Expression of Prospero, Snail and Krüppel homologues during spider neurogenesis

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Prospero:

„Do not infest your mind with	„Zerquäl dir nicht den Kopf und
beating on	grübel dich
The strangeness of this business;	In all die Seltsamkeiten.
at pick`d leisure	Bei mehr Muße,
Which shall be shortly single,	Die nun bald eintritt,
I`ll resolve you,	Lös ich selbst dir auf,
Which to you shall seem probable, of every	Wie`s dir verständlich scheinen soll, was sich
These happen`d accidents;	Hier zutrug an Geschichten.
till when, be cheerful,	Sei vergnügt
And think of each thing well“	Solang, und denk von allem gut“

From: „*The tempest*“, Shakespeare

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Abstract

The aim of the present study is it to understand neurogenesis of different arthropod groups in more detail. To gain insights into the evolution of the development of nervous systems in arthropods the spider *Cupiennius salei* is used as a comparative system to insects. The expression patterns of spider homologues which are known to play a role during insect neurogenesis are analyzed. Similarities and differences of these expression patterns are compared in two distantly related arthropod groups.

A spider homolog of *even skipped, Cupiennius even skipped*, and two *runt* homologues, *Cupiennius runt 1* and *2*, were cloned. Furthermore a spider homolog of *prospero, Cupiennius prospero*, was cloned. Protein expression and subsequent immunization experiments resulted in functional antibodies against Prospero, Snail and Krüppel spider homologues.

Results for *runt 1* and *even skipped* indicate that both genes play a role in neurogenesis of the spider, whereas *runt 2* is expressed in the peripherous nervous system.

There exist essential differences in the origin of the spider nervous system in comparison to insects. Different experiments were performed to localize cells of the developing central nervous system which are mitotically active. In the cell layers beneath the neuroectoderm only few cells are mitotically active. The results suggest that in the spider *Cupiennius* there do not exist mitotically active neuroblasts and ganglion mother cells like in insects. Instead groups of cells are recruited directly at the apical surface of the neuroectoderm for invagination.

Snail is expressed in invaginating cells. Experiments suggest that the expression of Snail starts after the begining of invagination and ends before cells retract their cytoplasmatic extensions completely. Expression of Snail was also found in cells associated to mechanosensitive hairs in the peripherous nervous system.

Prospero is also expressed in invaginating cells. Furthermore cells which completed invagination and nerve cells express Prospero at different levels. Results indicate that Prospero expressing cells invaginate and move into the direction of the developing neuropil. At the neuropil they differentiate into nerve cells. In cell layers beneath the neuroectoderm there are mitotically active cells expressing Prospero.

There is no evidence for asymmetric localisation of Prospero. Instead Prospero is segregated symmetrically into both daughter cells during mitotic division.

Krüppel-1 is expressed in many cells of the neuroectoderm and also in basal cell layers of the developing central nervous system. Transiently Krüppel-1 accumulates at higher levels in invaginating cells. These high expression levels show a dynamic change along the anterior posterior axis. Furthermore the invagination sites show a time delay between high levels of Krüppel-1 transcription and translation.

Invaginating cells show different expression levels of Snail and Prospero. The expression of Prospero is starting later and continues in cells which completed invagination. This argues in favor of a stepwise differentiation process of invaginating cells into nerve cells. There exists a similar sucession of the expression of Snail and Prospero homologues in *Cupiennius* as in *Drosophila*. Furthermore Snail and Krüppel homologues are expressed in comparable tissues of the neuroectoderm in both species. Results suggest that the Prospero, Snail and Krüppel spider homologues probably also play a role in the specification of nerve cells. The expression pattern of Prospero, Snail and Krüppel homologues in insects and *Cupiennius* argues in favor of a common ancestral process of nerve cell specification in the arthropod clade.

Zusammenfassung

Das Ziel der vorliegenden Studie ist es die Evolution der Neurogenese in verschiedenen Arthropodengruppen besser zu verstehen. Um Einblicke in die Evolution der Entwicklung von Nervensystemen der Arthropoden zu gewinnen wird die Spinne *Cupiennius salei* als komparatives Model zu Insekten benutzt. Die Expressionsmuster von Spinnenhomologen welche eine Funktion in der Entwicklung des Nervensystem der Insekten haben, werden analysiert. Die Gemeinsamkeiten und Unterschiede dieser Expressionmuster werden in Arthropodengruppen verglichen die weitläufig miteinander verwandt sind.

Ein Spinnenhomolog des Genes *even skipped*, *Cupiennius even skipped*, und zwei Spinnenhomologe des Genes *runt, Cupiennius runt 1* und *runt 2*, wurden kloniert. Außerdem wurde ein Spinnenhomolog des Genes *prospero*, *Cupiennius prospero* kloniert. Die Expression von Proteinen und anschließende Immunisierungsexperimente resultierten in polyklonalen Antikörpern gegen *Cupiennius* Snail, Krüppel-1 und Prospero.

Die Resultate für *runt 1* und *even skipped* ergeben daß beide Gene eine Rolle in der Spinnenneurogenese haben während Runt 2 im peripheren Nervensystem expimiert wird.

Bezüglich der Entstehung des Nervensystems der Spinne existieren im Vergleich zu den Insekten essentielle Unterschiede. Es wurden unterschiedliche Experimente durchgeführt um mitotisch aktive Zellen innerhalb des sich entwickelnden zentralen Nervensystems zu lokalisieren. In Zellschichten unterhalb des Neuroektoderms sind nur wenige Zellen mitotisch aktiv. Die Resultate zeigen daß in *Cupiennius* keine mitotisch aktiven Neuroblasten und Ganglionmutterzellen wie in Insekten existieren. Stattdessen werden Zellgruppen direkt an der apikalen Oberfläche des Neuroektoderms rekrutiert. Diese Zellen bewegen sich nach basal und bleiben über einen cytoplasmatischen Fortsatz, der später zurückgeszogen wird, mit der apikalen Oberfläche des Neuroektoderms verbunden. Dieser Prozeß wird als Invagination bezeichnet.

Snail wird in invaginiernden Zellen exprimiert.. Die Experimente deuten darauf hin daß die Expression von Snail vor der Invagination beginnt, und bendet wird bevor die invaginierenden Zellen ihre cytoplasmatischen Fortsätze von der apikalen Oberfläche des Neuroektoderms zurückziehen. Die Expression von Snail wurde auch in Zellen entdeckt die mit mechanosensitiven Haaren des peripheren Nervensystems assoziiert sind.

Auch Prospero wird in invaginierenden Zellen exprimiert. Außerdem wird Prospero in Zellen die komplett invaginiert sind und in Nervenzellen unterschiedlich stark exprimiert. Die Resultate deuten darauf hin daß Prospero exprimierende Zellen invaginieren und sich in die Richtung des sich entwickelnden Neuropils bewegen. Am Neuropil differenzieren sie zu Nervenzellen aus. Es existieren mitotisch aktive Zellen die unterhalb des Neuroektoderms lokalisierte sind und Prospero exprimieren. Es gibt keinen Beweis für die asymetrische Lokalisation von Prospero. Stattdessen verteilt sich Prospero Protein symetrisch auf beide Tochterzellen während der Mitose.

Krüppel-1 wird in vielen Zellen des Neuroektoderms und der basalen Zellschichten des sich entwickelnden zentralen Nervensystems exprimiert. Krüppel-1 wird vorrübergehend verstärkt in invaginierenden Zellen exprimiert. Diese verstärkte Expression weist eine dynamische zeitliche Veränderung entlang der anterioren-posterioren Achse auf. Außerdem existiert an den Invaginationsstellen eine Zeitverzögerung zwischen der starken Transkription und der Translation des Proteins.

Snail und Prospero werden unterschiedlich stark in invaginierenden Zellen exprimiert. Die Expression von Prospero beginnt später und setzt sich in Zellen die komplett invaginiert sind kontinuierlich fort. Dies deutet auf eine abgestufte Differenzierung der invaginierenden Zellen hin. Es gibt in *Cupiennius* eine vergleichbare Reihenfolge der Expression von Snail und Prospero wie in *Drosophila*. Außerdem werden in beiden Arthropodengruppen die Snail und Krüppelhomologe in vergleichbaren Geweben des Neuroektoderms exprimiert. Die Resultate weisen darauf hin daß die Spinnenhomologe von Prospero, Snail und Krüppel warscheinlich eine Rolle bei der Spezifizierung von Nervenzellen spielen. Der Vergleich der Expressionmuster der Genhomologe von Snail, Prospero und Krüppel zwischen *Cupiennius* und Insekten führt zu dem Schluß, daß in einem gemeinsamen Vorfahren der Arthropoden ein ursprünglicher Prozess bei der Spezifizierung von Nervenzellen schon vorhanden war.

Abbreviations and nomenclature

ASH	Achaete-scute homolog
BLC	Big Lateral Cluster
bps	base pairs
BrdU	5-Bromo-2`-deoxy-uridine
cDNA	complementary mRNA
CNS	Central Nervous System
DNA	Deoxyribonucleicacid
En	Engrailed-1
Eve	Even skipped
GMC	Ganglion Mother Cell
GST	Glutathion-S-Transferase
Kr	Krüppel-1
min.	Minutes
PCR	Polymerase chain reaction
PNS	Peripherous nervous system
Pros	Prospero
RNA	Ribonucleicacid
Sna	Snail

Names corresponding to genes are written in italics. Rezessive genes are written in small letters and dominant genes in capitals. DNA, RNA and proteins of all species are written normal and in capitals.

Introduction

1. Comparison of early steps in neurogenesis of different arthropods

Evolution produced a magnificant variety of nervous systems in the different arthropod groups (Whitington, 1995). Despite their differences, all arthropod nervous sytems share some general morphological features which are thought to be inherited from a common annelid-like ancestor (Gruner 1993; Nielson, 1995;). To gain detailed insights into the evolution of nervous systems it is necessary to study their development in different arthropods.

