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daughterless is essential for neuronal precursor differentiation but not for initiation of neuronal precursor formation in *Drosophila* embryo

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SUMMARY

The first steps of neuronal precursor formation require several genes that encode transcription regulators with the helix-loop-helix (HLH) motif, including the proneural genes of the *achaete-scute* complex AS-C (*achaete* (*ac*), *scute* (*sc*) and *lethal of scute* (*l'sc*)) and *daughterless* (*da*). The *da* protein dimerizes with AS-C products in vitro to form DNA-binding proteins. Previous studies have shown that the AS-C genes are expressed initially in discrete clusters of ectodermal cells (the proneural clusters) and then more strongly in the neuronal precursors that arise from these clusters and delaminate from the epidermal layer. In this paper, we studied the distribution of *da* protein with an antibody raised against Da. We found that Da is ubiquitously but non-uniformly distributed. Within the ectodermal layer, its level is neither elevated (as in the case of AS-C genes) nor reduced (as in the case of *emc* product) in the proneural cluster. It is, however, at higher levels in many neuronal precursors. We further studied the requirement of *da* in neuronal precursor development by using a variety of markers for neuronal precursors. Our results reveal the existence of at least two stages in neuronal precursor formation. *da* is not required for the initial appearance of nascent neuronal precursors but is required for these cells to express multiple neuronal precursor genes and to produce the normal number of neurons.

Key words: *Drosophila*, *daughterless*, neuronal precursor, *achaete-scute*

INTRODUCTION

Formation of neuronal precursors (the sensory organ precursors, or SOPs) in the peripheral nervous system (PNS) of the *Drosophila* embryo is known to involve multiple steps. The proneural genes *achaete* (*ac*), *scute* (*sc*) and *lethal-of-scute* (*l'sc*) of the *achaete-scute* complex (AS-C) are first expressed in clusters of ectodermal cells (the proneural clusters) at discrete locations, thereby endowing them with the potential to become neuronal precursors. One (or a few) cell from a proneural cluster is singled out, through the action of neurogenic genes, to become a neuronal precursor and to express the appropriate AS-C gene(s) at a higher level than the surrounding cells (for review, see Ghysen and Dambly-Chaudiere, 1989; Jan and Jan, 1990; Campos-Ortega and Jan, 1991; Artavanis-Tsakonas and Simpson, 1991; Campuzano and Modolell, 1992; Ghysen et al., 1993). All neuronal precursors then proceed to express several neuronal precursor genes, such as *prospero (pros)*, *couch potato* (*cpo*), *deadpan* (*dpn*) and *asense* (*ase*) (Doe et al., 1991; Vaessin et al., 1991; Bellen et al., 1992; Bier et al., 1992; Brand et al., 1993; Dominguez and Campuzano, 1993), which may control different aspects of neuronal differentiation. Using the expression of these neuronal precursor genes or of *lacZ* in certain enhancer trap lines (Ghysen and O'Kane, 1989) as specific markers for neuronal precursors, one finds subsets of neuronal precursors missing in loss-of-function mutants of AS-C, whereas supernumerary neuronal precursors appear in neurogenic mutants, consistent with their postulated roles in neuronal precursor formation.

Whereas deletion of AS-C removes most, but not all, of the PNS (Dambly-Chaudiere and Ghysen, 1987), null mutations of the *daughterless* (*da*) gene eliminates the entire PNS (Caudy et al., 1988a). Previous studies have shown that in *da* mutants no neuronal precursors for the PNS could be identified based on the then existing markers, i.e. *lacZ* expression in certain enhancer lines or BrdU incorporation (Ghysen and O'Kane, 1989; Bodmer et al., 1989). Moreover, *da*, like the genes of AS-C, encodes a transcription regulator of the basic helix-loop-helix (bHLH) class (Caudy et al., 1988b; Murre et al., 1989a), and an interaction between *da* and AS-C has been detected genetically (Dambly-Chaudiere et al., 1988). Consistent with these observations and the similar phenotypes of *da* and AS-C, the *da* protein dimerizes with AS-C gene products in vitro and forms specific DNA-binding proteins (Murre et al., 1989b; Vaessin et al., 1990; Cabrera and Alonso, 1991; Van Doren et al., 1991). Given that the *da* mRNA expression appears ubiquitous in the embryo, it has been proposed that *da* product is the ubiquitous factor that dimerizes with different AS-C gene products expressed in different subsets of proneural clusters, so that loss of function of either partner of the heterodimeric transcription factors leads to the elimination of neuronal precursors.

Besides *daughterless*, the gene *extramacrochaetae* (*emc*) also interacts with the AS-C genes. The *emc* product contains the helix-loop-helix (HLH) domain involved in dimerization but not the basic domain required for DNA recognition; it is thought to act as a negative regulator of AS-C by dimerizing with, thereby sequestering, the *ac*, *sc* or *da* proteins (Ellis et al., 1990; Garrell and Modolell, 1990; Van Doren et al., 1991). Although the *emc* mRNA appears to be ubiquitously expressed, its distribution is non-uniform. The regions where the *emc* protein is expressed at a lower level roughly correlate with where *ac* or *sc* proteins are expressed at higher level. It appears that the non-uniform distribution of the *emc* product contributes to the controlling of the number and position of neuronal precursors by regulating the effective level of *ac*, *sc* and *da* proteins (Cubas and Modolell, 1992; Van Doran et al., 1992).

In this study, we examine the expression patterns of *da* protein, in order to address the following issues. First, does the *da* protein show any regional distribution that might implicate the spatial regulation of *da* protein in the process of neuronal precursor selection? Second, given that the sc protein accumulates in neuronal precursors as they form, prior to the expression of most other neuronal precursor markers, it represents one of the earliest markers for neuronal precursors. By examining the *da* mutant phenotypes at different stages of neuronal precursor formation using Sc and the products of six neuronal precursor genes as markers, we attempted to determine more precisely the function of *da* during neurogenesis.

MATERIAL AND METHODS

Immunohistology

Polyclonal antibodies directed against the proneural proteins Da and Sc were raised in rabbits using the following peptides:

Da: CVNQANNGLPPHLQQQQQQQSQLGHAQLPQ
Sc: CDCTPDDEEILDYISLWQEQ

For immunization, these peptides were coupled to keyhole limpet hemocyanin (KLH) utilizing a cysteine residue that was added at the N-terminal end of the peptide.

Serum, from immunized rabbits, was diluted 1:100 in PBT+1% BSA (PBT: PBS+0.1% Triton X-100) and preadsorbed with 1/10 volume of wild-type embryos for 3 hours at room temperature on a shaker. The supernatant was recovered and stored at 4°C in the presence of 0.02% sodium azide. Antibody stainings were basically done as described in Bodmer and Jan (1987). Preabsorbed serum at final dilutions of 1:250 to 1:750 for anti-Sc and 1:2500 for anti-Da were used. In some cases, B-5 fixation was used for antibody stainings involving anti-Sc. Here embryos were fixed in B-5/Heptane for 20 minutes prior to devitellinization. For the detection of the *lacZ* gene product, anti-β-gal (Cappel) was used at a 1:5000 to 1:10000 dilution.

