 1993). Since the proneural cluster from which a single neural precursor emerges is small, the position of the neural precursor cells is mainly determined by the prepattern provided by the anterior-posterior and dorsal-ventral patterning genes (Fig. 9A).

The uniform expression of proneural and neurogenic genes in the endoderm suggests that a prepattern does not exist in this structure prior to the specification of the AMPs, ICPs and PMECs. This implies that the pattern of AMPs and ICPs is solely determined by the

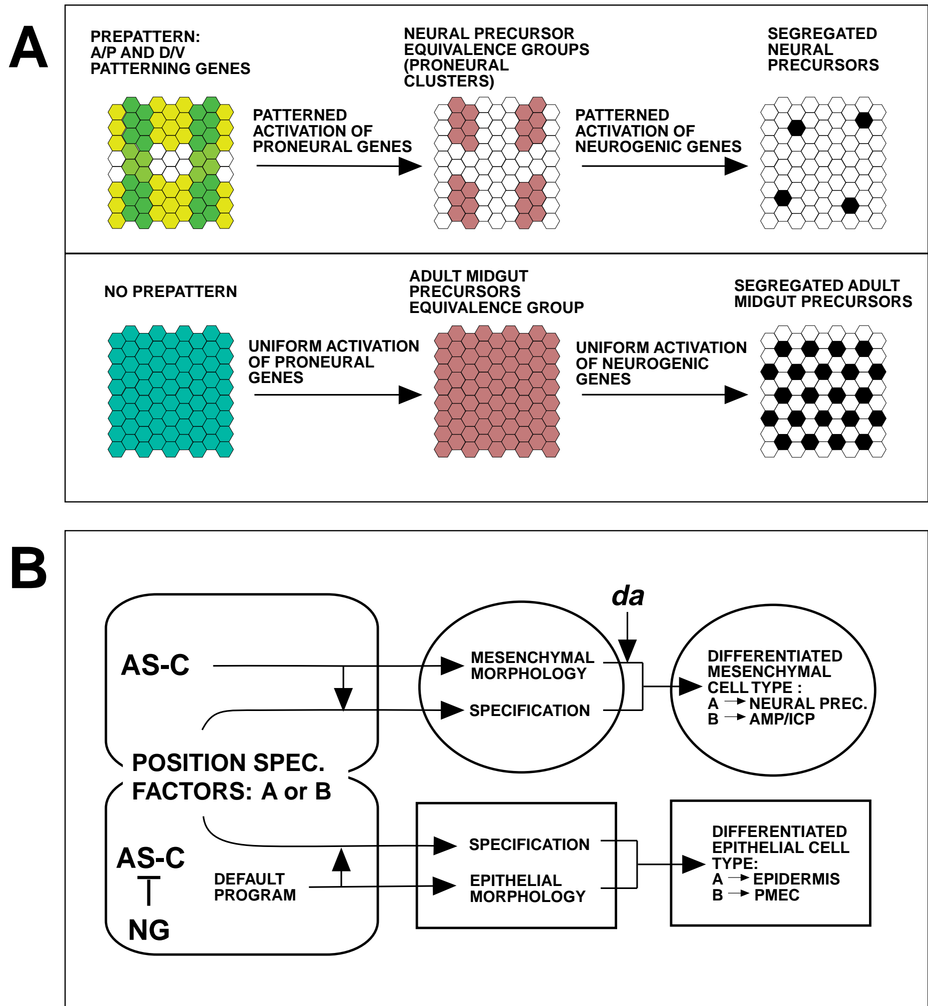

Fig. 9. (A) Comparison of the mechanisms controlling the pattern of neural precursors (upper panel) and AMPs/ICPs (lower panel). Prepattern generated by the anterior-posterior (A/P) and dorsal-ventral (D/V) patterning genes (green) leads to the expression of proneural genes in small groups of cells in the neuroectoderm called proneural clusters (red). Subsequent activation of the neurogenic genes in the proneural clusters and the interaction between neurogenic and proneural genes results in the selection and segregation of a single neural precursor from each cluster. In the endoderm, no prepattern exists. A uniform activation of proneural genes defines the AMPs/ICPs equivalence group from which many cells segregate. Selection and segregation is mediated by the interaction of neurogenic and proneural genes similar to that in the ectoderm. The resulting segregation pattern is stereotyped, with a single cell diameter spacing between segregating cells, a consequence of the limited range of the inhibitory signal provided by the membrane bound *Dl* protein. (B) Schematic of the proposed role of neurogenic/proneural genes in the segregation of epithelial versus mesenchymal cell morphologies associated with cell type specification. Neurogenic/proneural genes generate differences between neighboring cells of a given equivalence group. Cells in which AS-C is active will assume mesenchymal morphology. AS-C is required but not sufficient for cell type specification because it is involved in the specification of various cell types. Additional factors (position specific factors: A or B; e.g. segmentation or homeotic genes) are necessary for the determination of particular cell types. *da* acts together with the AS-C genes after the mesenchymal precursor has emerged to ensure the further differentiation of this cell. In those cells of the equivalence group where the neurogenic genes (NG) suppress AS-C activity a default program is activated that results in the formation (or maintanence) of epithelial cell morphology. Again, the default program has to cooperate with position specific factors to achieve specification. It is possible but not necessary that the position specific factors act together with the AS-C genes or the default program to promote mesenchymal or epithelial morphology, respectively (see text for further explanations).

