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Subcellular localization of Suppressor of Hairless in *Drosophila* sense organ cells during Notch signalling

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SUMMARY

During imaginal development of *Drosophila*, Suppressor of Hairless [Su(H)], an evolutionarily conserved transcription factor that mediates intracellular signalling by the Notch (N) receptor, controls successive alternative cell fate decisions leading to the differentiation of multicellular sensory organs. We describe here the distribution of the Su(H) protein in the wing disc epithelium throughout development of adult sense organs. Su(H) was found to be evenly distributed in the nuclei of all imaginal disc cells during sensory organ precursor cells selection. Thus differential expression and/or subcellular localization of Su(H) is not essential for its function. Soon after division of the pIIa secondary precursor cell, Su(H) specifically accumulates in the nucleus of the future socket cell. At the onset of differentiation of the socket cell, Su(H) is also detected in the cytoplasm. In this differentiating cell, N and deltex participate in the cytoplasmic retention of Su(H). Still, Su(H) does not colocalize with N at the apical-lateral membranes. These observations suggest that N regulates in an indirect manner the cytoplasmic localization of Su(H) in the socket cell. Finally, the pIIb, shaft and socket cells are found to adopt invariant positions along the anteroposterior axis of the notum. This raises the possibility that tissue-polarity biases these N-mediated cell fate choices.

Key words: lateral inhibition, neurogenesis, signal transduction, *Drosophila*, Suppressor of Hairless, Notch

INTRODUCTION

A family of transmembrane receptors, the Notch (N) receptors, participates in a conserved mechanism of intercellular signalling refered to as lateral inhibition that regulates cell differentiation and pattern formation (Artavanis-Tsakonas et al., 1995). The role of N in lateral inhibition has been studied in detail for the formation of the mechanosensory organs in *Drosophila*. A mechanosensory bristle of the adult fly is composed of two outer support cells, the trichogen (or shaft cell), which produces the stimulus-receiving shaft, and the tormogen (or socket cell), which generates the socket surrounding the base of the shaft, and two subepidermal cells, the neuron and the thecogen (or sheath cell). These four cells are generated from a single sensory organ precursor (SOP) cell via a fixed lineage (Hartenstein and Posakony, 1989). The SOP divides asymmetrically to give rise to two secondary precursor, pIIa and pIIb, which in turn divide asymmetrically to generate the shaft and socket cells, and the neuron and sheath cells, respectively. Lateral inhibition is thought to act at several steps in the bristle lineage. It is first required to select a SOP from proneural groups of cells with similar neural potential confered upon these cells by the expression of the proneural *achaete* and *scute* genes (reviewed in Ghysen et al., 1993). Lateral inhibition also participates in stably establishing alternative fates following SOP, pIIa and pIIb asymmetric divisions (Posakony, 1994; and references herein). During lateral inhibition, N activation by its ligand Delta (Dl) is thought to be relayed intracellularly by an evolutionarily conserved transcription factor, Suppressor of Hairless [Su(H)] (Bailey and Posakony, 1995; Fortini and Artavanis-Tsakonas, 1994; Lecourtois and Schweisguth, 1995; Schweisguth, 1995; Schweisguth and Posakony, 1992, 1994).

Su(H) may directly relay the signal of N activation from the membrane into the nucleus as Su(H) directly binds to a region of the intracellular domain of N that is known to be essential for N signalling activity (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995). However, the cellular compartment where this N-Su(H) interaction occurs is unknown. Two models for the N signalling pathway have been recently proposed. In a first model, referred to here as the 'processed coactivator' model, DNA-bound Su(H) is proposed to bind a processed form of N in the nucleus. This nuclear N protein would consist of (or part of) the intracellular domain that would be generated by the ligand-induced proteolytic cleavage of the N receptor (Lieber et al., 1993). This processed form of N would act as a transcriptional coactivator for Su(H). Consistent with this view, an activated form of murine N binds to DNA-bound Su(H) and can stimulate transcription in a Su(H)-binding-sites-dependent manner in human cells (Jarriault et al., 1995). This model implies that the intracellular part of N localizes in the nuclei of cells receiving lateral inhibition, which has not yet been

observed by immunodetection methods in *Drosophila* (Artavanis-Tsakonas et al., 1995). It also implies a processing of N, which has not yet been studied in detail in *Drosophila*. However, it has recently been suggested from transfection studies that a mouse N receptor deleted of its extracellular part but retaining its transmembrane domain is proteolytically processed to release an activated form of N that localizes into the nucleus (Jarriault et al., 1995; Kopan et al., 1996). In a second, non-exclusive, model, referred to here as the 'nuclear import' model, Su(H) binds to N intracellularly when N is not activated, i.e. does not bind Dl in the extracellular space. The binding of Dl to N somehow interferes with this N-mediated cytoplasmic retention of Su(H), resulting in its nuclear translocation. This view is supported by co-localization studies in transfected *Drosophila* S2 cells (Fortini and Artavanis-Tsakonas, 1994). This model implies that Su(H) localizes in the cytoplasm of cells sending the lateral inhibition signal.

In order to analyze the subcellular localization of Su(H) during N signalling, we have produced anti-Su(H) polyclonal antibodies. We show that Su(H) is evenly distributed in the nuclei of all proneural cluster cells during SOP determination. Soon after pIIa division, Su(H) specifically accumulates in the nucleus of the future socket cell. At the onset of differentiation of the socket cell, Su(H) is detected both in the nucleus and in the cytoplasm. Although cytoplasmic retention of Su(H) appears to be regulated by N, Su(H) does not colocalize with N at the apical-lateral membranes of the socket cell. Together, these observations are most consistent with the 'processed co-activator' model, in which a relocalization of Su(H) during N signalling is not an essential condition. Finally, we show that the pIIb, shaft and socket cells are vectorially orientated relative to the anteroposterior axis of the notum. This suggests that tissue polarity influences decisions controlling the fate of the SOP daughter cells.

MATERIALS AND METHODS

Drosophila stocks

Flies were cultured on standart yeast-cornmeal-sugar medium at 25°C. Flies of the genotype w^{1118} were used as wild types. The P[*lacZ*, ry^{+}] enhancer-trap transposon insertion A101 was used as a marker for sensory organ precursor cells and their progeny (Huang et al., 1991; Usui and Kimura, 1993). Mutant alleles of *Su(H)* and the *Su(H)* deficiency *Df(2L) TE35BC-GW24* were described previously (Schweisguth and Posakony, 1992). The *hs-Nintra* transformant line was kindly provided by T. Lieber and M. Young (Lieber et al., 1993). The *hs-deltex* transformant line was a gift of S. Artavanis-Tsakonas (Busseau et al., 1994). Mutations and chromosomes not described herein are described in Lindsley and Zimm (1992).

Heat-shock and temperature-shift conditions

Pupae were collected at puparium formation and transferred into small Petri dishes. These were immersed for 1 hour in a 37°C water bath at 23 hours after puparium formation (APF). N^{ts1} pupae were similarly transferred at 21 hours APF in a 31°C water bath. At 24 hours APF, pupae were immediately dissected.