In situ hybridizations

In situ hybridization to whole-mount embryos was done following the method of Tautz and Pfeifle (1989). Digoxigenin-labeled DNA probes were prepared with a Boehringer Mannheim kit according to manufacturer's protocol with the modification that 5 μg of random primer was added to the labeling reaction. Reactions were allowed to go on for 16-20 hours. Approximately 10% of a labeling reaction was used in each in situ hybridization experiment.

Antibodies and probes

Antibodies or DNA probes used are the following: anti-Dpn (Bier et al., 1992), anti-Ase (Brand et al., 1993), anti-Pros (Vaessin et al., 1991), *Cyclin* probe (Vaessin and Jan, unpublished), *scratch* probe (E. Bier, unpublished), *cpo* probe (Bellen et al., 1992).

Genetics

The mutation $Df(2L)da^{KX136}$ carries a small deletion that removes the entire *da* open reading frame (Caudy et al., 1988b). Therefore, embryos homozygous for this allele lack zygotic *da* function and the allele represents a true loss-of-function mutation.

da germ-line clones

da^- germ-line clones were induced as described in Brand and Campos-Ortega (unpublished data). To induce da^- germ-line clones, we irradiated (1000R) first instar female larvae that carried the *da* null mutations $Df(2L)da^{KX136}$ and $In(2L)da^{KX80}$ on the two second chromosomes and a X-chromosome carrying $Fs(1)ovo^D$, a P element that includes the wild-type *da* gene (da^+), integrated at chromosomal position 10B, and a newly induced *w* allele (Brand and Campos-Ortega, unpublished data). $Df(2L)da^{KX136}$ and $In(2L)da^{KX80}$ are amorphic mutations of the *da* gene that carry a 4 kb deletion and a breakpoint, respectively, in the *da* gene (Caudy et al., 1988b); animals homozygous for either allele do not have any detectable zygotic *da* RNA (Brand and Campos-Ortega, submitted) or protein (Fig. 1 and not shown). These females were crossed to males that carry the same da^+ X-chromosome and *da* null mutations on both second chromosomes. Only those females with germ-line clones that lack $Fs(1)ovo^D$ (and da^+) are expected to lay eggs. About 50% of all embryos are male and they lack both maternal and zygotic *da* activity. These male embryos develop beyond blastoderm stages because they do not require maternal *da* for proper dosage compensation (Cronmiller and Cline, 1987). As expected, embryos obtained from irradiated females fall into two main phenotypic groups that each makes up approximately 50% of the progenies. The first group formed a normal nervous system with no or only minor defects, as detected with antibodies that recognize all neurons. This group of embryos most likely corresponds to the female progenies that have received a wild-type *da* copy in the zygote. A second group shows a late neuronal *da* mutant phenotype that is more extreme than observed in zygotic loss-of-function mutations (Brand and Campos-Ortega, unpublished data), in addition to various morphogenetic defects. This group most likely corresponds to the male progenies that lack both maternal and zygotic *da* function.

The $Fs(1)ovo^D$ mutation (4D) is located more distal than the integration site of the wild-type *da* gene (10B) on the X-chromosome. Therefore, an individual recombination event between these two positions could result in functional germ-line cells (Fs^+) that would still carry a wild-type *da* gene. Occurrence of such an event, however, should be relatively rare due to the small distance between the $Fs(1)ovo^D$ mutation and the integration site of the P element carrying the wild-type *da* gene. Furthermore, embryos that would result from such a recombination event would not display the typical, strong morphogenetic defects associated with maternal and zygotic *da* loss of function.