Within arthropods our knowledge of neurogenesis is largely restricted to insects. The organisms which have been studied most thoroughly are locusts (Bate 1976; Bate and Grunewald, 1981) and the fruit fly *Drosphila melanogaster* (Hartenstein and Campos-Ortega 1984; Doe 1985). In *Drosophila* neurogenesis of the CNS (Central Nervous System) starts with the formation of proneural clusters in the neuroectoderm. The competence of cell clusters to attain a neural fate depends on the expression of proneural genes (Cabrera et al., 1987, Romani et al., 1989; Jimènez and Campos-Ortega 1990; Martin-Bermudo et al., 1991; Skeath and Caroll, 1992). In proneural cell clusters the expression of proneural genes becomes restricted to a single cell. This process of lateral inhibition is mediated by the neurogenic genes *Notch* and *Delta* (Lehmann et al., 1981; Lehmann et al., 1983).

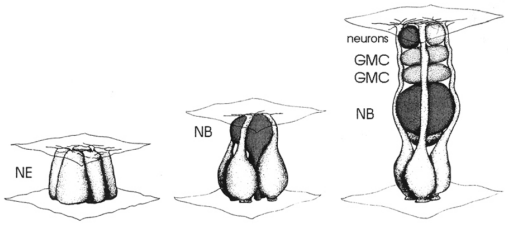


Fig. 1 Neuroblast delamination and differentiation. (From: Doe et al., 1998). Neuroblasts are formed from a cluster of neuroectodermal cells (NE). The neuroblast enlarges and delaminates towards the interior of the embryo. Asymmetric divisions of neuroblast stem cells give rise to ganglion mother cells (GMC`s), which divide once to produce sibling neurons or neuron/glial cell. Apical is on the top and basal on the bottom of the figure.

About 30 recruited stem cells per hemisegment then start to delaminate and form a two dimensional subepidermal array of so-called neuroblasts (Fig. 1; Doe and Goodman 1985; Doe 1992). Neuroblasts are larger and rounder than sourrounding cells (Doe et al., 1998). They are furthermore characterized by their capacity to divide asymmetrically, and give rise to smaller ganglion mother cells (GMC´s). The fate of each neuroblast is determined shortly after its enlargement and depends on the position of delamination (Doe and Goodman 1985; Doe et al., 1998). Every neuroblast expresses a different set of genes which specify the GMC cell fates (Broadus et al., 1995) The expression of different sets of molecular markers allows the identification of each neuroblast (Fig. 2).

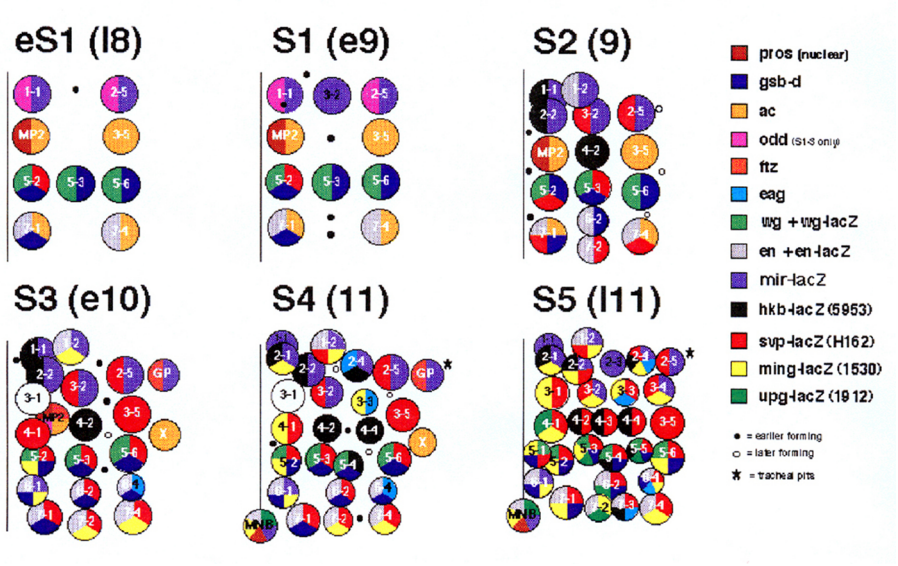


Fig. 2 Summary of molecular markers expressed in neuroblasts (From: Broadus et al., 1995). The expression of 15 molecular markers is indicated in different colors. Achaete (ac), Engrailed (en), Wingless (wg), Gooseberry-Distal (gsb-d), and Odd-Skipped (odd) represent protein patterns: Prospero (pros) represents nuclear protein localisation; mirror-lacZ (mir-lacZ), engrailed-lacZ (en-lacZ), fushi tarazu-lacZ (ftz-lacZ), seven up-lacZ (svp-lacZ), ming-lacZ, huckebein-lacZ (hkb-lacZ), wingless-lacZ (wg-lacZ), and unplugged-lacZ (upg-lacZ) represent ß-gal patterns; eagle (eag) is an RNA pattern. Early S1 NB´s at late stage 8 (eS1 (l8)). S1 NB`s at early stage 9 (S1 (e9). S2 NB´s at stage 9 (S2 (9)). S3 NB´s at stage 10 (S3 (e10)). S4 NB`s at stage 11 (S4 (11)). S5 NB`s at late stage 11 (S5 (L11)). Anterior, top; ventral midline, line; large circles, NB`s; small black spots, sites of NB formation at the next stage. Map and subsequent updates can be accessed over the internet at: http:// www.neuro.uoregon.edu/doelab//nbintro.html.

Neuroblasts delaminate in different developmental stages and are organized in columns and rows.

The invariant stem cell lineage of each neuroblast gives rise to a defined number of GMC´s which divide once more to produce characteristic pairs of neurons (Doe and Goodman, 1985). Early developing neurons (pioneer neurons) take a stereotypic route, which is determined by cues from the neuroepithelium and interaction of growth cones during their differentiation, and pioneer the axon pathways (Fig.3; Thomas et al., 1984).

The growth cones of later developing neurons follow the early developing axons. Cell body positions and axon outgrowths of several early developing neurons were found to be conserved between different insect species (Thomas et al., 1984; Whitington et al., 1996; Whitington and Bacon, 1998).

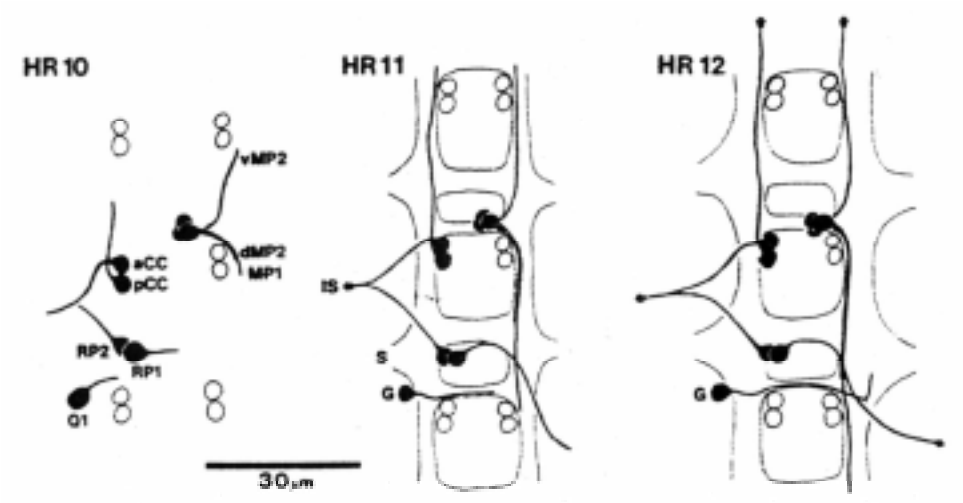


Fig. 3 Developmental time course of early developing neurons in *Drosophila*. (From: Thomas et al., 1984). Three contiguous segments are shown, marked by aCC and pCC cell bodies (open cell bodies). The first two axon fascicles in each of the longitudinal connectives are established by MP1, dMP1, pCC and vMP2. Cell Q1 (at 10 h) crosses the segment in what will become the posterior commisure; The G neurone crosses the segment between 11 and 12 h, grows past both the vMP2 fascicle and the MP1/dMP2 fascicle and selectively fasciculates with a more lateral bundle (The A/P fascicle) running within the connctive. Intersegmental nerve (IS); Segmental nerve (S).

Numbers and positions of neuroblasts are widely identical in different insect species (Bate, 1976; Doe and Goodman, 1985; Doe, 1992; Schmidt et. al., 1997).

A comparison between *Schistocerca* and a silverfish, which is a primary wingless insect, revealed that even in these distantly related species the neuroblast pattern is conserved (Truman et al., 1998). Differences were found in the relative positions and the timing of gene expression in neuroblasts between *Schistocerca* and *Drosophila* (Broadus and Doe, 1995).

Variable numbers of nerve cells between insect species and also in different segments of one species are mainly determined by different prolifertion rates of homologous neuroblasts (Truman et al., 1998).

The sister group of insects is not the myriapods, as was originally assumed, but the crustaceans (Friedrich und Tautz, 1995). In crustaceans, like in insects, basally located ganglion mother cells arise by the asymetric division of neuroblasts (Gerberding, 1997).

But in contrast to insects in crustaceans neuroblasts do not delaminate, and are not associated with specialised sheath cells as they are in insects (Scholtz, 1992; Doe and Goodman, 1985). Neuroblasts can also switch from a neuronal back to an epidermal cell fate (Dohle and Scholz, 1988). Comparative studies revealed that the segment polarity gene *engrailed* labels a similar set of NB´s in different crustacean species as in insects (Patel et al., 1989; Duman-Scheel and Patel, 1999). Furthermore similarities in axon growth, cell body positions, and expression of *engrailed* and the pair rule gene *even-skipped* in comparable subsets of pioneer neurons present strong arguments for homology and a common Bauplan of the CNS in both groups (Thomas et al., 1984, Whitington et al., 1993; Duman-Scheel and Patel, 1999). Comparison of pioneer neurons of different crustacean species with those seen in the silverfish *Ctenolepsima longicaudata* revealed differences in pathways taken by axons of homologous neurons and timing of axogenesis (Whitington, et al., 1996). But these differences are comparable in extent to those seen between winged insects and the primary wingless silverfish, providing further support for a common ancestral developmental program of the CNS in insects and crustaceans (Whitington, 1995; Whitington and Bacon, 1998).