Antibody production

Polyclonal antibodies were raised against a Su(H) polypeptide [Su(H)10-594] lacking the first 9 amino acids encoded by a *Dra*I-*Nde*I cDNA fragment. Su(H)10-594 was produced in *E. coli* using the pET3a expression vector (Rosenberg et al., 1987). About 0.3 mg of protein was gel-purified from the *E. coli* inclusion bodies fraction and used for immunization of three rats (Pocono Rabbit Farm). Two different Su(H) polypeptides, Su(H)10-594Δ139-196 (resulting from an internal *Xmn*I-*Sca*I in phase deletion) and Su(H) 10-196 (resulting from a ScaI 3′ end deletion), were used for boosting (0.17 mg and 0.05 mg, respectively, were injected per rat). Together, these two proteins fully overlap with Su(H)10-594, but migrate at a different position in SDS-PAGE. Thus, immunogenic *E. coli* proteins co-purifying with Su(H)10-594 should not be present in the fractions used for boosting. Two sera gave identical results both on western blots and in tissues (sera #22 and 24), and were used unpurified with the exception of the double-labelling experiments shown in Fig. 8 for which immunopurified antibodies from serum #24 were used. Immunopurification was carried out using the GST-Su(H)[444-594] fusion protein (Brou et al., 1994) blotted on nitrocellulose membranes as described in Harlow and Lane (1988). Anti-Su(H) antibodies were eluted at pH 2.8 in a glycine buffer.

Western blot analysis

Dechorionated embryos were individually staged, then frozen in an Eppendorf tube placed into a dry ice/ethanol bath. Dissected wing discs and nota were frozen as described above. Embryos and dissected tissues were crushed in Laemmli loading buffer (Harlow and Lane, 1988). Protein samples were separated by SDS-PAGE (8-10%) and electrotransferred onto immobilon-P membranes (Millipore). Membranes were then rinsed in Tris-buffered saline (TBS: Harlow and Lane (1988)), blocked in TBS, Tween 20 0.2% and dry milk 5%, and then incubated for 90 minutes with the anti-Su(H) sera (dilution 1:2000-1:5000) in TBS 0.1% Tween 20 (TBS-T). Prior to use, immune sera were preadsorbed overnight at 4°C against fixed embryos (vol 1/1, using a 1:10 serum dilution). Membranes were washed in TBS-T, incubated with anti-rat secondary antibodies coupled to phosphatase alkaline (Biosys; dilution 1:2000), washed in TBS-T, followed by BCIP/NBT staining.

Immunohistochemistry and in situ hybridization

Dechorionated embryos were fixed for 15-20 minutes using an heptane-4% paraformaldehyde, PBS 1×, EGTA 50 mM solution, devitellinized in 3:1 methanol/heptane, rinsed in methanol and routinely kept at −20°C in methanol. Following progressive rehydration in PBS, embryos were incubated with RNAse A (10 μg/ml) for 2 hours at 37°C. They were then blocked in PBS 1×, BSA 0.1%, Tween 0.1% (PBTw). Embryos were incubated overnight at 4°C with preadsorbed anti-Su(H) serum, diluted at 1:500-1:2000 in PBT. The FITC-conjugated anti-rat secondary antibodies (KPL) were preadsorbed overnight at 4°C against fixed embryos (dilution 1:10), and then diluted 1:150 in PBTw. Embryos were incubated with 5 μg/ml propidium iodide for 30 minutes during secondary antibodies washes and mounted in Citifluor (Citifluor Ltd).

Dissected imaginal discs and nota were fixed for 15 minutes in a 4% paraformaldehyde solution in PBS, rinsed in PBS, transferred into PBS 1×, BSA 0.1%, Triton X-100 0.1% (PBTr). Incubations with primary and secondary antibodies were as described above except that PBTr was used instead of PBTw. The following primary antibodies were used: rat anti-Su(H) (1:500-1:1000); rabbit anti-β-galactosidase polyclonal antibodies (Cappel, 1:2000); mouse anti-N polyclonal antibodies, directed against the intracellular domain of N (a gift of T. Lieber and M. Young; 1:500). The following secondary antibodies were used: FITC-conjugated anti-rat (KPL, 1:150); TRITC-conjugated anti-rat (Jackson's, 1:150); TRITC-conjugated anti-rabbit antibodies (Biosys, 1:150); FITC-conjugated anti-mouse (Jackson's, 1:150). In situ hybridization and immunostaining double-labelling experiments were carried out as previously described in Schweisguth and Posakony (1992). Confocal images were obtained on a MRC600 Biorad and a Leica TCS 4D confocal microscopes. Confocal and scanned optic images were processed with the Adobe Photoshop and NIH image programs.

RESULTS

Western blot analysis

Polyclonal antibodies were raised in rats against Su(H) polypeptides produced in *E. coli*. These antibodies specifically recognized the in vitro translated Su(H) protein (Fig. 1A, lanes 1, 2). In a stage 11 embryo, a major 75-80 kDa molecular species was seen at a migration position similar to the one observed for the in vitro translated product [Su(H)10-594 (Brou et al., 1994)] and consistent with the predicted size of Su(H) (594 amino acids) (Fig. 1A, lane 3). This band was also detected in total extracts prepared from third instar wing imaginal discs and from nota dissected from 24 hours APF pupae (Fig. 1A, lanes 5 and 6). This band was predominant in a nuclear fraction prepared from *Drosophila* SL2 cells, but could also be detected in the cytoplasmic fraction following crude fractionation (Fig. 1A; lanes 9, 10). Accumulation of this protein was greatly reduced in a stage 11 embryo homozygous for a deficiency covering the *Su(H)* locus (Fig. 1A, lane 4; see legend for details), while this protein accumulated at a high level in wing discs dissected from hs-Su(H) transformant larvae upon heat induction (Fig. 1A; lanes 7, 8). We conclude that this 75-80 kDa band corresponds to the major Su(H) species.

The Su(H) protein was detected in preblastoderm embryos (Fig. 1B, lane 1), consistent with its maternal function (Lecourtois and Schweisguth, 1995) and was present throughout embryogenesis (Fig. 1B). Whole-mount staining of wild-type embryos also revealed a low level of immunofluorescence or peroxidase immunocytochemical signal in all nuclei of stage 1-11 embryos (data not shown). However, the lack of an appropriate negative control, such as a protein null background [Su(H) was detected by western blot analysis in $Su(H)^{SF8}$, $Su(H)^{AR9}$ and $Su(H)^{IB115}$ mutant larvae *trans*-heterozygous for a $Su(H)^{-}$ deficiency (data not shown)], prevented us from analyzing in detail this weak nuclear signal. From stage 12 onward, a high level of Su(H) accumulation was observed in specific external sense organ cells (see below).

Localization of Su(H) in proneural cluster cells during SOP selection

To analyze the expression and subcellular localization of the Su(H) protein during macrochaete SOP specification, we studied by confocal microscopy the immunoreactivity of wing imaginal discs dissected from late third instar larvae. At this stage, all wing disc nuclei expressed the Su(H) protein at a low level (Fig. 2A). In order to more precisely examine the distribution of the Su(H) protein in the SOP relative to its neighbouring proneural cluster cells, immunodetection of Su(H) was carried out in the A101 enhancer-trap line, in which β-galactosidase is specifically accumulated in the nuclei of the SOPs and of their progeny cells (Huang et al., 1991). In double-labelling experiments using anti-Su(H) (green, Fig. 2B) and anti-β-galactosidase (red nuclear staining, Fig. 2C) antibodies, the anti-Su(H) signal was detected in the nucleus of both SOPs and neighbouring proneural cluster cells (see merge, Fig. 2D). No difference was observed in the distribution of Su(H) immunoreactivity between SOPs and neighbouring proneural cluster cells. A similar observation was made for microchaete SOPs in the developing notum at 14 hours APF, soon after the