RESULTS

The expression patterns of the *da* protein

For the characterization of the *da* protein product (Da) distri-

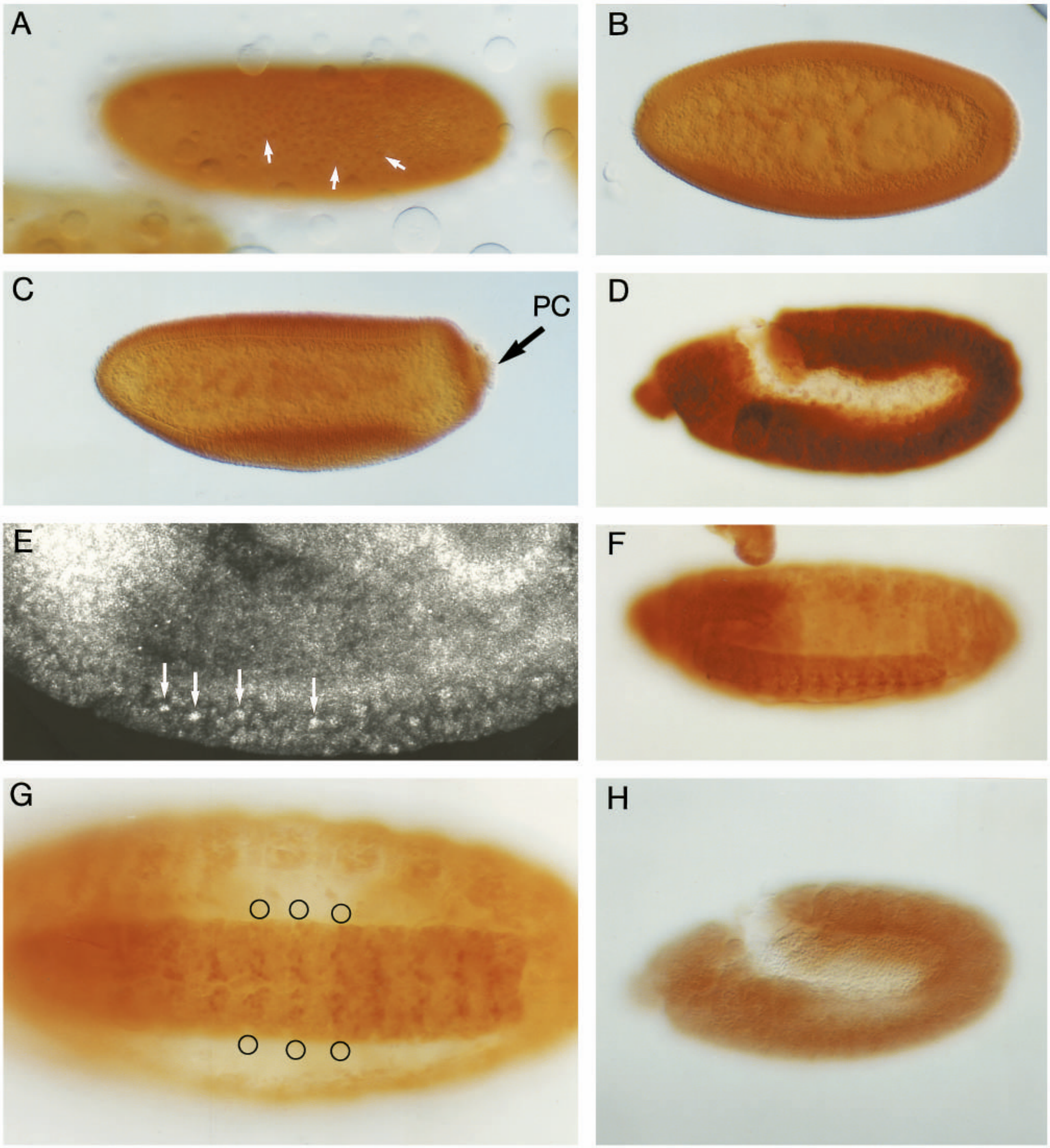

Fig. 1. *da* protein (Da) expression during embryogenesis. (A) Wild-type embryo during late cleavage stage. Da is detected in all nuclei. (some are indicated with arrows) (B) Early stage 5 wild-type embryo. At this stage, no distinct accumulation of Da in the nuclei is detectable. (C) During late stage 5 (not shown) and early gastrulation (stage 6), high levels of Da are detectable in the nuclei of all embryonic cells except for the pole cells (PC). (D) During the germ-band-extended stage, Da is expressed in high levels in all tissues. (E) Optical section of the ventral neurogenic region of stage 9 embryo. Using confocal microscopy, higher amounts of Da are detected in the delaminated neuroblasts (white arrows) as compared to the ectodermal cells. (F) Late stage 16 wild-type embryo. Higher levels of Da expression are evident in this embryo in the salivary glands (sg) and subsets of cells in the ventral cord (vc). (G) Higher magnification view of the ventral cord of a late stage 16 wild-type embryo. Distinct clusters of cells (circles) are positive for Da, while the majority of CNS cells at this stage express Da only at a very reduced level. (H) Embryo homozygous for a deletion of the *da* gene. No signal is detected.

bution, we raised polyclonal antibodies against a peptide with the C-terminal sequence of Da. Immunocytochemical staining by these antibodies was abolished in embryos homozygous for a small deletion that removes the *da* gene (*Df(2L)da*^{KX136})

(Caudy et al., 1988b), indicating that the staining is specific for the *da* protein (Fig. 1H).

In the wild-type embryo, nuclear staining was detected in most somatic cells, but not in pole cells (Fig. 1). A similar ubiquitous distribution is found for *da* RNA (Brand and Campos-Ortega, unpublished data). In preblastoderm embryos, presumably maternally supplied Da is present throughout the egg and is detected in all nuclei (Fig. 1A). This protein disappears shortly before blastoderm formation. During early blastoderm, no *da* protein is detectable (Fig. 1B). The Da level rapidly increases again shortly before onset of germ-band extension (Fig. 1C) and reaches the maximal level during stages 9 to 11 (staging according to Campos-Ortega and Hartenstein, 1985) when most neuronal precursors form (Fig. 1D). Optical sectioning, using confocal microscopy, shows that, at this stage, Da is present in ectodermal cells as well as in putative neuroblasts during their delamination and after completion of this process. The level of Da in the ectodermal layer appears to be fairly uniform; proneural clusters do not exhibit either elevated or decreased level of Da. Neuronal precursors, on the other hand, appear to have a somewhat higher level of Da as compared to their neighboring ectodermal cells (Fig. 1E). No somatic cell type could be identified that does not express *da* protein during some stages of embryogenesis. During germ-band retraction, *da* protein expression is reduced in most tissue types. Parallel to this overall reduction in the level of Da expression, a more complex pattern in the levels of Da expression becomes evident. For example, starting from stages 13(14), a subset of cells in the ventral side of the developing ventral cord of the CNS expresses the *da* protein at a distinctly higher level compared to other cells in the CNS (Fig. 1F,G). Higher levels of Da expression can also be observed in the salivary glands, parts of the gut and muscles until cuticle formation. Embryonic stages after cuticle formation (stage 16) were not analyzed.

The expression patterns of Da in imaginal discs

Appearance of proneural clusters and neuronal precursors for the adult PNS takes place over a much longer time span in the imaginal discs than in the embryos and has been characterized in detail in the wing disc (Huang et al., 1991; Cubas et al., 1991; Skeath and Carroll, 1991). As in the embryo, the level of *da* protein was found to be fairly uniform among all epidermal cells of the wing disc during all stages of sensory organ precursor (sop; sops are neuronal precursors in the PNS) formation. Thus, unlike the *emc* protein, the *da* protein does not show spatial regulation that might contribute to the localization of neuronal precursors. The level of Da is elevated in many neuronal precursors. In the wing disc, the two rows of sops along the presumptive wing margin exhibits a Da level higher than that in their neighboring epidermal cells (Fig. 2A). In the leg disc, the large cluster of sops that will later form the chordotonal organ clearly shows higher levels of Da (Fig. 2B). In the eye disc, some cells posterior to the morphogenetic furrows have elevated levels of Da (Fig. 2C). The position of these cells suggests that they are photoreceptors R8. In this context, it is interesting to note that the proneural gene for photoreceptors (*atonal*) has been found recently. *atonal* is expressed in morphogenetic furrow and in