Myriapods are probably a sister group of chelicerates (Friedrich and Tautz, 1995; Hwang et al., 2001) and more distantly related to crustaceans and insects (Whitington, 1995; Dohle, 2001). In contrast to insects and crustaceans myriapods do not have neuroblasts. Cells, which are produced by randomly oriented mitotic divisions in the neuroectoderm, invaginate in a dorsal direction (Whitington et al., 1991; Anderson, 1973).

Pioneer neurons do not form segmentally like in insects and crustaceans but in the brain from where they send out axons to the posterior body parts (Whitington et al., 1991).

In chelicerates no neuroblasts are found (Anderson 1973; Stollewerk et al., 2001). Previous assumptions that neuroblasts are present in different chelicerates (Winter, 1980; Mathew, 1956; Yoshikura, 1955) could be due to technical limitations at the time of studies. Neurogenesis by inward proliferation of neuroectodermal cells was observed in different chelicerate species. In the spider *Cupiennius salei* cells are recruited at the apical surface of the neuroectoderm and invaginate as cell clusters (Stollewerk et al., 2001). Interestingly, around 30 invagination sites, which show a stereotypic segmentally repeated pattern, are found in different spider species.

This number resembles the number of delaminating neuroblasts found in insects (Doe, 1992). Delamination and invagination are related processes. In both cases cell nuclei move basally whereas a cytoplasmatic extension remains connected to the apical surface. Later this extension is retracted. Furthermore, in the spider *Cupiennius salei* a pronneural gene, *CsASH1*, with high similarity to the genes of the acheate–scute complex of *Drosophila* was found to be involved in the recruitment of stem cells for invagination (Stollewerk et al., 2001). A second proneural gene, *CsASH2* was found to provide cells with a neuronal fate. Thus, apart from the lack of neuroblasts principal functions of pronneural genes are conserved between chelicerates and insects.

2. Expression of *snail* in *Drosophila*

In *Drosophila melanogaster* every neuroblast is provided with positional information and expresses a different set of genes which specify the cell fate (Doe, 1992). Some of these genes, so called pan-neural genes, are expressed in all neuroblasts (Bier et al., 1992; Roark et al., 1995). Snail and Worniu, two closely related zinc finger proteins have a pan-neural mode of expression in delaminating neuroblasts and show high functional redundancy whereas *escargot*, a third member of the snail family genes, has a more collaborative function (Ashraf et al., 1999; Manzanares et al., 2001). Early expression of *snail* is independent of proneural gene regulation (Ip et al. 1994). Embryos where all three genes are deleted show a severe phenotype of disrupted connectives and commisures.

Expression of marker genes in the developing CNS, such as *even-skipped* (eve), are absent or, as in the case of *fushi tarazu* (ftz), almost abolished (Ashraf et al., 1999).

Asymmetric localisation of Prospero protein and RNA are absent (Ashraf and Ip, 2001). Results show that *snail* together with *worniu* and *escargot* control two distinct mechanisms which mediate asymmetric segregation of determinants from neuroblasts into ganglion mother cells and the apical–basal spindle orientation during mitotic divisions. (Ashraf and Ip, 2001; Cai et al., 2001). One mechanism depends on *inscuteable* whereas the other one is *inscuteable*-independent (Kraut et al., 1996; Schober et al., 1999; Wodarz et al., 1999; Peng et al., 2000). *Drosophila snail* also plays a role in mesoderm formation (Leptin, 1991).

Neuroectodermal genes are repressed by Snail in the mesoderm, and so the boundaries between the two cell layers are defined by *snail* expression (Kosman et al., 1991; Ip et al., 1992). Furthermore *snail* expression was found in the midgut rudiment, the amnioproctodeum and the primordium of the Malphigian tubules (Alberga, et al., 1991).

3. Expression of the gap gene *Krüppel* in *Drosophila*

In *Drosophila* embryos the gap gene *Krüppel* is expressed in a central region of the syncytial blastoderm and later in a posterior cap of the cellular blastoderm (Knipple et al., 1985; Gaul et al., 1987). Expression of *Krüppel* plays a role in establishing thoracic and anterior abdominal segments, respectively, and the Malphigian tubules (Hoch et al., 1994; Wieschhaus et al., 1984). In *Drosophila* stage 8 embryos Krüppel is expressed along the entire neuroectoderm and persists until late stage 11 (Romani, et al., 1996). It is expressed in all neuroblasts with variable levels in a heterogenous spatio- temporal pattern. Krüppel is also found in a large fraction of the GMC`s and their neuronal and glial progeny where it persists until the end of embryogenesis. The *Krüppel* phenotype shows aberrant misrouting of axons in the commissures and connectives of the CNS, and defects in the specification of serotonin positive neurons, glial cells and alterations in the number of Gooseberry expressing cells (Romani, et al., 1996).

4. Expression of *prospero* in *Drosophila* and the homologous *prox-1* gene in vertebrates

The *prospero* gene has a pan-neural mode of expression in the developing central nervous system (CNS) of *Drosophila* (Hassan et al., 2000; Li and Vaessin, 2000). Prospero is expressed in all neuroblasts (Vaessin et al., 1991; Matsuzaki et al., 1992).

In neuroblasts Prospero protein is cytoplasmatic and does not enter cell nuclei (Vaessin et al., 1991; Matsuzaki et al., 1992) Prospero is localized at the basal cell cortex during mitosis and segregates asymmetrically into the GMC`s where it enters cell nuclei (Vaessin et al., 1991; Hirata et al., 1995; Spana and Doe, 1995; Matsuzaki et al., 1998; Srinivasan et al., 1998). In contrast to all the other neuroblasts in the unpaired midline neuroblast (MNB) and in the MP2, Prospero is localised in the nucleus from the beginning of NB formation (Broadus and Doe, 1995; Spana and Doe, 1995).

In the GMC`s *prospero* is also expressed (Doe et al., 1991; Chu-Lagraff et al., 1991; Matsuzaki et al., 1992; Spana and Doe, 1995). During mitotic divisions of the MP2 and the GMC`s Prospero is not localized cortically, but instead fills the cell, and is segregated symmetrically into the neurons where it remains expressed transiently (Broadus and Doe, 1995; Spana and Doe, 1995). Prospero is also localized in the nuclei of longitudinal glia and a lateral cluster of cells (Doe et al., 1991; Campbell et al., 1994).

The asymmetric localisation of Prospero and Staufen protein to the basal cell cortex of neuroblasts requires Miranda (Matsuzaki et al., 1992). Staufen mediates the asymmetric localisation and segregation of Prospero mRNA (Li et al., 1997; Broadus et al., 1998). Both processes, the asymmetric localisation and segregation of Prospero protein and mRNA, depend on the function of *inscuteable* (Kraut et al., 1996; Li et al., 1997)

Prospero is also expressed in the peripheral nervous system (PNS). It is expressed in the sensory organ precusor (SOP) and segregates asymmetrically into the IIb cell (Knoblich et al., 1995; Spana and Doe, 1995). After mitotic division of the IIb cell, it is transiently detected in the neuron and persists in the sheath cell.

Prospero loss of function results in severe misrouting of axons in the CNS and altered expression of several genes. Expression of *fushi tarazu* and *even-skipped* is reduced in GMC´s, absent in the CQ, aCC, pCC and RP2 pioneer neurons, and the number of *engrailed* expressing cells is increased (Doe et al., 1991).

The aCC and pCC neurons have an abnormal axon outgrowth and longitudonal connectives are absent (Doe et al., 1991). There is no outgrowth of motorneurons from the developing CNS and the progressive restriction of the pan-neural genes *deadpan* and *asense* is missing in *prospero* mutant embryos (Vaessin et al., 1991).

Prospero also upregulates the glia cell missing (gcm) gene and induces glial development (Akiyama-Oda et al., 1999; Akiyama-Oda, et al., 2000; Freeman and Doe, 2001). Similar to the CNS, *prospero* loss of function mutant embryos have axon pathfinding defects in the PNS, but the sensory organ identity is not altered (Doe et al., 1991; Vaessin et al., 1991).

These results suggest that Prospero acts as a factor for gene activation and repression, and that it is required for the correct specification of neural cell fates (Doe et al., 1991; Vaessin et al., 1991). In vitro experiments suggest that Prospero modulates the DNA binding activity of homeodomain proteins such as Even-skipped and Deformed (Hassan et al., 1997).

Prospero is also expressed in pair-rule stripes at the cellular blastoderm stage but the expression of several segmentation genes is normal in *prospero* mutant embryos (Doe et al., 1991).

Prox-1 homologs of zebrafish, chicken, mouse, human and the Prospero homolog of *Caenorhabditis elegans* share an atypical homeodomain with *Drosophila* Prospero (Oliver, et al., 1993; Bürglin, 1994; Tomarev et al., 1996; Zinovieva et al., 1996; Glasgow et al., 1998). In comparison to typical homeodomain proteins the Prospero homeodomain displays variations at the primary sequence level mainly in the helices 1 and 2, whereas the helix 3 has more conserved residues (Chu-Lagraff, et al., 1991). Furthermore, Prospero homeodomains have an insertion of three amino acids between helix 2 and 3 (Chu-Lagraff, et al., 1991). All *prospero* genes share a novel domain, the so called Prospero domain, localized between the homeodomain and the carboxyl terminus (Bürglin, 1994).

In vertebrates the *prox-1* gene is a neural-specific transcription factor, (Oliver, et al., 1993; Tomarev et al., 1996; Glasgow et al., 1998; Torii, et al., 1999) but asymmetric distribution of Prox-1 is not mentioned.

5. Expression of *even-skipped*, *runt* and *engrailed* genes during insect and crustacean neurogenesis

In *Drosophila* most of the segmentation genes are expressed during the development of the nervous system and determine differentiation of GMC`s and nerve cells (Doe 1988a,b; Patel et al., 1989a,b; Kania et al., 1990).

The best studied molecular markers of the developing CNS in different arthropod groups in comparative analyses are Even-skipped and Engrailed (Patel et al., 1989a,b; Duman-Scheel and Patel, 1999).

Even-skipped was characterized as a homeobox containing pair rule gene which is also expressed in the developing CNS of *Drosophila* (Frasch et al., 1987).