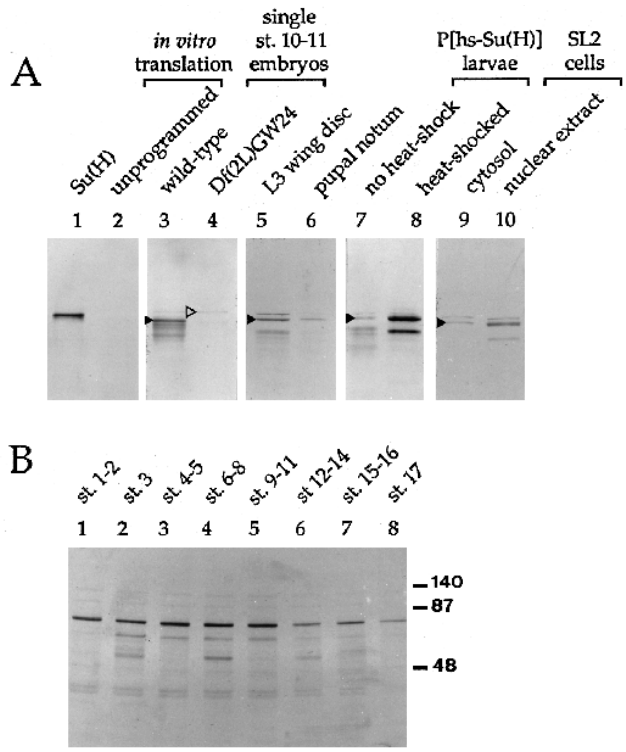

Fig. 1. Immunodetection of Su(H) on western blots. (A) Western blot analysis of: in vitro translated Su(H)[10-594] (Brou et al., 1994) (lane 1; no cross-reacting species was detected in the control lysate sample in lane 2); single stage 10-11 embryos from a Df(2L)GW24/CyO stock. A quarter of these embryos gave a staining pattern similar to the one shown in lane 4. These presumably correspond to embryos homozygous for the $Su(H)^{-}$ Df(2L)GW24 deficiency. All other embryos, which presumably carry one or two copies of Su(H), gave a staining pattern as the one shown in lane 3; wing disc dissected from a late third instar larva (lane 5); notum dissected from a 24 hours APF pupa (lane 6); wing discs dissected from P[hs-Su(H)]-4 larvae (lane 7: no heat-shock; lane 8: after one hour at 37°C); protein extracts prepared from *Drosophila* SL2 cells (lane 9: cytosolic fraction; lane 10: nuclear fraction). Su(H) is detected as a single 75-80×10^3 M_r protein in all lanes (see black arrowheads), except in lane 4. In addition, a variable number of faster migrating species were also observed (see lanes 3, 5, 7 and 8). These proteins appeared to be encoded by the *Su(H)* gene, as they were not detected when the locus is deleted (lane 4), and were conversely found in larger amounts when *Su(H)* was overexpressed in P[hs-Su(H)]-4 larvae (lane 8). The variability seen in their accumulation suggests that they correspond to breakdown products. In addition to the Su(H) 75-80×10^3 M_r protein and associated degradation products, a minor slower migrating species was often detected (open arrowhead in lane 4). This molecular species appeared unrelated to Su(H), since it was detected in mutant embryos (lane 4), did not accumulate upon overexpressing *Su(H)* (lane 8) and was not seen when immunopurified antibodies were used (not shown). (B) Accumulation of Su(H) during embryogenesis. Five precisely staged embryos were loaded per lane. Embryonic stages are indicated above the lanes. Staging is according to Campos-Ortega and Hartenstein (1985).

determination of the microchaete precursor cells. A relatively weak anti-Su(H) signal was detected in the nucleus of each epithelial cell (Fig. 2E). The microchaete SOPs (red nuclear

staining, Fig. 2F), accumulate the Su(H) protein in their nuclei at a comparable level (see merge, Fig. 2G). In a few cases, a significant immunofluorescence signal was also detected surrounding the SOP nucleus, i.e. in the cytoplasm, both in macrochaete and microchaete SOPs (arrowheads in Fig. 2D,G). Using this detection method, we did not observe any significant difference in the subcellular distribution and/or accumulation of the Su(H) protein between SOPs (which send the lateral inhibition signal) and their neighbouring cells (which receive this inhibitory signal). Likewise, following SOP division, no difference in immunofluorescence nuclear staining was observed between pIIa and pIIb (not shown). Thus, both selection of individual SOPs from proneural cluster cells and specification of the pIIa/pIIb fate, do not apparently correlate with differential expression and/or distribution of Su(H).

Specific accumulation of Su(H) in the nucleus and cytoplasm of the socket cell

We next examined Su(H) distribution during socket cell determination, for which a high level of *Su(H)* activity is known to be required (Schweisguth and Posakony, 1994). In the notum, the two microchaete secondary precursors divide asynchronously around 20-22 hours APF (Fig. 3A). The pIIa cell divides first to produce the two external accessory cells, the shaft- and the socket-producing cells (Fig. 3A,B). At this stage, prior to pIIb division, three SOP daughter cells were detected in the A101 enhancer-trap line. Remarkably, these three cells, i.e. the pIIb, shaft and socket cells, were reproducibly found aligned (Fig. 3D,F). In no cases did we observe a more compact 'three-cell cluster' in which each SOP daughter cell contacts the two other SOP daughter cells. The alignment of each 'three-cell clusters' roughly follows the anteroposterior axis of the fly: 85% of the clusters are orientated between ±40 degrees relative to the anteroposterior axis (Fig. 4).

Two types of 'three-cell clusters' were observed: in some cases no cell expressed a higher level of Su(H) (Fig. 3C). These 'three-cell clusters' were often surrounded by pIIa and pIIb cells that have not yet divided and were therefore interpreted as being newly born from pIIa division. In other cases, a strong anti-Su(H) signal was detected in one of these three cells. This cell always occupied the posterior-most position within the 'three-cell cluster' (Fig. 3D-G; no orientation values higher than ±90 degrees were observed in the analysis presented in Fig. 4). Thus, the 'three-cell clusters' are vectorially orientated relative to the anteroposterior axis of the notum. We conclude that the pIIa daughter cell located posteriorly in the 'three-cell cluster' accumulates a high level of Su(H).

The pIIb cell divides soon after (Fig. 3A) to generate the neuron and the sheath cell (Fig. 3B). Again at this early 'four-cell' stage, a single cell, always located posteriorly relative to the three other sensory organ cells, was detected expressing a very high level of Su(H). At 24 hours APF, this Su(H)-expressing cell was unambiguously identified as the socket cell (Fig. 3H-J), based upon its large size and the localization of its polyploid nucleus in the epidermal plane, i.e. above the shaft cell nucleus. Therefore, the cell that accumulates a high level of Su(H) at the 'three-cell stage' and occupies a posterior position within the 'three-cell cluster' could be identified as the socket cell.