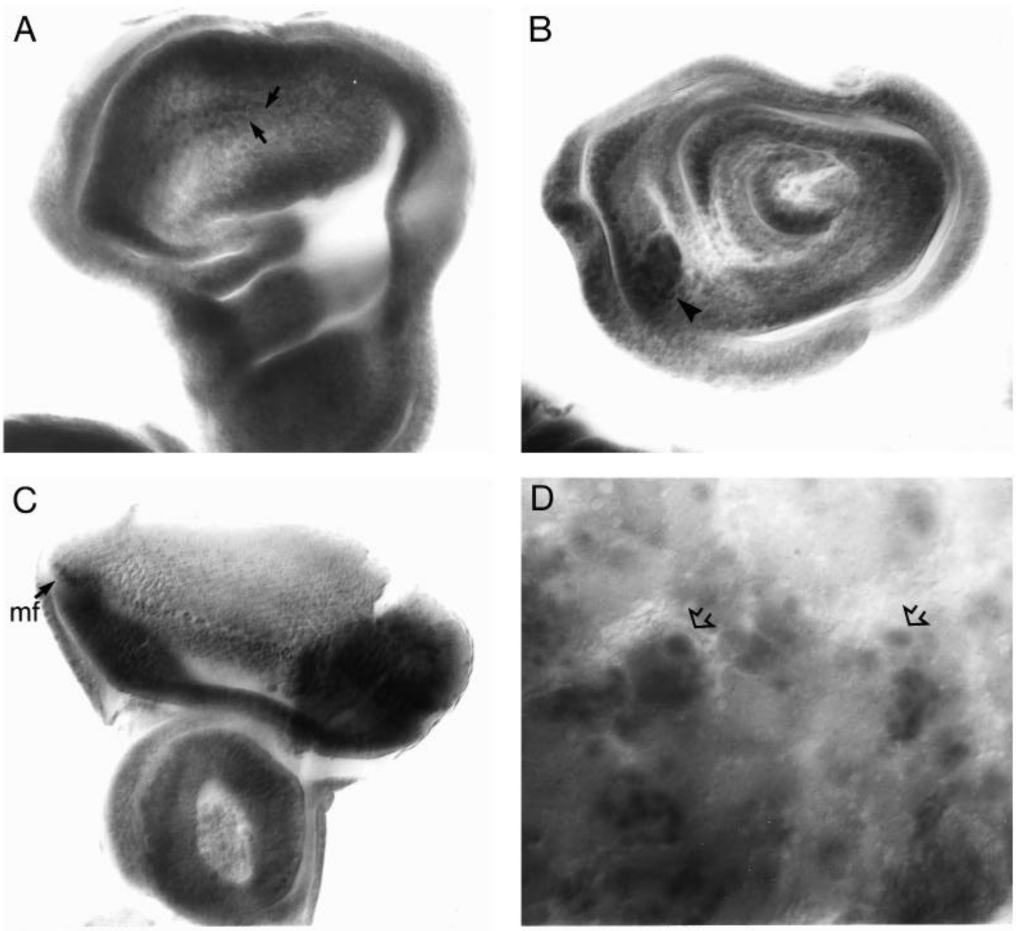

Fig. 2. Expression of Da in imaginal discs. (A) While rather uniform levels of Da expression are detected in the epidermal cells of the wing disc, a higher Da level is visible in two rows of neuronal precursors at the wing margin (arrows). (B) Similar to wing discs, a general low level of Da is detected in the epidermal cells of the leg disc. Higher levels of Da are expressed in a cluster of neuronal precursor cells that will give rise to chordotonal organs (arrow head). (C) In eye discs, cells posterior to the morphogenic furrow (mf) show elevated Da expression. (D) In the ventral region of the ventral cord of a third instar larva, Da is detected at elevated levels in neuroblasts located in this region as well as in progenies produced by these neuroblasts. Higher levels of Da are present in the neuroblasts themselves (open arrows) as compared to their progenies.

R8s immediate posterior to it (Jarman et al., unpublished data). The *atonal* gene product is a bHLH protein that dimerizes with Da in vitro and the heterodimer binds DNA (Jarman et al., 1993b). In the developing larval CNS, each neuroblast (NB) and the progenies derived from the same precursor as the NB (ganglion mother cells and neurons) form a cluster (Truman and Bate, 1988). NBs clearly have a higher level of Da as compared to progenies of NB (Fig. 2D).

The scute protein expression during embryogenesis

The proneural genes (*ac*, *sc* and *l'sc*) of the AS-C are required for the formation of subsets of the PNS and the CNS in the embryo. The *ac* protein expression patterns have been analyzed in both the embryonic CNS (Skeath and Carroll, 1992; Skeath et al., 1992) and PNS (Ruiz-Gomez and Ghysen, 1993). The *l'sc* protein expression has been described for early neurogenesis (Martin-Bermundo et al., 1991). The *sc* protein distribution has been previously examined only in the embryonic CNS (Skeath et al., 1992). We have raised polyclonal antibodies against a C-terminal peptide sequence of the *sc* protein, as an early marker for some of the forming neuronal precursors. These antibodies gave rise to nuclear staining in wild-type embryos but not in sc^{10-1} mutant embryos (Fig. 3L). The sc^{10-1} mutation causes a truncation of the predicted protein product, deleting 183 amino acids from the C-terminal half of the *sc* protein (Villares and Cabrera, 1987). The absence of staining indicates that our antibodies against the *sc* protein (Sc) are specific. Similar to the other genes in the AS-C (Cabrera et al., 1987; Romani et al., 1987), Sc expression closely reflects the *sc* transcript pattern during embryogenesis. Sc first appears shortly before the onset of gastrulation and is present in longitudinal stripes, the highest level of expression coincides with the border between the ventral neurogenic region and the mesoderm anlage (Fig. 3A). Expression of Sc in proneural clusters of the CNS is evident during early germ-band extension, in regularly spaced arrays of 4-8 ectodermal cells in the ventral neurogenic region (Fig. 3B). These proneural clusters subsequently become elongated in shape and form narrow bands of cells spanning the width of the ventral neurogenic region (Fig. 3C). The Sc expression in proneural clusters is then restricted to neuroblasts that delaminate from the ectodermal layer (Fig. 3D); the expression in the surrounding cells of the proneural cluster is reduced drastically at this stage.

Proneural clusters for the PNS begin to express Sc at stage 9 (Fig. 3E-I). Sc is first found in the proneural cluster for the P cell, which gives rise to chordotonal organs in the posterior compartment (Ghysen and O'Kane, 1989) (Fig. 3E), and then becomes restricted to the P cell itself (Fig. 3F). Slightly later, Sc expression in an anteriorly located proneural cluster becomes restricted to the neuronal precursor (sop) that is derived from this cluster, the A cell (Fig. 3F). Subsequently, Sc is expressed in a proneural cluster dorsal to the P cell (Fig. 3G), and then in other proneural clusters and neuronal precursors (Fig. 3H,I). Sc expression in neuronal precursors of the PNS disappears during late stage 11/early stage 12 (Fig. 3J) while the developing stomatogastric nervous system and a subset of cells in the hindgut begins to express Sc. Low levels of Sc are found in sensory organs at stage 14 (Fig. 3K). After late stage 14, Sc can no longer be detected.

The Sc expression pattern in *da* mutant embryos

Because Sc expression is readily detected in both CNS and PNS neuronal precursors as they arise from their proneural clusters, it allows us to examine the requirement of the *daughterless* (*da*) gene during the earliest stages of neuronal precursor formation. The initial Sc expression in proneural clusters and neuronal precursors appear normal in *da* mutant embryos deficient for the entire *da* transcription unit (*Df(2L)da*^{KX136}).

Identification of *da* mutant embryos was possible because their heterozygous parents carried a second chromosome balancer with an enhancer trap *lacZ* construct (Fig. 4A); embryos that showed no *lacZ* expression were homozygous for the *da* mutation. These *da* mutant embryos had normal Sc expression patterns between stage 9 and 11. Sc is still expressed in proneural clusters in the ventral neurogenic region and then restricted to the neuronal precursors for the CNS (Fig. 4B). Similarly, in the PNS, Sc first appears in the proneural clusters for the P cell and A cell (Fig. 4C), and then becomes restricted to these neuronal precursors (Fig. 4C,D).