Furthermore it was the first molecular marker used to trace cell lineages from identified GMC`s, corresponding to defined neuroblasts, and to identified neurons (Doe, 1992). The described aCC and pCC neurons are Even-skipped positive (Thomas et al., 1984; Doe et al., 1988a,b; Patel et al., 1989c) and develop from the Even-skipped expressing GMC1-1a (Doe, 1992). The Even-skipped positive motoneuron RP2 (Thomas et al., 1984; Patel et al., 1989c) develops from the Even-skipped expressing GMC 4-2a (Doe, 1992). The 6-cell U/CQ group and the corresponding GMC`s also express Even-skipped (Doe, 1992; Patel et al., 1989c) and an Even-skipped expressing latereral cluster of so called EL neurons is present (Broadus, et al., 1995). It was shown that temperature sensitive inactivation of Even-skipped protein during neurogenesis alters the outgrowth of axons in neurons which express Even-skipped in the wildtype (Doe et al., 1988b). So *even-skipped* is important for the determination of the final fate of nerve cells. Even-skipped expression was shown to be conserved between *Schistocerca* and *Drosophila* (Patel et al., 1992). Furthermore, neurons which were found to be identical between insects and crustaceans on the basis of morphological criteria (Thomas et al., 1984; Whitington et al., 1993) were also found to express Even-skipped in both arthropod groups (Duman-Scheel and Patel, 1999).

The segment polarity gene *engrailed* is expressed posteriorly in all row 6 and 7 neuroblasts, the anterior neuroblast 1-2, and the midline neuroblast of Drosophila (Broadus et al., 1995). Engrailed is also expressed in subsets of neurons (Cui and Doe, 1992).

It was shown that Engrailed controls the glial/neuronal cell fate decision (Condron et al., 1994) and that it interacts with *patched*, *gooseberry* and *wingless* (Bhat and Schedl, 1997) during neurogenesis. The pattern of Engrailed expression is conserved between different species of insects and crustaceans (Duman-Scheel and Patel, 1999; Patel et al., 1989a,b).

The *Drosophila* pair-rule gene *runt* is expressed in the developing CNS and PNS (Kania et al., 1990; Duffy et al., 1991; Dormand and Brand, 1998).

All *runt* genes contain a conserved sequence of 128 amino acids, the Runt-domain, which is responsible for the DNA binding properties and heterodimerization of the protein, and identifies a family of transcriptional regulators (Kagoshima et al., 1993; Tsai and Gergen, 1994). Runt is expressed in subsets of neuroblasts, GMC`s and neurons in the developing CNS (Kania et al., 1990; Duffy et al., 1991; Dormand and Brand; 1998).

The Even-skipped expressing EL and CQ-neurons and their corresponding Even-skipped expressing GMC`s also express Runt (Duffy et al., 1991). In addition Runt is expressed in the MP1 neuron and the midline glia, and its activity in neuroblasts was found to be obligatory for the expression of Even-skipped in the EL neurons (Dormand and Brand, 1998). The expression of *runt* in the developing CNS was not yet compared between different arthropod species and it remains unclear if *runt* plays a role in neurogenesis in arthropod groups other than insects.

6. The aim of the present work

To understand the evolution of nervous systems in different phyla and their relationships to each other it is necessary to understand their development in distantly related arthropods. Chelicerates form a basal group within the arthropod clade. Studying spider neurogenesis provides insights into a comparable basal mode of development within the arthropods.

Expression patterns of pan-neural genes between different arthropod phyla have never been compared. Especially this group of genes, with its universal mode of expression in neuroblasts, could allow the identification of cells which give raise to neurons in the developing CNS of distantly related arthropods.

Furthermore, genes which are expressed in neurons of insects and crustaceans can be used as molecular markers to determine if expression of corresponding spider homologs is present in identical cells. The experiments performed focus mainly on the following questions: In which cell populations are the genes expressed? Does a cell genealogy comparable to insect neurogenesis exist in the spider? Which characteristics of gene expression are conserved between spiders and other arthropod groups?

Material and methods

1. Molecular methods and data processing of obtained sequences

Initial PCR experiments with degenerate primers were based on the alignment of known amino acid sequences for *even-skipped, runt,* and *prospero* genes. The data were obtained from the NCBI database of the National institute of health, U.S.A. (NIH), and processed with the programs „Gene Jockey“ and „DNASIS“. A cDNA library was used as template for initial PCR`s. The cDNA`s were made by reverse transcription of RNA from *Cupiennius salei* spider embryos of different developmental stages. For subsequent RACE-PCR eperiments cDNA libraries with linkers at the 5`and 3`prime ends were generated as described for „Marathon cDNA-Amplification kit“ (Fa. Clontech). Sequences obtained from the initial PCR experiments served as templates for RACE-PCR`s. Amplified DNA`s were cloned into the vector p-Zero (Fa. Invitrogen). Sequencing was performed with an ABI 377XL (Fa. Applied biosystems). Obtained sequences were analysed with the data processing programs mentioned above, and also with „Sequence navigator“. To compare obtained sequences with genes which were present in the NCBI database the programs „BLASTn“ and „BLASTX“ were used. „DNASIS“ also was used to determine the expected molecular weights of the GST-fusion proteins (see below). The obtained sequences for Even skipped, Runt-1 and Runt-2 are shown in the supplement and are also published (Damen et al., 2000). The sequence accession numbers are AJ252155 for Even skipped, AJ272529 for Runt-1, and AJ272530 for Runt-2. The unpublished sequence of Prospero is shown also in the supplement.

2. Fixation and and preperation of spider embryos

Embryos were dechorionated for about 1 min. with a 50% Klorix solution. After several washes with water, embryos were fixed in a solution of 50% Heptan and 50% PBST (with 0.1 % Tween 20) and 200 µl 37% Formaldehyde. Fixation was always performed at room temperature on a wheel. Embryos were stored at –20°C in Methanol. Embryos fixed and stored like this were used for in situ hybridisation experiments and also hybridisations with subsequent antibody stainings.

A variation was the fixation in 100% Heptan with 200µl 37% Formaldehyde overnight.

This method can be recommended for in situ hybridisation experiments but not for subsequent antibody stainings. Antibody staining experiments with embryos fixed in this way were unsucessful.

Another variation of fixation was performed for pure antibody staining experiments: Embryos were fixed in 100% Heptan with 200µl 37% Formaldehyde for only one hour instead of overnight. Note that for a successful fixation with this method it is necessary to shake the Heptan-Formaldehyde solution vigorously for at least 1 hour. Embryos also have to be mixed more in the fixation solution than in the two other procedures, and so the wheel has to be at a steeper angle. Embryos were then stored in Ethanol instead of Methanol, because the actin cytoskeleton of cells dissociates from cell membranes in the presence of Methanol. Embryos fixed and stored in this way were used exclusively for antibody staining experiments.

3. In situ hybridisations

Fragments of DNA`s, cloned into the vector p-Zero were used for the transcription of Digoxigenin labelled antisense RNA with the „DIG RNA labelling kit“ (Roche). The digoxigenin labelled antisense RNAs were used for in situ hybridisation experiments as described (Damen, et al., 1998). If necessary, after the color reaction of the in situ hybridisation, embryos were transferred into PBS-Triton buffer and a normal antibody staining experiment was performed (see below).

Fluorescent in situ hybridisations were performed with the „HNPP/Fast red detection set“ (Roche) and the „Alkaline phosphates substrate kit“ (Fa. Vektor).

4. Constructs for protein expression

To express proteins for immunization, DNA fragments were cloned into the Sma I restriction site of the expression vector pGEX-2T (Provided by Ruth Grosskortenhaus; Originally from Pharmacia). Digestion with Sma I produces blund ends. The pGEX-2T expression vector has the lac I promotor and the lac I^q repressor. The desired gene fragment is cloned in frame with the open reading frame of GST (Glutathione–S-Transferase). Protein expression is induced with IPTG. The protein is expressed as a GST-fusion protein. The correct reading frame was checked by sequencing.

As all clones with exception of snail consisted of several smaller overlapping race fragments it was necessary to reamplify cDNAs for cloning into the expression vector. The fragment of snail used for cloning into the expression vector was obtained by reamplification of an existing clone.

The following sequences were amplified for in frame cloning: For Snail (provided by Monika Retzlaff; Retzlaff, 1996), bps 9-1191; For Krüppel-1 (Complete sequence provided by Wim Damen; Sommer et al., 1992; Retzlaff, 1996), bps 24-1425; For Engrailed-1 (provided by Wim Damen; Damen et al., 1998), bps 136-822; Two fragments of the Prospero cDNA were amplified for in frame cloning: bps 2106-2655 (Prospero 1) and bps 30-2655 (prospero 2); For Even skipped bps 21-858. The exact sequences cloned for protein expression are shown in the supplement.

The E. coli strain C43 (Provided by Ruth Grosskortenhaus; Miroux et al., 1996) was used for cloning. Sequencing showed that the fragment was inserted the wrong way around into the expression vector in all clones. This may be due to toxic properties of the proteins. The lac repressor (lac I^q) on the expression vector does not repress the promotor totally. So even in the absence of IPTG some protein is expressed. The corresponding bacterial clones grow only slowly. This problem was overcome by the use of the E. coli strain AD494 (Provided by Robert Wilson; Derman et al., 1993) which has a genomic lac I^q repressor. Gained plasmids were checked by sequencing and then recloned into the strain C43.

5. Expression, purification and handling of proteins

As the proteins are expressed as GST fusion proteins they can be purified in a simple way with Glutathion which is bound to a matrix. Experiments have shown that from 1L of bacterial culture between 1-2mg of fusion protein can be purified. Protein purification was done as follows:

A single clone is grown in a 200ml overnight culture at 37°C.

1L LB-medium is added to the 200ml overnight culture. For faster growth of bacterial culture it is recommended to warm up the 1L of LB-medium to 37°C before use.

Ampicillin with a final concentration of 50µg/ml and IPTG with a final concentration of 0.1 mM are added to the 1200ml culture.

The culture starts to grow at OD_{600} 0.2 to 0.5. Growing of the culture is stopped after about 1-2 hours at OD_{600} 0.8 to 1.5.

Bacteria are cooled on ice for 30 min.

One ml bed volume (As the suspension consits of 50% matrix and 50% Ethanol with water so about 2ml of the fresh suspension have a bed volume of 1ml) of Gluthathionresin (Fa. Novagen) can bind about 5mg of protein. For easier handling the use of 0.5-1ml bed volume of GST resin is recommended.