The subcellular distribution of Su(H) in the socket cell appeared to vary between 20 and 24 hours APF. At the 'three-cell stage', the strong anti-Su(H) signal was mostly nuclear (Fig. 3E-G), while it was later seen both in the nucleus and in the cytoplasm (Fig. 3H-J). This might reflect a redistribution of Su(H) from the nucleus to the cytoplasm, or simply an increase in the volume of the socket cell cytoplasm. Socket cells of macrochaetes also accumulates the Su(H) protein at a very high level, both in its nucleus and its cytoplasm, until at least 48 hours APF (see Fig. 7). In the pupal eye, one cell per

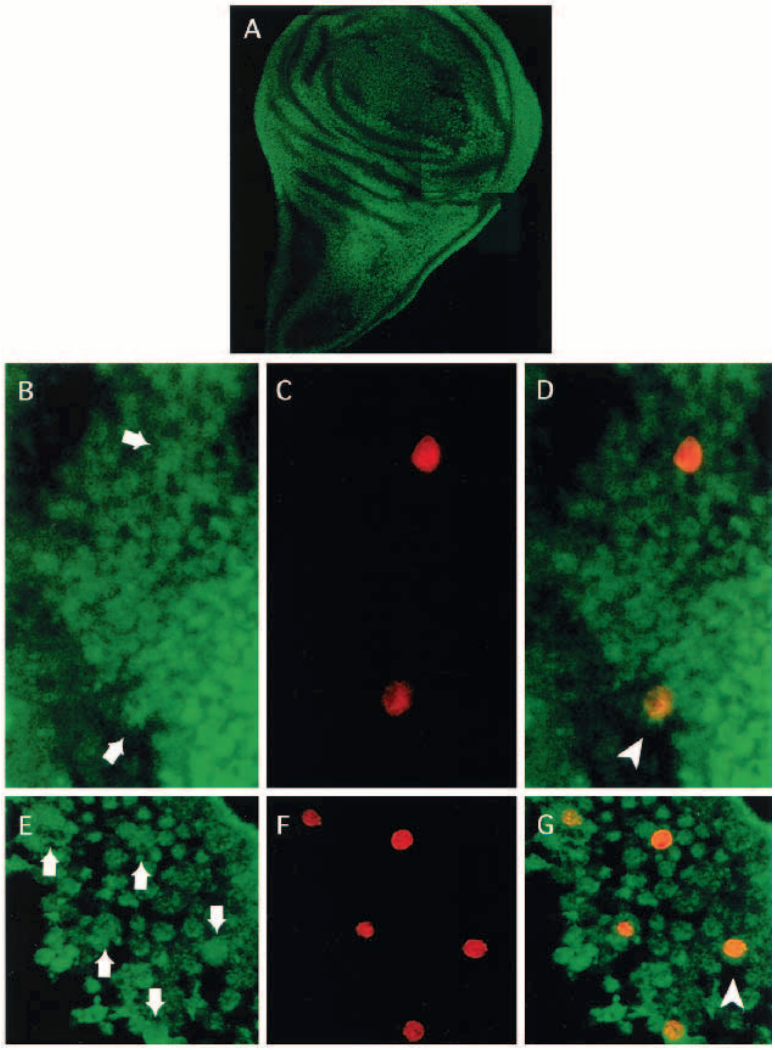

Fig. 2. Uniform distribution of Su(H) immunoreactivity during SOP selection. (A) Wing imaginal disc from a late third instar larva: Su(H) immunoreactivity appears to be uniformly distributed. (B-D) Su(H) immunoreactivity (green in B,D) in a wing disc region where the pDC and aPA SOPs have been specified (arrows in B). (E-G) Su(H) immunoreactivity in the notum of a 14 hours APF pupa (green in E,G; arrows in E indicate microchaete SOPs). Expression of β-galactosidase in SOP nuclei is seen in red (C,D,F,G). No difference in the distribution of Su(H) immunoreactivity was observed between SOP and neighbouring proneural cluster nuclei. Cytoplasmic signal could also be seen in SOPs (arrowheads in D,G).

ommatidium exhibited a strong Su(H) immunoreactivity at 24 hours APF (Fig. 3K). Its position relative to the cone cell nuclei (arrows in Fig. 3L) suggests that it is the socket cell of the interommatidial bristle. Immunoreactivity was seen both in the cytoplasm and in the nucleus. This is clearly seen in Fig. 3M, where the optical plane crosses the eye epithelium with an angle relative to the plane of the tissue, thus revealing both cytoplasmic and nuclear staining.

Several conclusions could be drawn from these observations. First, the Su(H) protein accumulates in one only of the two pIIa daughter cells, the socket cell. Second, at the 'three-cell stage', the Su(H) protein is abundant in the nucleus, where it is thought to act as a transcription factor to regulate the shaft/socket alternative fate decision. Third, this future socket cell occupies a posterior position. This in turn implies that the central cell of the 'three-cell cluster' is its sister cell, the future shaft cell, and that the pIIb cell is the anterior-most cell. Finally, Su(H), a DNA-binding protein, also localizes to the cytoplasm of the differentiating socket cells, both in the notum and in the eye.

Cell-specific expression of Su(H) in the embryonic peripheral nervous system

The accumulation of Su(H) in the socket cell cytoplasm observed in pupae prompted us to analyze its subcellular localization in embryonic sense organ cells. A strong anti-Su(H) signal was detected in a single outer accessory cell per external sense organ, as shown for the thoracic 2-abdominal 1 segments of a stage 16 embryo (Fig. 5A-C). This pattern of Su(H) accumulation first appeared in late stage 12 embryos. At this stage, two cells per abdominal segments expressed Su(H) at a high level. The Su(H) protein was then mostly found in the nucleus (Fig. 5D-F). At stage 16, anti-Su(H) immunoreactivity was seen both in the nucleus and in the cytoplasm, as shown for the two dorsal hair support cells of an abdominal dorsal cluster (Fig. 5G-I) and for the support cell of the dorsomedial sensory cone in the abdominal 8 segment that extends a long cytoplasmic process (Fig. 5J-L). The 'socket-like' morphology of the two support cells shown in Fig. 5G suggests that Su(H) is expressed in the socket cell of the larval external sense organs.