proneural and neurogenic genes. The result is a simple pattern of rather regularly spaced segregating cells. The fact that ICPs and AMPs are separated by a single cell diameter is consistent with the inhibitory signal provided by the Dl protein acting as a membrane bound and not as a diffusible factor (Vässin et al., 1987; Kopczynski et al., 1988; Fehon et al., 1990). We propose that the control of the segregation of the AMPs and ICPs from the PMECs represents a simple, two step process in which a uniform activation of the proneural genes is followed by a uniform activation of the neurogenic genes (Fig. 9A).

Similar requirement of neurogenic and proneural genes in ectoderm and endoderm

Our findings show a striking similarity between the endoderm and neuroectoderm with respect to the expression pattern and mutant phenotype of the proneural and neurogenic genes. In both tissues the genes of the neurogenic/proneural group are required for the segregation of different cell fates where the cells that depend on proneural gene function make up a rather small number compared to the cells that require neurogenic gene function. In both tissues *l'sc*, *sc* and the E(SPL)-C are activated uniformly in the epuivalence groups that give rise to neural precursors or AMPs/ICPs, respectively. Later, the expression of *l'sc* and *sc* increases in the segregating cells and decreases in the remaining cells of the equivalence groups (PMECs or epidermal precursors; Cabrera et al., 1987; Martìn-Bermudo et al., 1991; Vässin et al., 1994; this work).

Furthermore, the genetic and molecular interactions between neurogenic and proneural genes appear to be similar in endoderm and neuroectoderm. In neurogenic proneural double mutants (Brand and Campos-Ortega, 1988; Heitzler and Simpson, 1991; this work) the proneural phenotype prevails and the development of both epidermis and PMECs is restored. This suggests that neurogenic gene function is mediated through the proneural genes in both the neuroectoderm and the endoderm. Similar to the proneural clusters in the neuroectoderm (Brand and Campos-Ortega, 1988; Skeath and Carroll, 1992), the neurogenic genes repress proneural gene expression in the endoderm as indicated by the increased transcript level of *sc* in *Dl* and E(SPL)-C mutants. The uniform high level of *sc* expression in the endoderm of neurogenic mutants indicates a lack of both mutual and lateral inhibition similar to that in the neuroectoderm (Simpson, 1990; Ghysen et al., 1993).