Two volumes of about 500ml bacterial culture are centrifuged at 10.000g. The pellet is resuspended in 40ml PBS, 10mM DTT, and 0.2mg/ml Lysozym. The lysate is cooled on ice for 30 min.

4.5ml 10% Triton X, 450µl 1M $MgCl_2$, and 20µl of DNAse A (Boehringer) are added to the lysate followed by carefull shaking for 40 min. at room temperature.

Alternatively this step of cell lysis can also be performed with the „Bug Buster“ solution (Fa. Novagen) and the corresponding procedure.

Rests of bacterial cells and insoluble proteins are pelletted by centrifugation for 20 min. 16.000g at 4°C.

A purification column (Columns of the „bug buster purification kit“, Fa. Novagen) is loaded with o.5 –1ml bedvolumes of the GST –matrix which is provided as a 50% resin with 20% Ethanol.

The column is equilibrated with 5 bedvolumes of PBS. This and the following steps with the columns are always performed by gravity flow through.

The cell extract is warmed up to room temperature.

The cell extract from the centrifugation step is loaded on the equilibrated column

The column is washed with 10 bedvolumes of PBS.

The washed protein from the column is eluted with 3 bedvolumes of elution buffer (500mM Tris–HCl; 100mM reduced Glutathion; pH 8.0)

An alternative to the use of columns is to bind, wash and elute the protein by batch purification in a 50ml centrifuge tube. This has the advantage that it is faster than the use of columns, but yielded quantities are sometimes lower and the pelletation of the GST resin does not work very well. The protocol is performed as follows:

The cell extract gained by centrifugation at 16.000g is warmed up to room temperature.
The cell extract is incubated by shaking carefully for 30 min. with 0.5-1ml bed volume GST matrix.
The cell extract is pelletted by centrifugation at 500g for 5 min. and washed with 10ml PBS.
Washing and pelletting are repeated two times.
The matrix is incubated with elution buffer (3 bedvolumes of the GST matrix) for 10 min. shaking carefully at room temperature and the centrifuged again.
The matrix can be used several times after stepwise washing with 10 bed volumes of:
1. 50 mM Tris pH 8.0 + 0.5 M $NaCL_2$.
2. 100 mM NaAcetat pH 4.5 + 0.5 M $NaCL_2$
3. 1x Washbuffer (PBS) The matrix is stored in 20% Ethanol at 4°C

This protocol was also used for smaller bacterial cultures of about 100ml to control protein expression. The described protocol was adapted to these lower quantities. In some cases the yielded protein concentrations gained with 100ml cell cultures were very low and invisible on coomassie stained protein gels. This problem was overcomed by the concentration of proteins with „Ultrafree columns (Fa. Millipore). The gained purified proteins were stored in the elution buffer at –20°C and controlled on protein gels. Protein quantities were determined by comparison with defined standards of Albumin. These rough estimates were always oriented on towards the fragment of a protein gel which had the desired molecular weight. For immunization, desired volumes of proteins were lyophilized in a vacuum centrifuge by centrifugation for several hours.

Lyophilised proteins were sended to Fa. Eurogentec (Herstal, Belgium) for immunization. For every boost or immunization of a rabbit about 100µg and for a rat about 25µg of protein were used.

6. Immunization schedule (Fa. Eurogentec)

Immunization, boosting and sampling were performed by Eurogentec (Herstal, Belgium). After the first immunization, animals were boosted 3 times at time intervals of two weeks. Pre-immune sera were taken before the first immunization. Test samples were taken 10 days after boost 2. A large blood sample was taken 10 days after boost 3. Final bleeding occurred 1 month after the last boost. Alternatively animals were boosted again in time intervals of two weeks with large samples taken 10 days after the last boost.

7. Sera and the western blot experiments

Sera sent on dry ice by Eurogentec were stored at -20°C. To avoid repeated thawing and freezing large quantities of sera were stored as aliquots. Sera in use was stored at 4°C with a 0.02% Sodium-Azid solution. Because Sodium-Azide is degraded slowly, after 6 month fresh Sodium-Azid solution was added.

Western blot experiments were performed with a „Trans-Blot SD Semi-dry Transfer cell“ (Fa. BIO RAD). As a power supply the „Power PAC 200“ (Fa. BIO RAD) which has a higher electrical resistance than the „Power PAC 300“was used.

The latter cannot be used for western blot experiments. The blot itself was performed under recommended conditions and with the corresponding blotting buffer. Here is a detailed protocol:

The PVDF membrane and two pieces of blotting paper (extra thick blotting paper from BIO RAD) are cut to the size of 8.5 x 5.5cm for one minigel.

The PVDF membrane is covered with 100% Methanol to reduce the hydrophobizity. Then the PVDF membrane is transfered into the transferbuffer. It is equilibrated in the transferbuffer for 30 min.

The protein gel (Only the resolving gel) is equilibrated for 15 min. in the transferbuffer. Before building the blotting sandwich the two blot papers are also soaked with the transferbuffer

First a blotting paper on the platinanode is placed then the PVDF-membrane, the protein gel and finally again a blotting paper. The air bubbles can be eliminated by rolling a pasteur pipette over every sheet.

The blot is performed at a constant 15V for 20 minutes with 220mA as a limit. (When blotting two minigels only change the limit for the electric flow through: Instead of 220mA put 400mA)

After blotting, the PVDF membrane is dried at room temperature until it is totally white. Drying has the purpose of fixing the protein on the membrane which then cannot be washed away. Dry membranes can also be stored for at least several months. Before transfering a peace of dry membrane into PBS buffer or another solution it must be rehydrated in 100% methanol.

The membrane can be signed with a pen and is cut in small stripes of about 0.5cm for the staining procedures. All following incubation and washing steps are performed on a shaker. Incubation steps are performed by shaking very slowly, whereas washing steps are performed by vigorous shaking.

If several proteins were blotted with one gel and it is important to know the relative positions or if it is not clearly which side of the membrane the protein was blotted on, one fast possibility to find this out is the staining with ponceau-S-solution. This staining is reversible. A rehydrated stripe of PVDF membrane is incubated for at least 15 minutes in a 0.1% ponceau-S-solution, and rinsed briefly with PBS and air dried.

For the following staining procedure a rack with 10 chambers for the single stripes is used. It is advantagous to put the stripes with the side where the protein was blotted on to the top. Every chamber is 8cm large, 1cm wide, and 0.9cm deep. 3ml buffer is sufficient to cover one stripe in one chamber Before incubation with diluted sera or the antibody against GST the stripes are blocked in a PBS solution with 5% fat free milk powder for at least 30 minutes.

The incubation of diluted sera or the anti GST antibody is also performed in this solution of PBS with 5% fat free milk powder overnight at 4°C shaking slowly.

After removing the incubation solutions, the stripes are washed 3 times with PBST (PBS with 0.1% Tween 20) for 15 minutes by vigorous shaking.

The secondary alkaline phosphatase conjugated antibody is diluted 1:1000 in PBS and incubated for 2 hours at room temperature. After this incubation step the stripes are washed as before in PBST. Then the membranes are washed for few minutes in the staining solution (2.5ml 1M $MgCl_2$; 1.0ml 5M $NaCl_2$; 5ml 1M Tris pH 9.5; in 50ml $water_{bidest}$)

Staining of stripes is performed in a petri dish in 10ml staining solution with 45µl NBT and 35µl X-Phosphate. Note that for comparisons the staining times of corresponding stripes should be identical. To finish the staining reaction the stripes are rinsed in PBS and air dried.

The transferbuffer consists of 48mM Tris; 39mM Glycin; 20% Methanol; 3.75ml 10% SDS (1.3mM) in 1000ml $water_{bidest}$. The buffer should have a pH between 9.0- 9.4. Never adjust the pH! This changes the electric flow through.

8. Antibody stainings

For antibody staining experiments the sera and primary antibodies against phosphorylated Histon 3 were diluted 1:500. The Cy-5 and Cy-3 conjugated secondary antibodies were diluted 1:1000. Secondary antibodies were stored in aliquots in the dark at –20°C. Yoyo-1 was diluted 1:2000 for stainings of DNA. Sera and primary antibodies were incubated in volumes of 2ml. For Phalloidine-rhodamine stainings one batch of the substance (Fa. Mobitec) was dissolved in 1.5ml Methanol and stored as 20µl aliquots at –20°C. For usage the methanol of one aliquot was evaporated in a speed vac and dissolved in 500µl PBST (PBS with 0.1% Triton X). Secondary antibodies and Yoyo-1 dilutions were adapted to these volumes of 500µl and incubated in the same batch. All incubations were performed on a wheel in the dark overnight at 4°C. Washing steps were also performed on a wheel but at room temperature. For washing, batches of 2ml were used with a wheel at a steeper angle than in the case of incubations. Washing was performed in a time interval for at least 2– 4 hours. The PBST buffer was changed after every 30 minutes.

Washing was performed after incubation with primary antibodies and after incubation with secondary antibodies plus fluorescent dyes.

To avoid fading of fluorescent dyes and antibodies the embryos were prepared immediately after washing using a binocular microscope. The germ layers were dissected from yolk with two very fine paint brushes in PBST with 50% Glycerol. After several transfers into fresh PBST with 50% Glycerol to wash the yolk away, the germ layers were put on a slide. The cover slips were fixed with nailpolish. Stainings were analyzed on a laser scanning microscope (LSM).

Non-fluorescent antibody staining procedures were identical to those described for fluorescent secondary antibodies. To develop the signal secondary antibodies conjugated with alkaline phosphatase were used. Levamisol was added to the staining buffer (5ml 1M Tris pH9.5; 2.5ml 1M $MgCl_2$; 1ml 5M $NaCl_2$; and 0.5ml 10% Tween 20 in 50ml $water_{bidest}$) to a final concentration of 25mM. Levamisol blocks the activity of endogenous alkaline phosphatases (It does not block the activity of intestinal alkaline phosphatases) which can cause extreme backround levels in antibody staining experiments with spider embryos. Analyses were performed on a light microscope.