This socket cell-specific accumulation of Su(H) contrasts

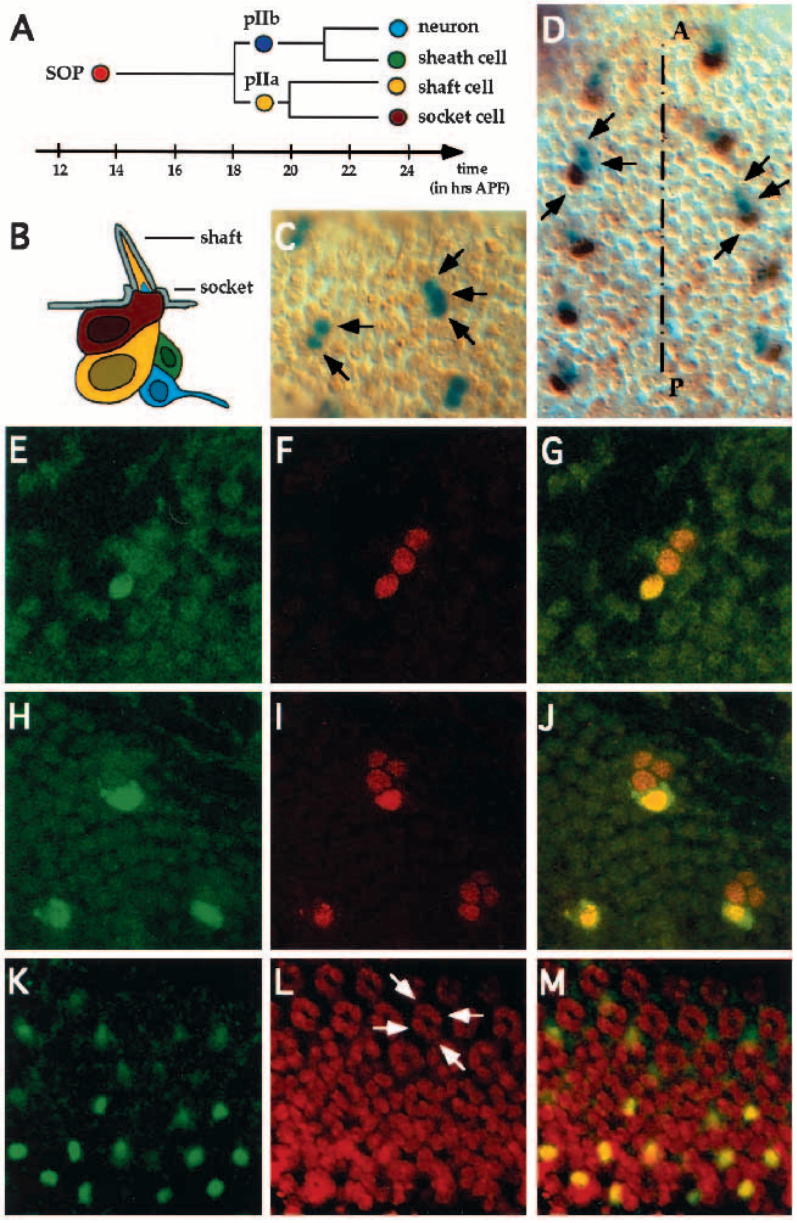

Fig. 3. Specific accumulation of Su(H) in the socket cell. (A) Pattern of division of microchaete SOPs. Note that developmental times (with a variation of ±1 hour) that we have observed in our culture conditions, using the A101 strain, differs slightly from the one reported previously for a Canton S strain (Hartenstein and Posakony, 1989). (B) Schematic representation of a differentiated microchaete. (C,D) Su(H) immunoreactivity in the notum was revealed by peroxidase conjugate (brown staining) at 19 hours (C) and 21 hours (D) APF. Sensory organ cells were identified in the A101 enhancer-trap line using β-galactosidase activity staining (blue staining). Soon after division of the pIIa cell ('three-cell cluster' indicated with three arrows in C) no strong Su(H) immunoreactivity was detectable. Note that pIIa has not divided yet at neighbouring sense organ positions (pIIa and pIIb are indicated with two arrows in C). (D) At the 'three-cell stage', sensory organ cells are aligned roughly following the anteroposterior axis (indicated by a dashed line) and that the Su(H)-positive cell (brown staining) is positioned at the posterior-most position. (E-J) Su(H) (in green: E,G,H,J) and β-galactosidase (in red: F,G,I,J) immunoreactivity in the notum of 19 (C-G) and 24 hours (H-J) APF pupae. At the 'three-cell stage' (E-G), the posterior-most cell began to accumulate Su(H). In the merge shown in G, this signal appeared to be mostly nuclear (in yellow). At 24 hours APF, a very high level of Su(H) immunoreactivity was seen in the nucleus (in yellow) and in the cytoplasm of the socket cell (see merge in J). (K-M) Su(H) immunoreactivity (in green: K,M) in the pupal eye at 24 hours APF. Nuclei were identified using propidium iodide staining (in red: L,M). Cells that display strong Su(H) immunoreactivity are located around each ommatidia identified by the apical four cone cell nuclei (arrows in L). Su(H) immunoreactivity was detectable both in the cytoplasm (top) and in the nucleus (in yellow; bottom). In C-J, anterior is at the top.

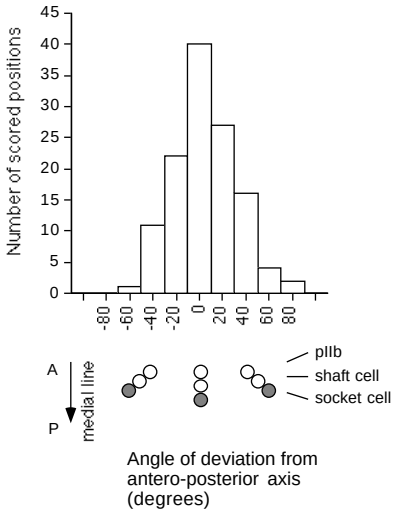

Fig. 4. Anteroposterior orientation of the 'three-cell clusters'. Histogram showing the anteroposterior orientation of sensory organ cells at the 'three-cell stage'. The orientation of 150 'three-cell clusters' located in the anterior half of rows 1-3 (where the latter are parallel to the midline) was measured from 25 nota dissected from A101 pupae (orientation values shown in abscissa; numbers of clusters shown in ordinate). These preparations were stained for β-galactosidase activity staining (to identify the 'three-cell rows') and Su(H) (to identify the socket cell in each cluster). These 'three-cell clusters' are schematized underneath the histogram. The midline was taken as a reference for the anteroposterior axis. Positive (negative) values are arbitrarily given to clusters having their socket cell being furthest (closest) to the midline.

with our previous report indicating that *Su(H)* transcripts were found in both shaft- and socket-secreting cells in embryos and pupae (Schweisguth and Posakony, 1992). A possible difference between mRNA and protein distribution was reexamined in double-labelling experiments. Cytoplasmic *Su(H)* transcripts were detected by in situ hybridization (blue alkaline phosphatase staining), while the Su(H) protein was revealed by antibody staining (brown peroxidase staining). At embryonic stage 14, each nucleus accumulating the Su(H) protein is clearly surrounded by a single ring of cytoplasm stained in blue (Fig. 6A). No other juxtaposed cell was detected expressing high levels of *Su(H)* transcripts. In the notum of a 24 hours APF pupa, the socket cell, which has a crescent shape (see Figs 7, 8), appears to be the only sense organ cell that accumulates a high level of *Su(H)* transcripts. We conclude that a single accessory cell, identified as the socket cell, has an elevated level of *Su(H)* transcript and Su(H) protein.

Cytoplasmic retention of the Su(H) protein is regulated by Notch in the socket cell

Transfection studies had previously shown that Su(H) can bind to the intracellular domain of the N transmembrane receptor (Fortini and Artavanis-Tsakonas, 1994). This observation led us to investigate whether N is involved in the cytoplasmic localization of the Su(H) protein in the differentiating socket cell of macrochaete bristles at 24 hours APF. This cell offers a unique opportunity to investigate this problem since this cell can be easily identified, possesses a large polyploid nucleus and a clearly detectable cytoplasm, and since the Su(H) protein is normally found both in the cytoplasm and in the nucleus of this cell (see Fig. 7A). The role of N in regulating the subcellular distribution of Su(H) was first studied using a thermosensitive mutant allele of *N* (N^{ts1}) that behaves as a strong hypomorphic allele at restrictive temperature (31°C). At permissive temperature (25°C), the Su(H) protein was found in the cytoplasm and in the nucleus of macrochaete socket cells in N^{ts1} homozygous pupae (Fig. 7D). This distribution was similar to the one seen in wild-type pupae (Fig. 7A). By contrast, the Su(H) protein was mostly detected in the socket cell nucleus when pupae were kept at restrictive temperature for 3 hours prior to dissection (Fig. 7E). This heat treatment had no effect upon the subcellular distribution of Su(H) in wild-type control pupae (not shown). This shows that the cytoplasmic localization of Su(H) depends upon a wild-type N receptor (see diagram in Fig. 7F). Since the N^{ts1} mutation corresponds to a missense mutation at EGF repeat 32 in its extracellular part (Xu et al., 1992), this mutation probably affects indirectly the ability of N to regulate the distribution of Su(H). No significant change in the amount and distribution of N immunoreactivity was observed in N^{ts1} pupal nota at 24 hours APF (not shown), indicating that this mutation does not affect the transport and/or stability of N. It is possible that abnormal folding of the extracellular domain down-regulates the ability of N to retain, directly or indirectly, Su(H) in the cytoplasm.