The first deviation from the normal Sc expression patterns was detected in *da* mutant embryos after the neuronal precursors delaminate. Sc was readily detectable in the delaminated neuronal precursors in the CNS (Fig. 4G) as well the PNS (Fig. 4F), and remained in these neuronal precursors for an abnormally prolonged period. Unlike the wild-type embryos, the *da* mutant embryos showed Sc expression in several neuronal precursors for the PNS even during late stage 11 (whereas in wild-type Sc expression is extinguished at this stage, see Fig. 3J) (Fig. 4F); this expression was no longer detectable at stage 12. The fate of the neuronal precursors after they cease expressing Sc is unclear. Previous studies indicate that they do not divide (Bodmer et al., 1989). It is possible that some of them die, as substantial cell death has been detected in the CNS (starting at stage 11) and the lateral epidermal regions that harbor the PNS (starting at stage 13) (Brand and Campos-Ortega, 1988). Although Sc expression was still evident in the developing stomatogastric nervous system of the *da* mutant embryos, no further Sc expression was found at later stages of embryogenesis. It is known that some of the cells that normally express Sc later during embryogenesis, e.g., cells of the sensory organs of the PNS, never appear in *da* mutant embryos (Caudy et al., 1988a).

It is unlikely that the normal Sc expression patterns in *da* mutants during early embryogenesis is due to maternal contribution of the *da* gene product, because maternally supplied *da* protein was not detectable after blastoderm formation in homozygous *Df(2L)da*^{KX136} embryos. Moreover, Sc expression was still evident in homozygous *da* mutant embryos derived from germ-line clones (Fig. 4H), even though these embryos lacked both maternal and zygotic *da* gene function and showed defects in germ-band retraction and certain aspects of mesoderm invagination. These morphogenetic defects result in a distortion of the developing embryo. Nevertheless, in embryos lacking maternal and zygotic *da* function, Sc-positive cells arise at approximately normal positions and developmental stages as expected for neuronal precursors. Apparently normal formation of neuronal precursors is also evident from the expression pattern of hunchback (hb) protein, a marker for neuroblasts (Brand and Campos-Ortega, unpublished data). Thus, loss of maternal and zygotic *da* function results in an

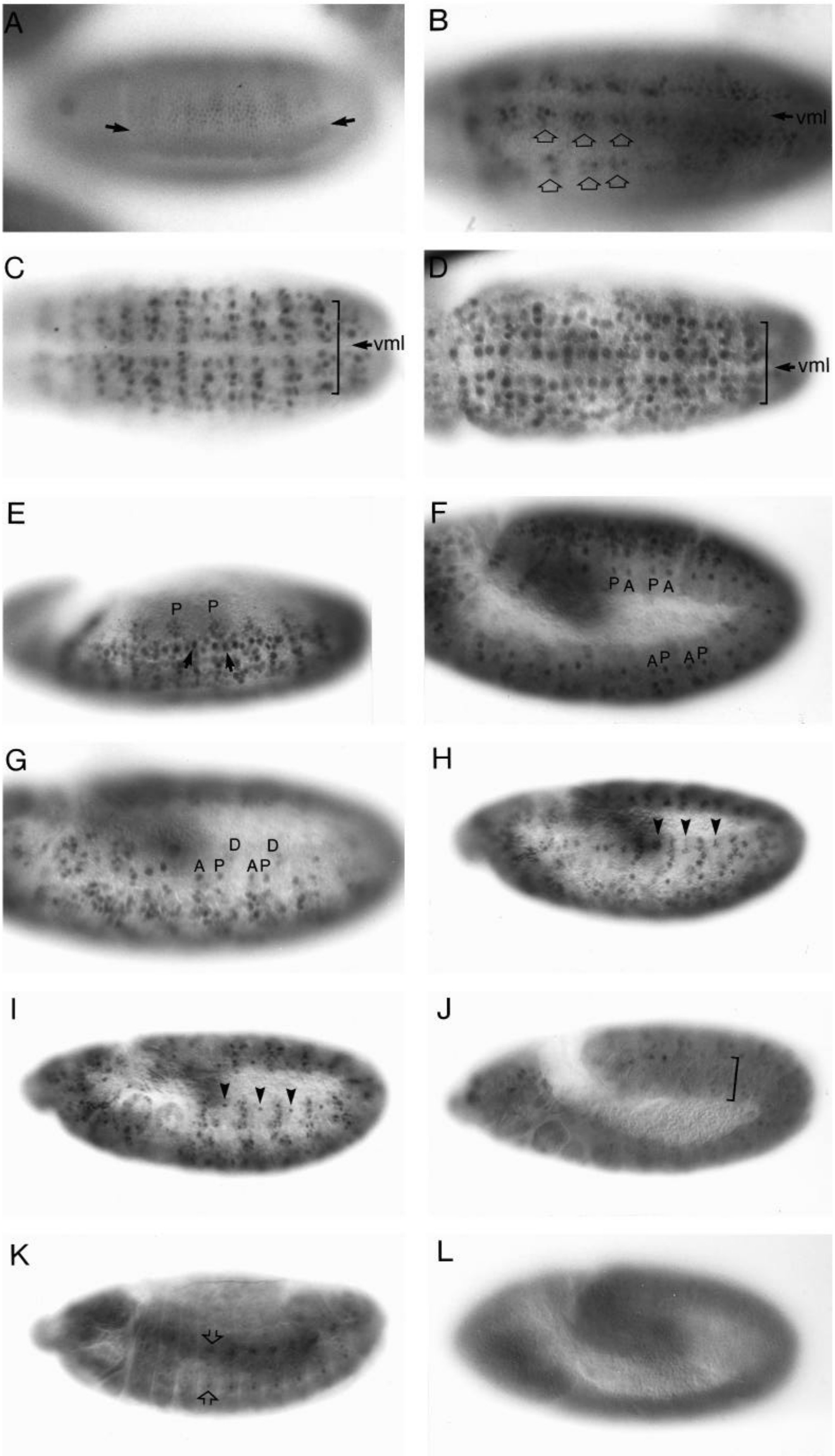

Fig. 3. Expression of Sc during wild-type embryogenesis. (A) During early stage 6, Sc is expressed in the ventral neurogenic region in regularly spaced patches. No signal is detected in the invaginating mesodermal anlage. The border between the mesodermal anlage and the ventral neurogenic region is indicated with arrows. (B) Ventrolateral region of a late stage 6/early stage 7 embryo. Two rows of cell clusters, containing 4-8 Sc-positive cells per cluster (open arrows), are on each side of the ventral midline (C) At stage 8, Sc is expressed in narrow rows of neuroectodermal cells. Bracket indicates the extent of the ventral neurogenic region. (D) The expression of Sc is restricted to the delaminated neuroblasts in the ventral neurogenic region (bracket) in stage 9 embryos. Low level of expression remains detectable in the ectodermal cell layer. (E) During stage 9, the first proneural cluster for the PNS, corresponding to the P cluster (P) starts to express Sc. Arrows indicate CNS neuroblasts at the dorsal border of the ventral neurogenic region. (F) Lateral view of part of stage 10 embryo. Sc is expressed in the first sensory organ precursors, P and A sops. (G) Shortly after the formation of the P and A sops, a proneural cluster forms dorsal (D) to the P sop (H) Lateral view of a stage 11 embryo. One narrow band of Sc-positive cells, which consists of proneural cluster cells for several sensory organ precursors, as well as one individual proneural cluster (arrowheads), is visible in each body segment. (I) Lateral view of an embryo at a slightly later stage as shown in H. Sc expression in the single proneural cluster has become refined to a single sop (arrowhead), while the restriction of Sc expression in the Sc-positive band of cells is just starting. (J) In a late stage 11/early 12 wild-type embryo, Sc protein expression rapidly disappears from the developing sensory organ precursors in periphery (bracket). (K) During stage 14, transient expression of Sc in a subset of PNS cells (open arrows) is detectable. (L) Embryo hemizygous for *sc*$^{10-1}$ mutation, no signal is detected. vml, ventral midline