9. Labeling experiments with BrdU

Embryos were injected with BrdU like previously described for RNAi (Schoppmeier and Damen, 2001). To detect the labeled cells an BrdU labeling kit (Fa. Roche) was used. To detect the primary monoclonal antibody, mouse-Ig-anti BrdU, the protocol was modified. A Cy3 conjugated antibody goat anti Ig-mouse was used (Dianova no.:115-165-164). The staining with this secondary antibody was performed like normal (see antibody stainings).

Results

1. Expression of GST fusion proteins and control of expressed proteins and corresponding antibodies

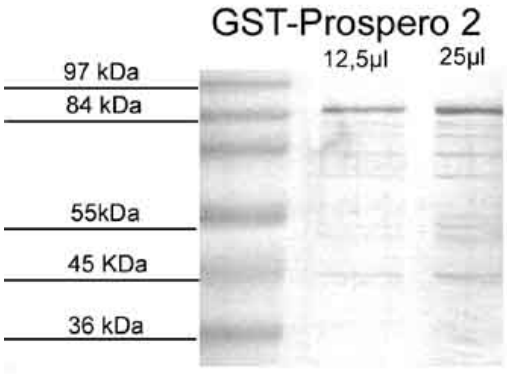
After cloning DNA fragments in frame into the expression vector pGEX-2T, it was possible to express and purify the GST-fusion proteins for immunization. As a control of the expressed proteins and the corresponding antibodies western blot experiments were performed (see Material and Methods).

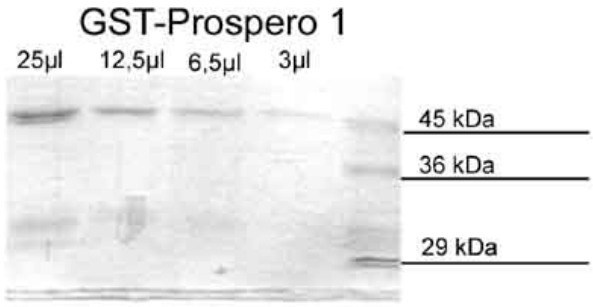
1.1. Protein expression

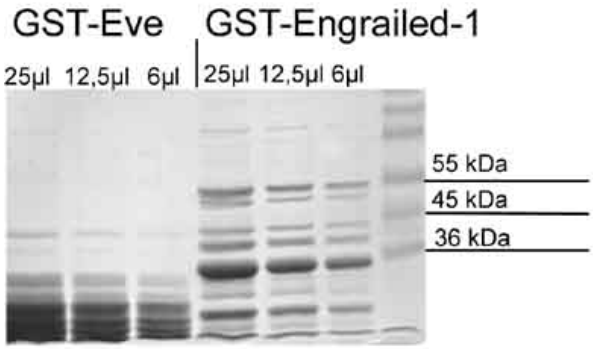
For every construct the expression and purification of proteins results in fragments of different molecular weights (Fig. 4). For the *prospero* gene two constructs were made: the smaller one is called Prospero 1 and the larger one is called Prospero 2 (see material and methods; constructs for protein expression). While for the Prospero 1 and Engrailed-1 GST-fusion protein fragments of the expected molecular weights of about 48 and 54 kDa seem to be present (Fig. 4; GST-Engrailed-1, GST-Prospero 1), for Prospero-2 and Even skipped this is obviously not the case. The biggest fragments between 36 and 45 kDa for Even skipped (Fig. 4; GST-Eve) and between 84 and 97 kDa for Prospero 2 (Fig. 4; GST-Prospero 2) do not correspond to the expected molecular weights of about 58 kDa for Even skipped and 124 kDa for Prospero 2. The GST-Krüppel-1 fusion protein is expected to have a molecular weight of about 78 kDa. The GST-Snail fusion protein is expected to have a molecular weight of about 70 kDa. In the case of the GST fusion proteins for Krüppel-1 and Snail the biggest part of purified proteins has an unexpectedly low molecular weight beneath 36 kDa (Fig. 4; GST-Snail, GST-Krüppel-1).

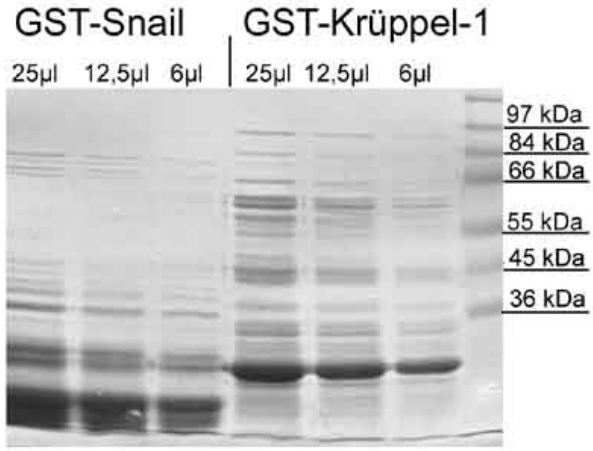
To test if these fragments with unexpected low molecular weights are degraded fragments the purified GST-fusion proteins were blotted and an antibody against GST was probed (Fig. 5a,b). This experiment was performed for all purified proteins with exception of GST-Prospero 2.

Fig. 4 Coomassie stained minigels are shown for the different purified GST fusion proteins as indicated. Different dilution series were loaded as indicated. The sizes of the marker fragments are indicated to the right.


GST-Prospero 2
12,5µl
25µl
97 kDa
84 kDa
55kDa
45 KDa
36 kDa


GST-Prospero 1
25µl
12,5µl
6,5µl
3µl
45 kDa
36 kDa
29 kDa


GST-Eve
GST-Engrailed-1
25µl 12,5µl 6µl
25µl 12,5µl 6µl
55 kDa
45 kDa
36 kDa


GST-Snail
GST-Krüppel-1
25µl 12,5µl 6µl
25µl 12,5µl 6µl
97 kDa
84 kDa
66 kDa
55 kDa
45 kDa
36 kDa

To avoid false positive results unpurified bacterial protein of the same bacterial strain containing the identical expression vector pGEX-2T, without any cloned DNA, was also blotted with the same gel (Fig. 5; Bact. protein). This clone expresses GST alone.

The antibody against GST crossreacts with several bacterial proteins (Fig. 5; Bact. protein). Experiments show that in all purified GST-fusion proteins distinct fragments with different molecular weights which do not have a corresponding fragment within the bacterial proteins exist (Fig. 5). Experiments reveal that for every construct GST-fusion proteins of different molecular weights exist. Probably the proteins are partially degraded by the activity of proteases. Therefore contaminations, due to insufficient purification of GST-fusion proteins, contribute only weakly to the protein patterns on gels.

The expressed proteins were purified and lyophilized. The whole purified proteins were used for immunizations. Isolation of single protein fragments by excision of acrylamide gel slices was performed for GST-Prospero 1. For the first three boosts the gel slices were directly used.

1.2. Control of polyclonal antibodies

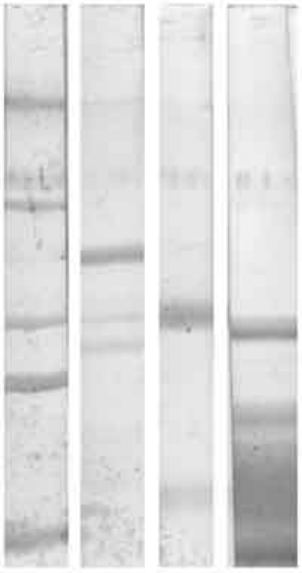
The following experiments all were performed with the antisera and not with purified antibodies. The GST fusion proteins were always purified before use. To control the reactivity of antibodies the purified proteins were blotted.

The antisera were probed and compared with the pre-immunsera (Fig. 6). The results are shown for the antisera against the fusion proteins of GST-Engrailed-1, GST-Snail and GST-Prospero 2 . Results for all probed antisera and pre-immunsera are almost all identical. In all cases the animals show a clear immunological reaction. The antisera show clearly stronger antibody responses to the blotted proteins than the corresponding pre-immunsera. In the case of GST-Prospero 2 for one animal there is also a stronger response of the pre-immunsera with the protein than normal (Fig. 6, animal 2). A comparison with the corresponding antiserum reveals that the reactivity of the pre-immuneserum is still clearly weaker.

To test if the second and subsequent boosts with the same protein improve the antibody responses the different antisera obtained from one animal were compared (Fig. 7).

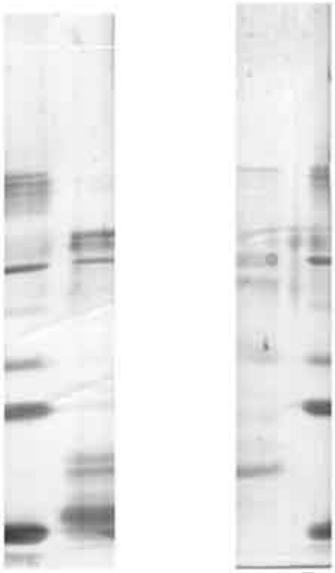
Fig. 5 Western blots of purified GST fusion proteins and unpurified bacterial proteins (containing only the pGEX-2T expression vector). An antibody against GST is probed with different blotted GST fusion proteins and unpurified bacterial proteins. Dilution of the primary antibody against GST: 1: 200. Dilution of the secondary antibody: 1: 1000. Staining time: About 15 minutes.

Probed: Mouse anti GST



Blot: Bact. protein | GST-Eve | GST-En | GST-Pros

Probed: Mouse anti GST



Blot: Bact. protein | GST -Sna | GST -Kr | Bact. protein

For GST-Prospero 1 the antisera of only one animal show stronger antibody responses after boost 4 and 5 (Fig. 7: Animal 1, boost 4,5). The antiserum after the fourth boost shows a weaker response than the antiserum after the fifth boost.

Antisera of earlier boosts and the antisera from the second animal do not show any response at all (Fig. 7; GST-Prospero 1, animal 1, boost 2, 3; Animal 2, boost 2-5).

In the case of GST-Snail, GST-Krüppel-1 and GST-Prospero the antisera of one animal show a stronger antibody response after the third boost than before (Fig. 7; GST-Snail, animal 1; GST-Krüppel-1, animal 2; GST-Prospero 2, animal 2). The antisera of the other three animals do not show a clear improvement of their response in this experiment. No differences were found between the antisera obtained after the last boost and thoose obtained 3 weeks later after the final bleeding (Fig. 7; final bl.).