We next analyzed the distribution of Su(H) following overproduction of the intracellular domain of N [N^{intra}: residues 1790-2703 (Lieber et al., 1993)]. This form of N is sufficient for Su(H) binding in the yeast interaction-trap assay (Fortini and Artavanis-Tsakonas, 1994), although it apparently lacks residues important for the binding of murine Su(H) to mNotch (Tamura et al., 1995). This truncated protein contains nuclear localization signals that lead to its accumulation in the nucleus (Lieber et al., 1993), and in particular in the socket cell nucleus (not shown). The expression of N^{intra} was driven by the *hsp70* heat-shock promoter. In non-induced conditions (25°C), the Su(H) protein was detected in the nucleus and in the cytoplasm of the macrochaete socket cells of hs-N^{intra} pupae (Fig. 7G), as in wild-type pupae (compare with Fig. 7A). Following a 1 hour-long 37°C heat-shock, the Su(H) protein was seen predominantly accumulated in the nucleus of the macrochaete socket cells of hs-N^{intra} pupae (Fig. 7H). This heat-shock treatment had no significant effect upon the subcellular distribution of the Su(H) protein in wild-type control pupae (Fig. 7B). We propose that the binding of Su(H) to N^{intra} in the nucleus competes with the mechanism(s) responsible for the cytoplasmic localization of Su(H) (Fig. 7I).

Lastly, we assayed the effect of overexpressing the cytoplasmic protein deltex (dx) that binds the ankyrin repeats of N. This domain maps near, or overlaps with, the binding site of N for Su(H) (Fortini and Artavanis-Tsakonas, 1994; Tamura et al., 1995). The expression of dx was induced in hs-dx pupae at 24 hours APF. In non-induced conditions (25°C), the Su(H) protein was again normally distributed in both the cytoplasm and nucleus of the macrochaete socket cells (Fig. 7J). Following a 1 hour-long 37°C heat-shock, the Su(H) protein was predominantly localized into the socket cell nuclei (Fig. 7K). A similar effect of dx overexpression on Su(H) localization has recently been reported in transfected S2 cells (Matsuno et al., 1995). These data show that accumulation of dx prevents the cytoplasmic retention of Su(H). One possibility is that dx directly interferes with the putative binding of Su(H) to N in the cytoplasm, therefore increasing the pool of cytoplasmic Su(H) available for nuclear import (Fig. 7L).

Together, these data indicate that N regulates the cytoplasmic retention of Su(H) in the socket cell, possibly by direct N-Su(H) protein-protein interaction. The functional importance

of this N-regulated import of Su(H) into the nucleus cannot be directly investigated in the socket cell (whose fate remained unchanged in these experiments). Indeed, since adoption of the socket fate requires a high level of *Su(H)* activity, the fate of the socket cell is not expected to be modified by an increase of nuclear Su(H). Finally, although these changes in Su(H) localization have been implicitly interpreted as resulting from the nuclear import of cytoplasmic Su(H), we cannot exclude that they are instead associated with a decreased stability of cytoplasmic Su(H).

Su(H) does not co-localize with N at the apicolateral membrane

The Su(H) protein appears to be distributed evenly in the cytoplasm of the socket cell. By contrast, N localizes to the apicolateral point of contact between epithelial cells (Fehon et al., 1991). Indeed, N colocalized with the F-actin at the apex of both socket and epidermal cells (not shown). This observation led us to detail the relative distribution of N and Su(H) in the socket cell. Horizontal optical sections of either macrochaete or microchaete socket cells close to the apical surface of the epithelium, where N immunoreactivity is at its highest (using antibodies that recognize the intracellular domain of N), revealed that cytoplasmic Su(H) did not co-localize with N (Fig. 8A-C). This was also clearly seen on vertical sections (Fig. 8D-F). This indicates that Su(H) does not significantly interact with N at these specific sites.

DISCUSSION

Is differential expression of Su(H) required for alternative cell fate choices?

The present study suggests that alternative cell fate decisions occur in conditions where *Su(H)* is not differentially expressed. We have shown that the level of Su(H) accumulation was similar in all the nuclei of the imaginal disc at the late third larval stage, both during SOP and pIIa/pIIb specification. That the adoption of alternative cell fate does not necessarily rely on differential levels of *Su(H)* gene products is consistent with the observation that maternally derived *Su(H)* activity fully compensates for the lack of zygotic activity during early neurogenesis (Lecourtois and Schweisguth, 1995; Schweisguth and Posakony, 1992). In these mutant embryos, each cell presumably receives a comparable amount of maternal Su(H), and yet neurogenesis occurs normally.

Soon after pIIa division, a high level of Su(H) protein (and transcript) was detected in the socket cell. This cell-specific expression was seen early in the differentiation of the sense organ, before pIIb division. Interestingly, a high level of *Su(H)* activity is known to be both necessary and sufficient for the adoption of the socket fate: decreasing *Su(H)* activity results in the transformation of the socket cell into a second shaft cell, while increasing the level of Su(H) produces the opposite cell fate change (Schweisguth and Posakony, 1994). Thus, specific accumulation of Su(H) in the pIIa daughter cell, which is thought to receive lateral inhibition, may contribute to establish the shaft/socket cell decision. However, at an early 'three-cell stage', none of the two pIIa daughter cells displayed a significantly elevated level of Su(H) accumulation, suggesting that there is a time delay between pIIa division and Su(H) accumulation. Moreover, the socket cell was always located posteriorly within the cluster suggesting that the differential expression of Su(H) is the consequence of a pre-existing asymmetry (see below). Thus, differential expression of Su(H) may not be a primary event in the shaft/socket choice but may rather stabilize a pre-established difference between sister cells. Even so, differential levels of Su(H) can influence the establishment of distinct cell fates (Schweisguth, 1995).

How is the shaft/socket cell choice reproducibly biased?

The orientation of the 'three-cell clusters' along the anteroposterior axis and the position of the socket cell within this cluster were found to be reproducibly defined in the developing notum. One consequence of this alignment is that the socket cell probably does not contact the pIIb cell, thus restricting intercellular signalling between these two cells. This arrangement also raises a number of questions regarding positional information in the notum (Lawrence, 1966).

First, what controls the alignment of the pIIb, shaft and socket cells within each 'three-cell cluster'? It is possible that this alignment is primarily determined by the orientation of the cleavage plane at SOP and pIIa divisions. It would thus be of interest to examine how the orientation of the mitotic spindle is controlled during these divisions.

Second, how is the posterior position of the socket cell within the 'three-cell cluster' specified? One possibility is that pIIb may influence the shaft/socket choice. In this view, direct cell contact with pIIb might induce an asymmetric cell division by polarizing the distribution of cell fate determinants, such as numb (Rhyu et al., 1994), in the dividing pIIa. Alternatively, the posterior position of the future socket cell may be controlled by positional clues independent of pIIb, such as tissue polarity (Gubb, 1993).

Lastly, the posterior position of the socket cell suggests that, following SOP division, pIIa is located posterior to its sister pIIb. By contrast to the dividing pIIa that is directly juxtaposed to pIIb, the dividing SOP is evenly surrounded by epithelial cells. Therefore, the orientation of the asymmetric SOP division likely results from the anteroposterior planar polarity of the notal epithelium.