The similarity between the neuroectoderm and the endoderm extends to the function of *da*. In *da* mutants all neural precursors segregate from the neuroectoderm but do not continue to develop and degenerate (Brand and Campos-Ortega, 1988; Vässin et al., 1994). In the endoderm of *da* mutants we find evidence that AMPs and ICPs die soon after they have segregated. Expression of *ase,* which is restricted to the AMPs and ICPs, is not seen in AS-C or *da* mutant embryos (Brand et al., 1993; own observations) suggesting that the bHLH protein encoded by *da*, which cooperates with the bHLH factors of the AS-C in order to control target gene expression (e.g. Murre et al., 1989), might also do so in the endoderm.

Proneural genes participate in a combinatorial mechanism of cell type specification

It has been speculated that, in contrast to the neurogenic genes (see Introduction), the proneural genes are specifically required to promote neural developmental pathways (for review see Ghysen and Dambly-Chaudière, 1988; Jan and Jan, 1993a). This notion, regarding the genes of the AS-C, is inconsistent with the results described here which show that specification of endodermally derived ICPs and AMPs, two cell types unrelated to the nervous system, require proneural gene activity. Since the function of the neurogenic and proneural genes is apparently not related to the terminal fate of the involved cells the question arises of how the specific cell fate decisions are made. The proneural genes have to act in concert with other factors to commit cells to a particular developmental pathway (Fig. 9B). Candidates for these additional factors are genes that are expressed in a tissue-specific or positional-specific manner as, for example, the anterior-posterior and dorsal-ventral patterning genes in the neuroectoderm and the gene *serpent* (*srp*) in the endoderm. In *srp* mutants, the endoderm is homeotically transformed into ectodermal portions of the gut (Reuter, 1994). The expression of proneural genes plus *srp* may specify the ICPs and AMPs, and endoderm cells in which proneural genes are repressed by lateral inhibition and which only express *srp* would develop as PMECs.

Neurogenic genes and proneural genes may promote epithelial versus mesenchymal development

In both neuroectoderm and endoderm, the neurogenic genes promote the development of epithelial cells (epidermis and PMECs, respectively), whereas the proneural genes are required for the development of mesenchymal cell types (neural progenitors and AMPs/ICPs, respectively). The hypothesis that neurogenic and proneural genes promote the development of epithelial versus mesenchymal cell morphology, respectively (Fig. 9B), is corroborated by the functional characterization of homologs of the neurogenic and proneural genes in vertebrates. The expression of an activated form of the *Xenopus N* homolog, *Xotch*, causes an increase in epithelial neural tube tissue and abolishes the development of the mesenchymal neural crest (Coffman et al., 1993). Uniform activation of *N* in *Drosophila* leads to the loss of mesenchymal neural precursors and presumably to an increase in epithelial neuroectodermal cells (Struhl et al., 1993; Rebay et al., 1993; Lieber et al., 1993). Furthermore, the *myoD* family of transcriptional regulators is structurally related to and operates apparently in a similar manner to the *Drosophila* proneural genes (Jan and Jan, 1993a). Rudnicki et al. (1993) found that *myoD* together with *myf-5*, a second bHLH gene of the *myoD* family, promotes the segregation of mesenchymal myoblasts from the epithelial somite.

Epithelial and mesenchymal cells are the two basic components of animal embryos (e.g. Bard, 1990; Tepass and Hartenstein, 1994a). The segregation of a homogeneous cell population into these two cell types occurs repeatedly in each germlayer throughout embryonic development. The data reported here and the results of others suggest that the activity of the neurogenic and proneural (and possibly the *myoD* family) genes controls this segregation process. The genetic program that leads to the differentiation of an epithelial cell structure is suppressed in an embryonic cell in which proneural genes are active and a mesenchymal program is realized. In adjacent cells that derive from the same equivalence group and in which neurogenic genes suppress proneural gene activity, an epithelial program is realized by default.

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