To compare the affinities of distinct antisera between each other they were probed at different dilutions with the corresponding blotted proteins (Fig. 8). For GST-Prospero 1 and 2, GST-Snail and GST-Krüppel the antiserum of the animal with the strongest response after boost 3 was chosen (see Fig. 7). In the following table the results of Figure 8 are summarized:

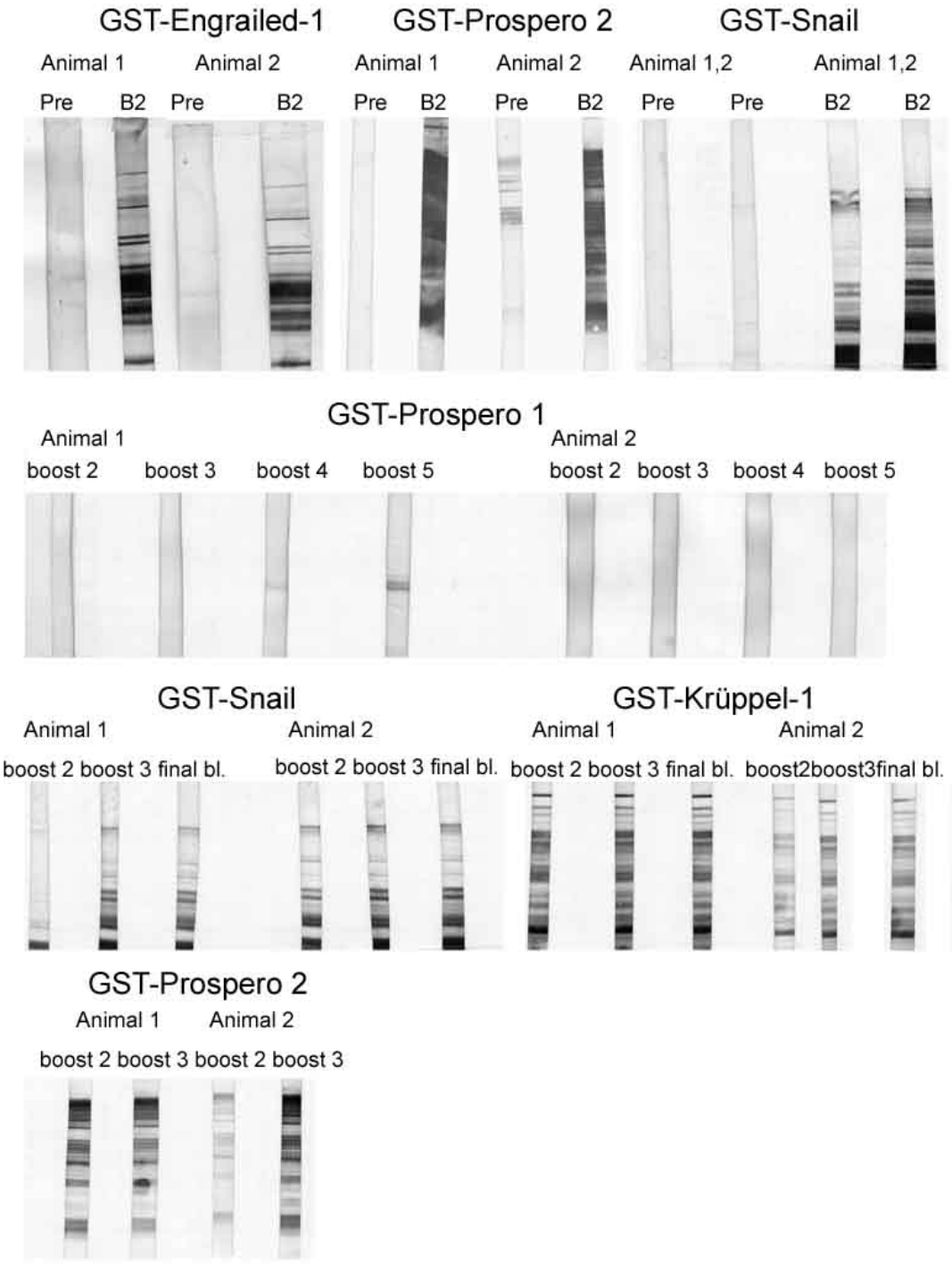
Dilution:	**1:2000**	**1:5000**	**1:10.000**	**1:20.000**	**1:40.000**	**1:100.000**
Prospero 2	++	+++	+++	+++++	+++++	----------
Snail	++	+++	+++	++++	++++	++
Krüppel	++	+++	++	+++	+++	+
Engrailed	++	++	++	++	++	----------
Eve	+	+	+	+	+	----------
Prospero 1	-	-	-	-	-	----------

Note that in the table only the columns are comparable, but not the rows. (+) Signal is present. Identical numbers of (+) mean that no obvious differences in the strength of the signals are visible. Different numbers of (+) mean that it is possible to distinguish the strength of the signals clearly for corresponding antisera. (-) There is no visible signal at all. (--------) Experiment was not performed.

At higher dilution ranges of antisera beginning with 1:20.000 it is possible to distinguish between the different affinities of the obtained antisera clearly (Fig. 8).

Fig. 6 Comparison of pre-immunsera and corresponding antisera after the second boost on blotted proteins of GST-Engrailed-1, GST-Prospero 2 and GST-Snail. For both animals (Animal 1 and 2) the pre-immunsera (Pre) and the antisera obtained after boost 2 (B2) are probed. Dilution of antisera and pre-immunsera: 1: 500. Dilution of the secondary antibody: 1: 1000. Staining time: About 10 minutes.

Fig. 7 Antisera obtained after subsequent boosts are probed with corresponding proteins of GST-Prospero 1, GST-Snail, GST-Krüppel-1 and GST-Prospero 2. Antisera of both animals are tested (Animal 1 and 2). The antisera of different boosts are probed (boost 2-6). The antisera of the final bleedings (final bl.) are probed. Antisera against GST-Prospero 1 were diluted 1:10.000 and the others 1: 20.000. Dilution of the secondary antibody: 1: 1000. Staining time: About 10 minutes.


GST-Engrailed-1
Animal 1
Animal 2
Pre
B2
Pre
B2
GST-Prospero 2
Animal 1
Animal 2
Pre
B2
Pre
B2
GST-Snail
Animal 1,2
Animal 1,2
Pre
Pre
B2
B2
GST-Prospero 1
Animal 1
boost 2
boost 3
boost 4
boost 5
Animal 2
boost 2
boost 3
boost 4
boost 5
GST-Snail
Animal 1
boost 2 boost 3 final bl.
Animal 2
boost 2 boost 3 final bl.
GST-Krüppel-1
Animal 1
boost 2 boost 3 final bl.
Animal 2
boost2boost3final bl.
GST-Prospero 2
Animal 1
Animal 2
boost 2 boost 3 boost 2 boost 3

The antisera from the animal which was immunized and boosted with protein of GST-Prospero 2 shows the highest affinity. In the case of GST-Snail and GST-Krüppel-1 these affinities were lower but still strong. Finally the antisera of the animals which were immunized and boosted with proteins of GST-Engrailed and GST-Even skipped showed the lowest affinities. In the case of GST-Prospero 1 no signal was obtained. In a further experiment the antisera corresponding to GST-Krüppel and GST-Snail were diluted 1:100.000 (Fig. 8; GST-Snail, GST-Krüppel-1).

Like in the case of a dilution at 1:40.000 the antisera corresponding to GST-Snail showed a stronger response than the antisera corresponding to GST-Krüppel.

1.3. The antibodies in embryo stainings

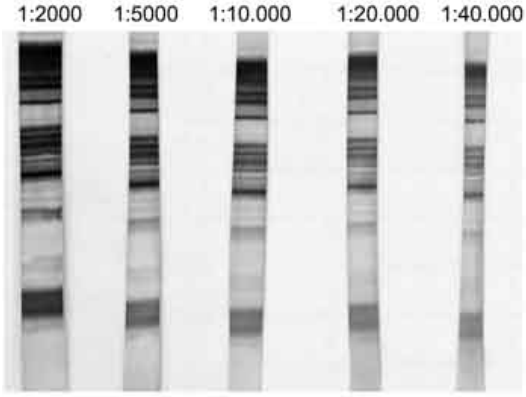
Only the proteins of GST-Snail, GST-Krüppel-1 and GST-Prospero 2 yielded two antisera each which are functional in embryo staining experiments. These are the antisera against the three larger fusion proteins (Fig. 4). Larger proteins potentially have more immunogenic sites than smaller ones. This may challenge a stronger imunogenic response yielding in antisera with higher affinities. However, experiments have shown that the antisera against GST-Snail, GST-Krüppel-1 and GST-Prospero 2 have higher affinities than those ones against GST-Engrailed-1, GST-Even skipped and GST-Prospero 1 (Fig. 8). The antisera against GST-Prospero 1 was made against a stretch of 182 amino acids.

The 164 carboxyterminal amino acids belong to the Prospero homeodomain and the Prospero domain which are conserved between different species. So, this protein provides only few imunogenic sites. This may be one reason why the yielded antisera against GST-Prospero 1 after boost three shows no detectable signal (Fig. 8,GST-Prospero 1).

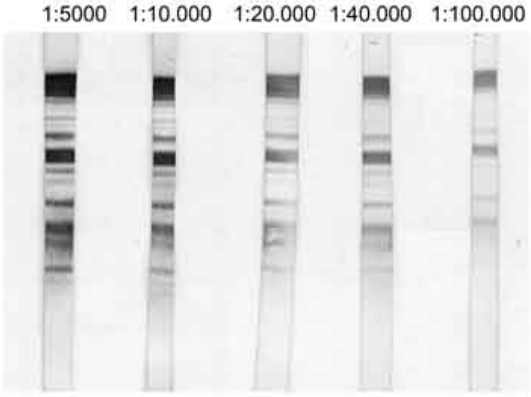
For the three fusion proteins, which were produced first, corresponding antisera of all 6 animals against GST-engrailed-1, GST-Even skipped and GST-Prospero 1 never showed any positive result in staining experiments with spider embryos. After boost 5 the immunizations of animals with GST-Prospero 1, GST-Engrailed-1 and GST-Even skipped were stopped.