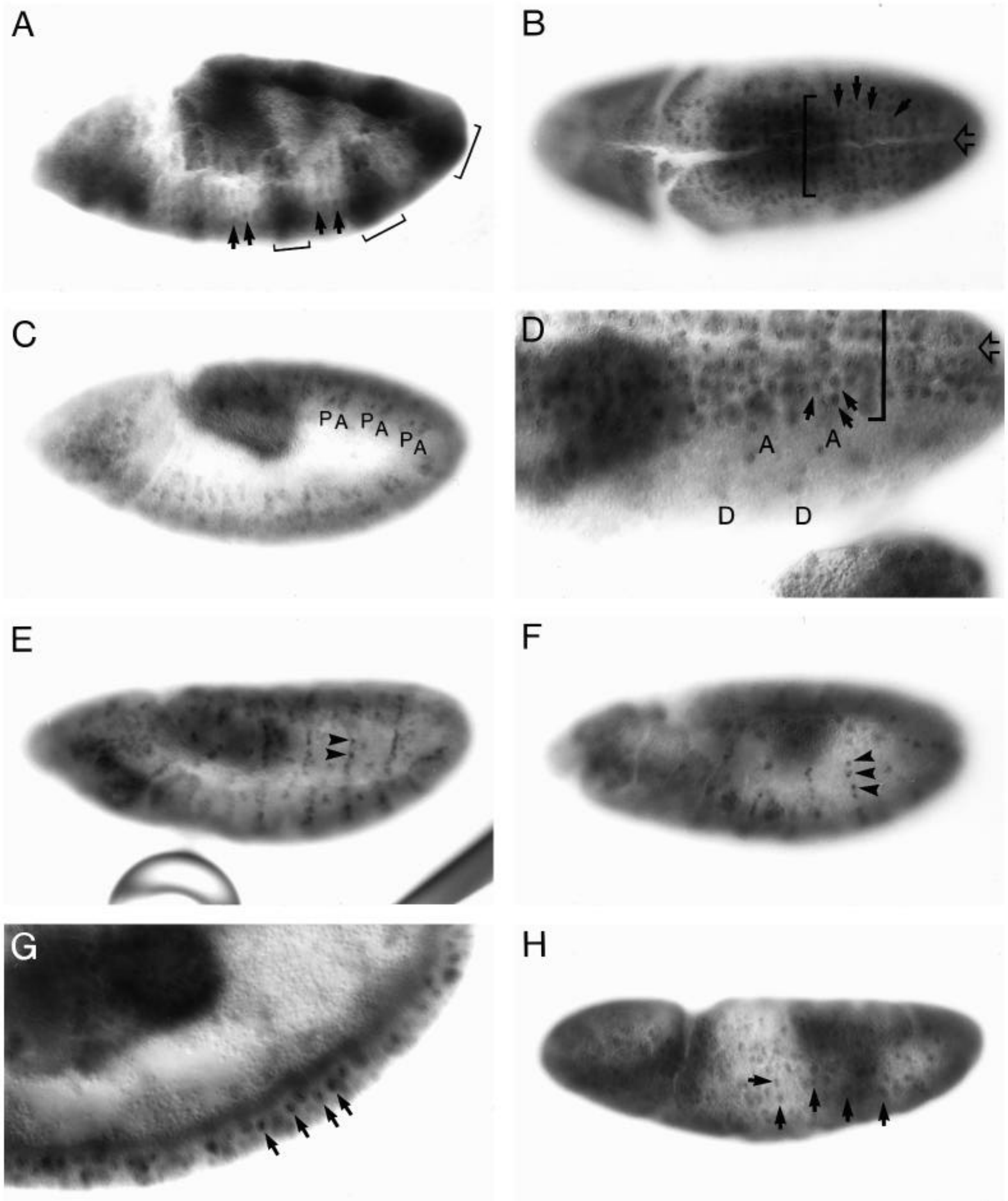

Fig. 4. Sc expression in the absence of Da. (A) The *CyO*-Blue balancer was used in these experiments to distinquish embryos homozygous for the *da* loss-of-function allele *Df(2L)da*^{KX136} from embryos that carry *da*$^{+}$ that is provided by the balancer chromosome. Lateral view of a stage 9 embryo carrying the *CyO*-Blue balancer chromosome. Double staining with anti-Sc and anti-β-gal reveals the expression of Sc as well as the *lacZ* expression of the marked balancer chromosome. Brackets indicate areas of *lacZ* expression while arrows indicate Sc expression in single neuroblasts. (B) Ventral view of a stage 10 *da* mutant embryo. Sc expression is evident in the newly formed neuronal precursors. Bracket indicates area where neuroblasts are forming. Arrows highlight some neuroblasts. Open arrow indicates midline (C) Formation of proneural cluster and restriction of Sc expression to individual sop in the lateral region of a stage 9 *da* mutant embryo. In the abdominal region, the P and A proneural clusters and/or the newly formed sops are indicated. In several of the posterior abdominal segments, restriction of Sc expression to the P sop is already visible, while in most other segments only the corresponding proneural cluster is stained. (D) Dorsal view of some abdominal segments of an early stage 10 *da* mutant embryo. Indicated here are the A sop as well as a newly formed sop (marked as D) located dorsally to the P sop. Sc expression in the P cell is no longer detected at this stage. Bracket indicates ventral neurogenic region. Several neuroblasts in this area are highlighted with arrows. Open arrow indicates midline (E) Formation of proneural clusters in the lateral region of a stage 11 *da* mutant embryo. Increased expression of Sc in individual cells within elongated clusters of Sc-expressing cells is visible (arrowheads). In addition, various stages of restriction of Sc expression in the single, dorsally located cluster are evident. (F) Restriction of Sc expression to individual subepidermal cells in the periphery of a late stage 11 *da* mutant embryo (arrowheads). (G) Lateral view of ventral neurogenic region of *da* mutant embryo. Sc expression can be detected in delaminated neuroblasts. Arrows highlight some neuroblasts. (H) Expression of Sc in the absence of maternally supplied Da. While embryos originating from germ-line clones that are homozygous for loss-of-function *da* mutations are defective in gastrulation, expression of Sc is apparent. Arrows highlight some Sc-positive cells.

approximately normal number of neuroblasts that express both *sc* and *hb* protein.