Regulation of the cytoplasmic localization of Su(H) by N in the socket cell

In the differentiating socket cell, we have shown that cytoplasmic Su(H) does not specifically co-localize with N at the apicolateral membrane, suggesting that cytoplasmic Su(H) does not directly interact with N. In apparent contradiction with these data, N was shown to regulate the cytoplasmic retention of Su(H) in this cell. Interpretation of these paradoxical data depends on whether cytoplasmic Su(H) interacts directly or indirectly with membrane-bound N molecules.

If Su(H) does not directly interact with N at the apical plasma membrane, at least in the socket cell, it implies that dx overexpression and changes in *N* activity can both interfere with a yet unknown mechanism responsible for the cytoplasmic retention of Su(H). In this view, Su(H) could be prevented from interacting with membrane-bound N by dx, which was shown to co-localize with N at the apical surface of the wing disc epithelium (Diederich et al., 1994). Consistent with this

hypothesis, binding of Su(H) and dx to N appears to be exclusive in transfected S2 cells (Matsuno et al., 1995). Thus, in the socket cell, N localized at the apical-lateral membrane may bind dx but not Su(H).

Alternatively, if Su(H) directly interacts with N at the membrane, it implies that the dynamic equilibrium between membrane-associated and cytoplasmic Su(H) is largely in favor of the accumulation of Su(H) in the cytoplasm. Assuming that N receptor activation triggers the nuclear translocation of Su(H), direct interaction of a limited amount of Su(H) molecules with N at the membrane could displace this dynamic equilibrium between cytoplasmic, membrane-associated and nuclear pools of Su(H) in favor of nuclear Su(H). This would then result in a reduced amount of cytoplasmic Su(H). If Su(H) directly binds to membrane-bound N, it might modulate N receptor activity by regulating the ability of its intracellular domain to bind additional proteins, such as dx (Diederich et al., 1994) or dishevelled (dsh) (Axelrod et al., 1996). Membrane-associated Su(H) might also modulate the hypothetical proteolytic cleavage of N since the possible site of cleavage maps near, or overlaps with, the binding site of N for Su(H) (Kopan et al., 1996; Tamura et al., 1995).

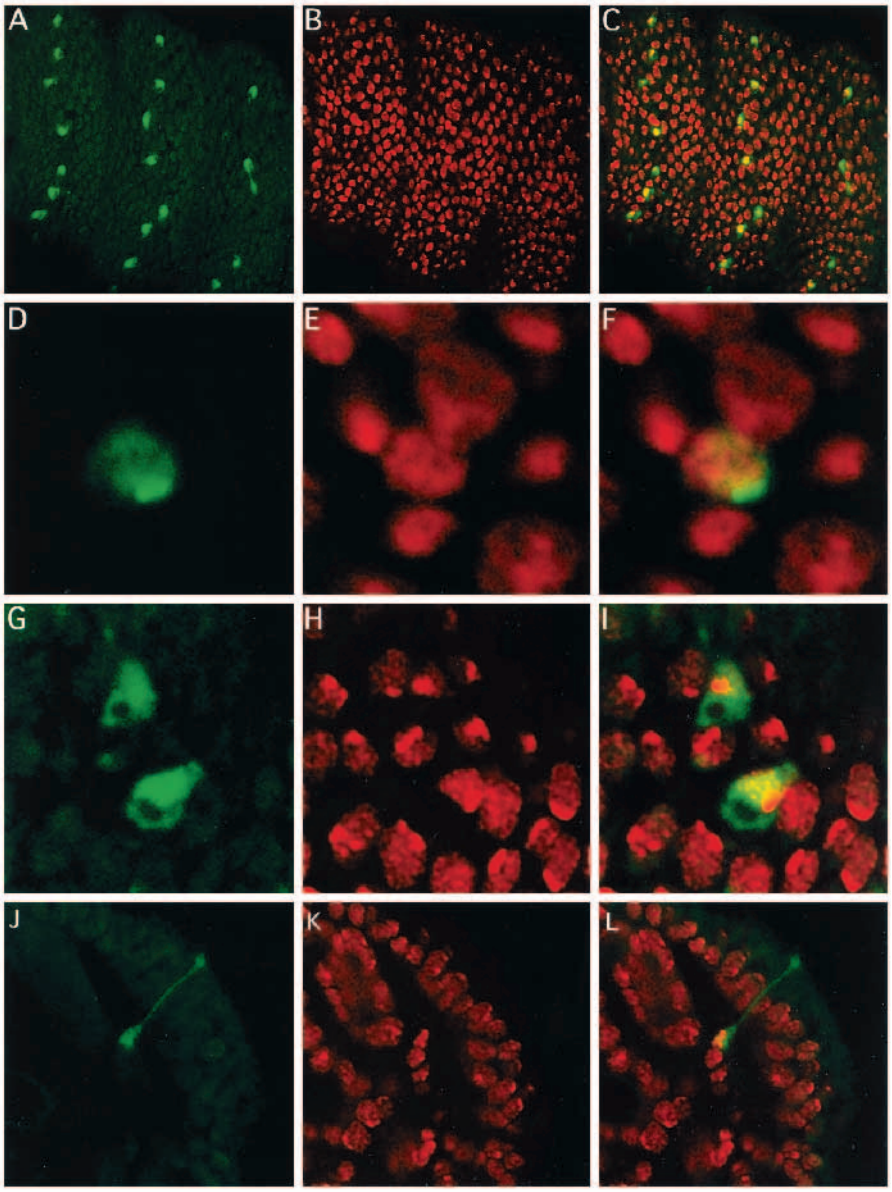

Fig. 5. Cell-specific accumulation of Su(H) in the embryonic PNS. In all panels, Su(H) immunoreactivity is in green (A,D,G,J), and nuclei (propidium iodide DNA staining) is in red (B,E,H,K). Merged images are shown in C,F,I,L. (A-C) Thoracic 2-Abdominal 1 segments of a stage 16 embryo. A single accessory cell per external sensory organ expressed a high level of Su(H) immunoreactivity (ventral-most sensory organs are not shown). (D-F) High magnification view of a dorsal cluster external sensory organ at stage 12. Su(H) immunoreactivity appears to be mostly located in the nucleus. (G-I) Su(H) immunoreactivity in two lateral external sensory organs [dc2 and dh1: see Campos-Ortega and Hartenstein(1985) for nomenclature] at stage 16. Su(H) immunoreactivity is detected in both the nucleus and the cytoplasm. Note the socket morphology of the Su(H)-positive cells. (J-L) The cytoplasmic and nuclear localization of Su(H) at stage 16 embryo is clearly observed in the support cell of the dorsomedial sensory cone.

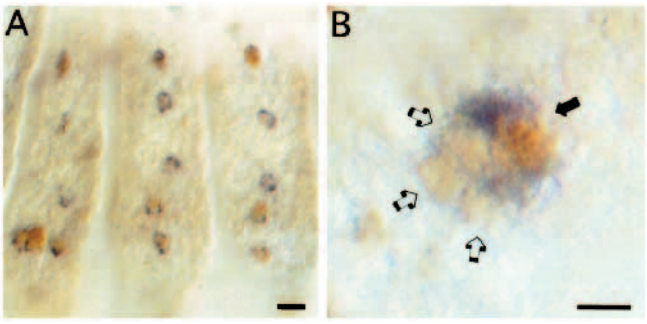

Fig. 6. A high-level of Su(H) transcripts in a single sensory organ cell. *Su(H)* transcript localization was revealed by *in situ* hybridization (blue alkaline phosphatase staining). Su(H) protein distribution was detected by brown peroxidase immunostaining. (A) Lateral view of the Thoracic 1-Abdominal 2 segments of a stage 14 embryo. Cells that accumulate a high level of cytoplasmic transcripts are the only ones that also accumulate a high level of proteins. (B) Microchaete cells at 24 hours APF. Note the socket-like morphology of the cell accumulating a high level of Su(H) transcripts (black arrow). The other three cells do not appear to accumulate a high level of Su(H) protein and/or transcript (open arrows). Scale bar, 10 μm (A) and 5 μm (B).