Alterations of expression of neuronal precursor genes in *da* mutant embryos

Shortly after a neuronal precursor is singled out from the proneural cluster, as evident from its elevated expression of proneural gene(s) (e.g. *scute*) and delamination from the ectodermal layer, it begins to express a number of neuronal precursor genes. This group of genes is expressed in all neuronal precursors but not in the surrounding ectodermal cells and is likely to control different aspects of neuronal precursor differentiation (Vaessin et al., 1991; Bellen et al., 1992; Bier et al., 1992; Brand et al., 1993; Jarman et al., 1993a; Dominguez and Campuzano, 1993). Having found that loss of *da* function had little effect on the initial Sc expression in proneural clusters and neuronal precursors, we then asked if *da* function is required for the expression of five neuronal precursor genes: *prospero* (Doe et al., 1991; Vaessin et al., 1991), *deadpan* (Bier et al., 1992), *asense* (Dominguez and Campuzano, 1993; Brand et al., 1993), *cyclin A* (Lehner and O'Farrell, 1989) and *scratch* (E. Bier, unpublished), and a

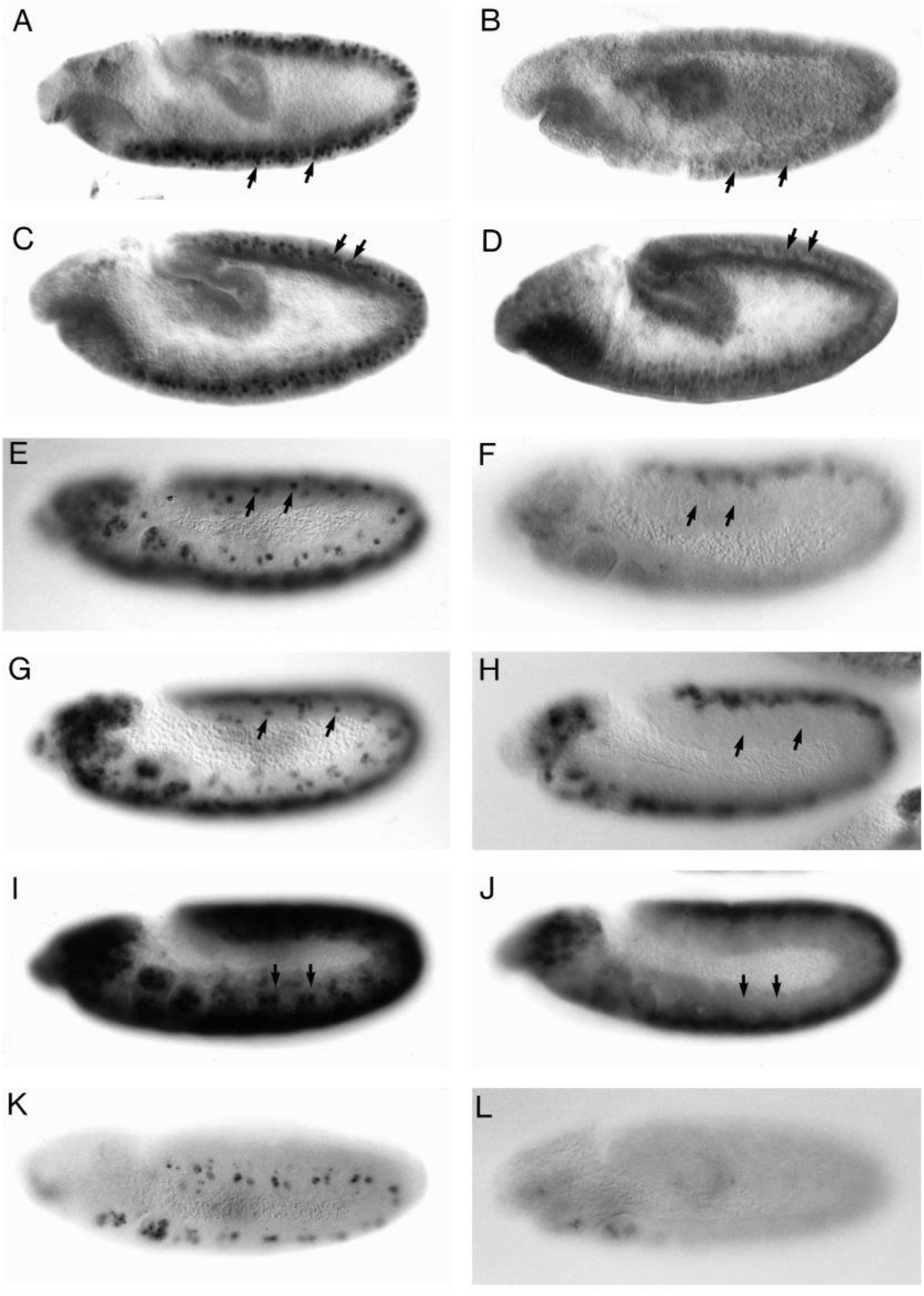

Fig. 5. Neuronal precursor gene expression in wild-type (A,C,E,G,I and K) and homozygous *da* mutant embryos (B,D,F,H,J and L). (A,B) Expression of *dpn* protein (Dpn) in neuroblasts of wild-type (A) and *da* mutant (B) embryos. While in the wild-type embryo Dpn is detected in all delaminated neuroblasts (arrows), no expression is detected in delaminated neuroblasts in the homozygous *da* mutant embryo (arrows). (C,D) *ase* protein (Ase) expression in wild-type (C) and *da* mutant (D) embryos. A significant reduction in the expression of Ase is evident in neuroblasts (arrows) of *da* mutant embryos. (E,F) RNA expression of *scratch* in wild-type (E) and *da* mutant (F) embryos. No expression of the *scratch* transcript is detectable in sensory organ precursors, some of which are indicated by arrows, and only reduced levels of *scratch* RNA is detectable in the neuronal precursor of the CNS. (G,H) RNA expression of *prospero* in a wild-type (G) and *da* mutant (H) background. No expression of *prospero* is detected in sensory organ precursors, while the expression in neuronal precursor cells of the CNS is reduced. Arrows indicate where some of the sops are located in normal embryo. (I,J) RNA expression of *cyclin A* in wild-type (I) and in *da* mutant (J) embryos. Similar to the other neuronal precursor genes, *cyclin A* does not show detectable expression in the periphery of *da* mutant embryos at stages where *cyclin A* is expressed specifically in sensory organ precursors, some of which are indicated by arrows. Reduced expression of *cyclin A* is detectable in the precursors of the CNS. The high background present in both the wild-type and mutant embryos is due to the presence of high levels of maternally supplied *cyclin A* transcript. (K,L) RNA expression of *couch potato* (*cpo*) in wild-type (K) and *da* mutant (L) embryos. *cpo* is only expressed in the sensory organ precursor, but not in the precursor of the CNS. In K, *cpo* expression during the initial stage of sensory organ formation is shown, when the first two sensory organ precursors express the *cpo* transcript. No *cpo* expression is detectable in the periphery of *da* mutant embryos (L).