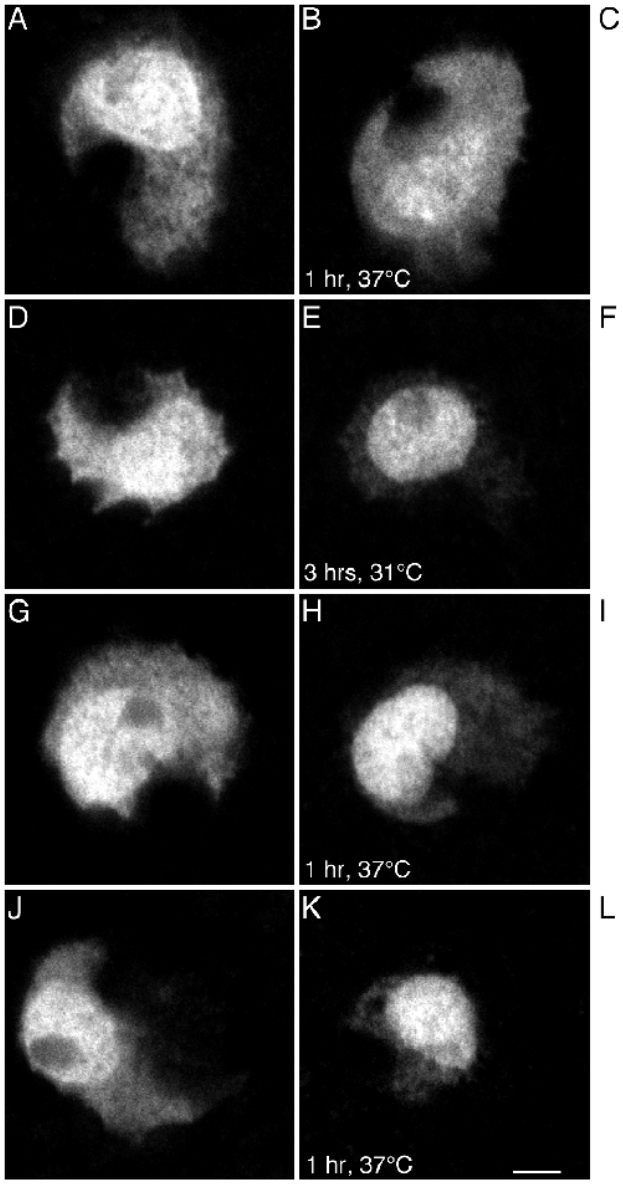

Fig. 7. Notch regulates the cytoplasmic retention of Su(H). Su(H) immunoreactivity in the socket cell of scutelar macrochaetes at 24 hours APF. Control wild-type flies at 25°C (A) and after 1 hour at 37°C (B): the relative distribution of Su(H) in the nucleus and in the cytoplasm is not temperature-dependent. N^{ts1} flies at 25°C (D) and after 3 hours at 31°C (E): cytoplasmic localization of Su(H) appears to require the wild-type N protein. *hs-*N^{intra} flies at 25°C (G) and after 1 hour at 37°C (H): expression of N^{intra} reinforces nuclear localization of Su(H). *hs-dx* flies at 25°C (J) and after 1 hour at 37°C (K): expression of dx appears to also induce the nuclear translocation of Su(H). Each of the diagrams shown in C, F, I and L depicts one possible interpretation of the changes in subcellular localization of Su(H) (see also Discussion). Scale bar, 4 μm.

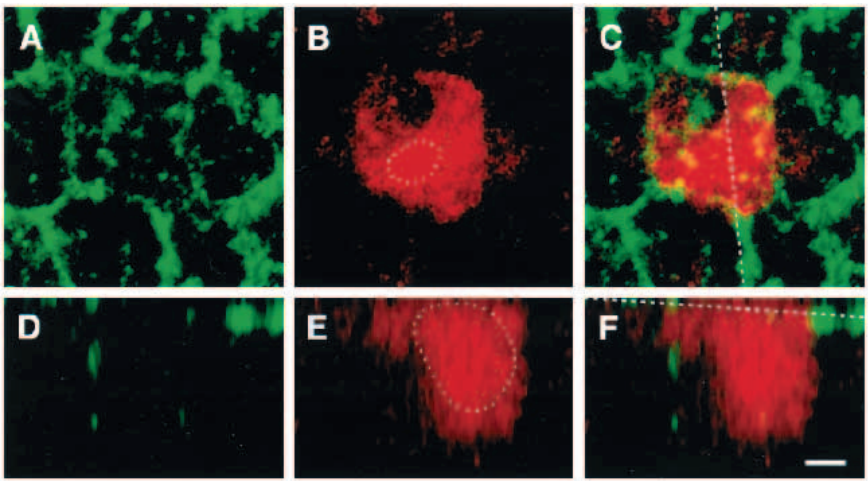

Fig. 8. Notch and Su(H) proteins do not co-localize in the differentiating socket cell. Optical sections of a microchaete socket cell at 24 hours APF notum showing N and Su(H) immunoreactivity (green and red, respectively). (A-C) Horizontal section crossing the apex of the socket cell (the plane of section is shown in F as a dashed line). Anterior is at the top. (D-F) Vertical section of the same cell constructed from 19 horizontal sections (the plane of this reconstruction is indicated by a dashed line in C). Anterior is to the left. (A,D) N immunoreactivity was predominantly found at the apicolateral membranes in both epidermal and socket cells. (B,E) Su(H) immunoreactivity was distributed uniformly in the socket cell cytoplasm, as well as in the nucleus (outlined by a dotted line). (C,F) Su(H) did not specifically accumulate in the cellular compartments showing a high level of N protein accumulation. Scale bar, 2 μm.

'Nuclear import' versus 'processed coactivator' hypotheses

Our observations constitute the first in vivo evidence that Su(H), a transcription factor, can localize in the cytoplasm. Our results further indicate that N participates in the cytoplasmic retention of Su(H) in vivo. These observations are in agreement with the 'nuclear import' model, which required a cytoplasmic localization of Su(H) and proposes that Su(H) translocates from the cytoplasm to the nucleus in response to N activation (Fortini and Artavanis-Tsakonas, 1994). This model also suggests that membrane-bound N receptors directly bind Su(H). Such a co-localization of Su(H) with N at the membrane was not seen in the socket cell. Most importantly, this model further predicts a difference in the nucleocytoplasmic distribution of Su(H) between cells that receive lateral inhibition and those that send it. Such a difference was not detected during SOP selection in the wing disc. This is certainly not because subcellular localization was investigated after cell decision was taken; at the time A101 is expressed, the E(spl) gene products accumulate under the control of Su(H) in cells directly juxtaposed to the SOP (Bailey and Posakony, 1995; Jennings et al., 1995; Lecourtois and Schweisguth, 1995). It is formally possible that only a fraction of the cytoplasmic pool of Su(H), which is not specifically identified using our antibodies, translocates into the nucleus in response to N signalling. Still, we consider that the observation that Su(H) is evenly distributed in the nuclei of all proneural cluster cells during SOP determination is more consistent with the 'processed co-activator' model for which the regulated nuclear import of Su(H) is not a necessary condition.

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