PNS-specific neuronal precursor gene, *couch potato* (Bellen et al., 1992).

The expression of all six genes was reduced or eliminated in *da* mutant embryos (Fig. 5). Of these six genes, *couch potato* is normally expressed only in neuronal precursors for the PNS (Bellen et al., 1992), and this expression was abolished in *da* mutant embryos (Fig. 5L). Likewise, the expression of *deadpan* and *asense* in both CNS and PNS neuronal precursors (Bier et al., 1992; Brand et al., 1993) was drastically reduced or eliminated (Fig. 5B,D). The expression of *prospero*, *scratch* and *cyclin A* was reduced in neuronal precursors for the CNS and undetectable in neuronal precursors for the PNS (Fig. 5F,H,J). In contrast to the dramatic reduction of the early expression of neuronal precursor genes, the late expression of these genes was not significantly altered in the parts of the CNS that remained in the *da* mutant embryo, indicating that the early and late expression of these neuronal precursor genes is regulated differently.

DISCUSSION

The formation of neuronal precursors in *Drosophila* involves the function of several genes that encode transcription factors with the HLH motif. While the distributions of the AS-C products and the *emc* protein are non-uniform, resulting in the formation of neuronal precursors at locations of high AS-C expression and low *emc* expression, we found that the level of *da* protein in both embryo and imaginal discs is rather uniform among the ectodermal cells prior to and during neuronal precursor segregation and then is elevated at least in some neuronal precursors. Further, we show that neuronal precursors form in embryos with neither maternal nor zygotic *da* activity, express *sc* protein, and delaminate from the ectodermal layer. The failure of these precursors in *da* mutant embryos to divide normally and to give rise to the correct number of neurons is correlated with their inability to express properly a battery of neuronal precursor genes. The functional role of the *da* gene in neuronal precursor formation, and the potential significance of the expression pattern of the *da* protein, are discussed below.

The time and context of *da* action

Our finding of delaminating neuronal precursors in the PNS of *da* mutant embryos is unexpected, given that previous studies using BrdU or the A37 enhancer trap line as markers have failed to reveal any traces of such precursors in *da* mutants (Ghysen and O'Kane, 1989; Bodmer et al., 1989). However, our results are not in contradiction with the earlier results, rather the availability of additional neuronal precursor markers has revealed the existence of two stages of neuronal precursor development. The *da* gene is not required for a 'nascent neuronal precursor' to form and delaminate from the epidermal layer. Without *da* function, however, a nascent neuronal precursor cannot proceed to become a neuronal precursor and express neuronal precursor genes properly. While the markers used in previous studies label those neuronal precursors that are derived from nascent neuronal precursors in a *da*-dependent manner, we are now able to detect nascent neuronal precursors in both wild-type and *da* mutant embryos due to their high levels of Sc expression.

It is not known whether AS-C is required to single out the neuronal precursors for the larval PNS from proneural clusters in the embryo, although mosaic analysis of the imaginal disc indicates that cells with higher levels of AS-C activity are more likely to become neuronal precursors (de Celis et al., 1991; Cubas et al., 1991). If such a requirement exists in the embryo, our results suggest that this requirement may be fulfilled in the absence of *da* function, since nascent neuronal precursors appear transiently in *da* mutant embryos. Instead of forming heterodimer with the *da* protein, the AS-C products in the proneural clusters might form homodimers or dimerize with other proteins besides Da; an elevated level of these transcription factors is sufficient to commit the cell to the fate of a nascent neuronal precursor transiently, but not to sustain the cell as a neuronal precursor in the absence of *da* activity. Although our results suggest that *da* is not required for a nascent neuronal precursor for the PNS to be singled out from a proneural cluster in the embryo, we do not know whether the same is true in imaginal discs. This issue needs to be addressed in the future by examining whether proneural clusters form in da^- clones and if so, whether nascent neuronal precursors are singled out from such clusters.

The progressive commitment of a neuronal precursor

Multiple stages of neuronal precursor development can now be defined based on their expression of different proneural and neuronal precursor genes as well as their requirement for *da* and the proneural genes of the AS-C (Fig. 6). From the proneural cluster of cells that express some of the AS-C genes and acquire the potential to become neuronal precursors, the cell that shows the highest level of AS-C expression delaminates and becomes a nascent neuronal precursor, which probably begins to express *hunchback* (Brand and Campos-Ortega, unpublished data). The *da*, and presumably AS-C, activities are then required for this nascent neuronal precursor to progress into a neuronal precursor, which expresses neuronal precursor genes such as *prospero*, *deadpan*, *couch*

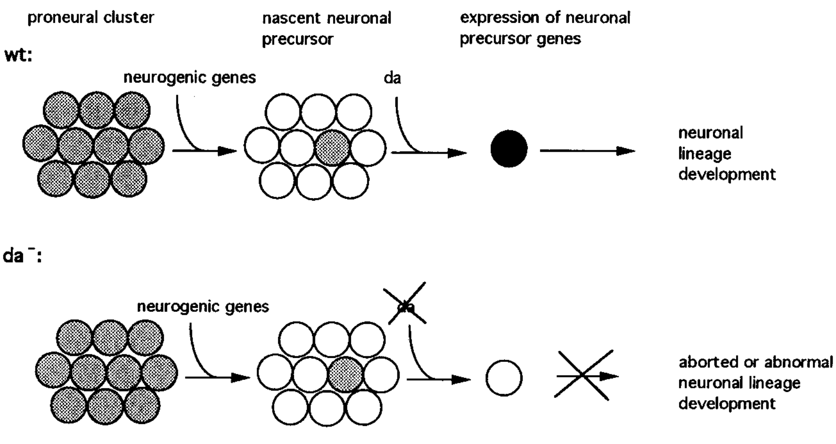

Fig. 6. Model of *daughterless* function in neuronal precursor formation. *da* gene function is not essential for the formation of the proneural cluster, nor for the subsequent restriction of AS-C expression to the nascent neuronal precursor cell. Once the nascent neuronal precursor has formed, *da*, together with the *AS-C* genes, is required to activate, or enhance, the expression of neuronal precursor genes, thereby allowing the initiation of neuronal lineage and development. In a *da* mutant background, the absence or reduction of expression of neuronal precursor genes hinders division and further differentiation of neuronal precursors.

potato, *scratch*, *asense* and *cyclin A*. These neuronal precursor genes are required for the neuronal precursor to divide normally and to give rise to neurons with proper features of neuronal differentiation. In the absence of the ubiquitous *da* protein, a number of neuronal precursor genes fail to be expressed in the PNS; the nascent neuronal precursors for the entire PNS fade away rather than giving rise to nervous tissues. In the CNS, those neuronal precursor genes either fail to be expressed or are expressed at much reduced levels, leading to a much reduced CNS (Caudy et al., 1988a; Jimenez and Campos-Ortega, 1990). Since different neuronal precursors express different subsets of proneural genes, Da functions as a common co-factor which controls the expression of a common set of genes shared by all neuronal precursors, thereby defining neural tissue type.

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