Fig. 8 Antisera obtained after boost 3 are probed at a dilution range between 1:2000 and 1:100.000. The GST fusion proteins of Prospero 2, Snail, Krüppel-1, Engrailed-1, Even skipped and Prospero 1 were blotted and their corresponding antisera are probed. In all 6 dilution series the strength of the staining signals with the highest molecular weights are compared between the different antisera. Note that GST-Engrailed and GST-Even skipped were not blotted over the whole stripes at an antisera dilution of 1:2000. Positive staining for both antisera at this dilution is indicated only at the right sides (arrows) of the two stripes. Dilution of the secondary antibody: 1: 1000. Staining time: 16 minutes.

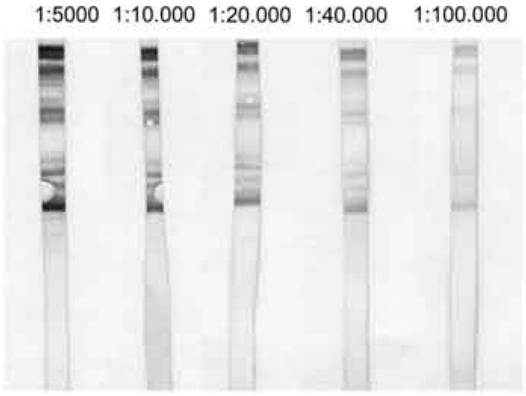
GST-Prospero 2



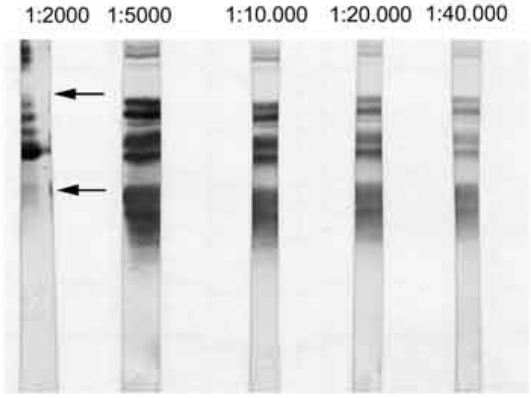
GST-Snail



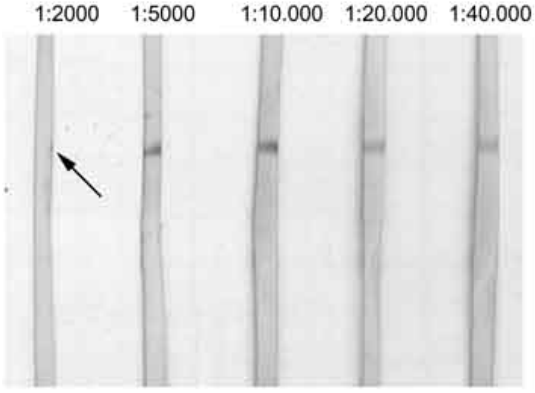
GST-Krüppel 1



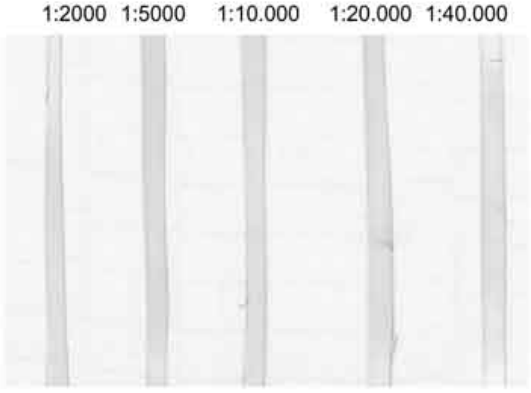
GST-Engrailed 1



GST-Even skipped



GST-Prospero 1



2. Antibody stainings, in situ hybridisations and sequences

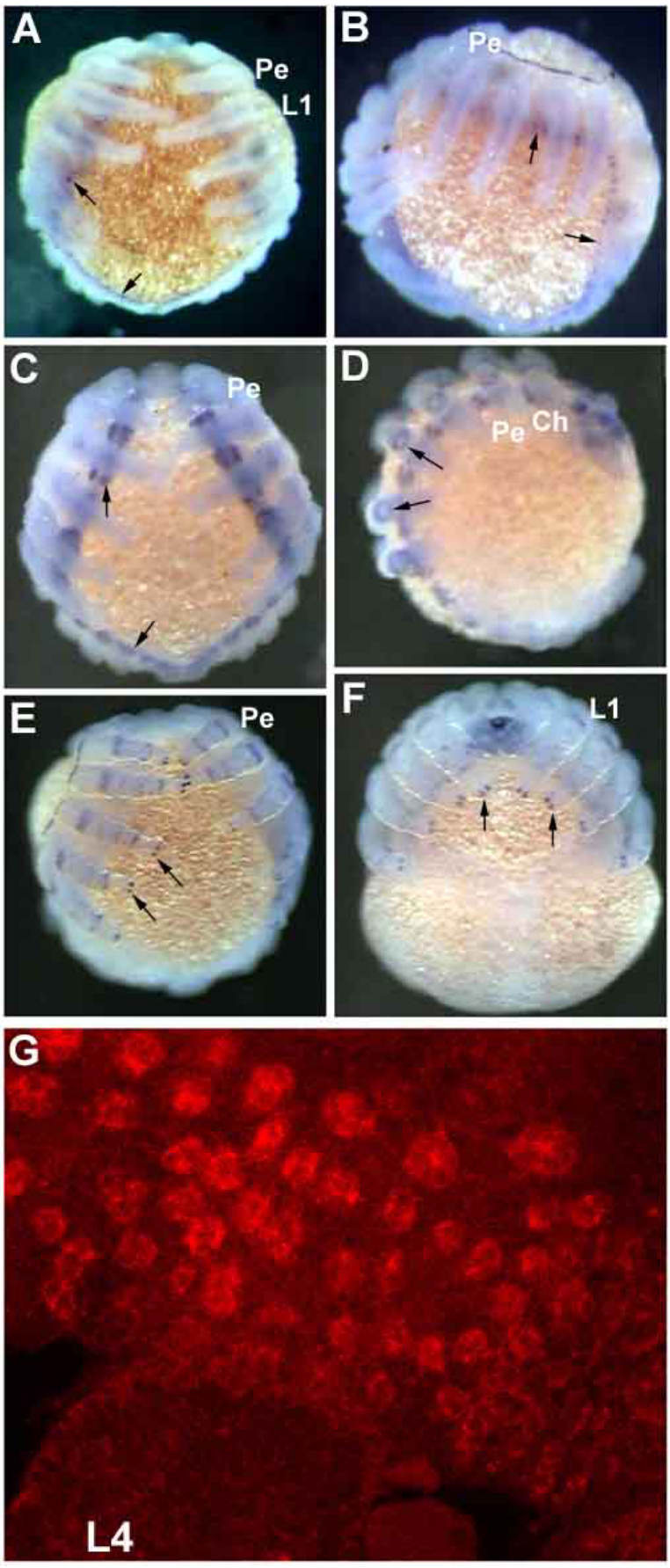
The earliest visible sign of neurogenesis in the spider *Cupiennius salei* is the formation of invagination sites (Stollewerk et al., 2001). An invagination site consists of cells whose nuclei move basally whereas cytoplasmatic extensions remain connected to the apical surface during invagination. These cells have a bottle shaped form and give raise to neurons of the developing CNS, as was shown by staining with Rhodamine-phalloidin. This is a dye which stains actin, so mainly the actin-rich cell cortex is stained and cell shapes are visible. As the membranes of cytoplasmatic extensions in invaginating cells are packed to higher densities, actin rich dots can be visualized with Rhodamine-Phalloidin at the apical surface of the neuroectoderm. To reveal the pattern of Snail, Krüppel-1 and Prospero expressing cells in embryos of *Cupiennius salei*, polyclonal antibodies against the proteins were used. Staining with Cy-coupled secondary antibodies was combined with phalloidin-rhodamine stainings. Snail, Krüppel-1 and Prospero expressing cells were visualized with the confocal laser-scanning microscope (LSM) and with non-fluorescent antibody stainings. Furthermore transcription of Snail, Krüppel-1, Prospero, Even-skipped and Runt-1+2 were analyzed by whole mount in situ hybridizations.

2.1. Expression of *even-skipped* and *runt-1* and *2*

In situ hybridisations show that *even-skipped* is expressed in a segmentally repeated pattern of a few cell clusters over the entire developing CNS (Fig. 9A,B, arrows). *Even-skipped* is also expressed dorsally and plays a role in the segmentation of spider embryos (not shown). The dorsal expression probably corresponds to the developing heart.

Initial PCR-experiments identified two genes with a Runt domain in *Cupiennius*, *runt-1* and *runt-2*. *Runt-1* is expressed strongly in the entire developing CNS (Fig. 9C, arrows) and in a ring shaped structure of the developing pedipalps and walking legs (Fig. 9D, arrows). The latter expression pattern is surprising because a function for *runt* genes in leg development is not known for other arthropods. *Runt-1* also plays a role in segmentation.

Fig. 9 In situ hybridisations show the expression of *even skipped* (A,B), *runt-1* (C,D) *runt-2* (E,F) and *prospero* (G). Fotos were taken from embryos about 200 hours after egg laying (A-E), and about 230 hours after egg laying (F). (A,B) The expression of *even-skipped* is visible in the region of the developing CNS (arrows). (C) *Runt-1* is strongly expressed in the developing CNS (arrows). (D) *Runt-1* is also expressed in a ring shaped structure of the walking legs and the pedipalps (arrows). (E,F) *Runt-2* is expressed in the developing PNS of the appendages. (G) Fluorescent in situ hybridisation. Confocal micrograph visualizes the expression of *prospero* in the developing CNS. *Ch*, chelicere; *Pe*, pedipalps; *L1*, *L4*, walking legs 1 and 4.


A
Pe
L1
B
Pe
C
Pe
D
Pe
Ch
E
Pe
F
L1
G
L4

The second *runt* gene, *runt-2* is expressed in the PNS of the walking legs and the pedipalps (Fig. 9E,F, arrows). It may also play a role in the development of other tissues in the appendages of *Cupiennius*.

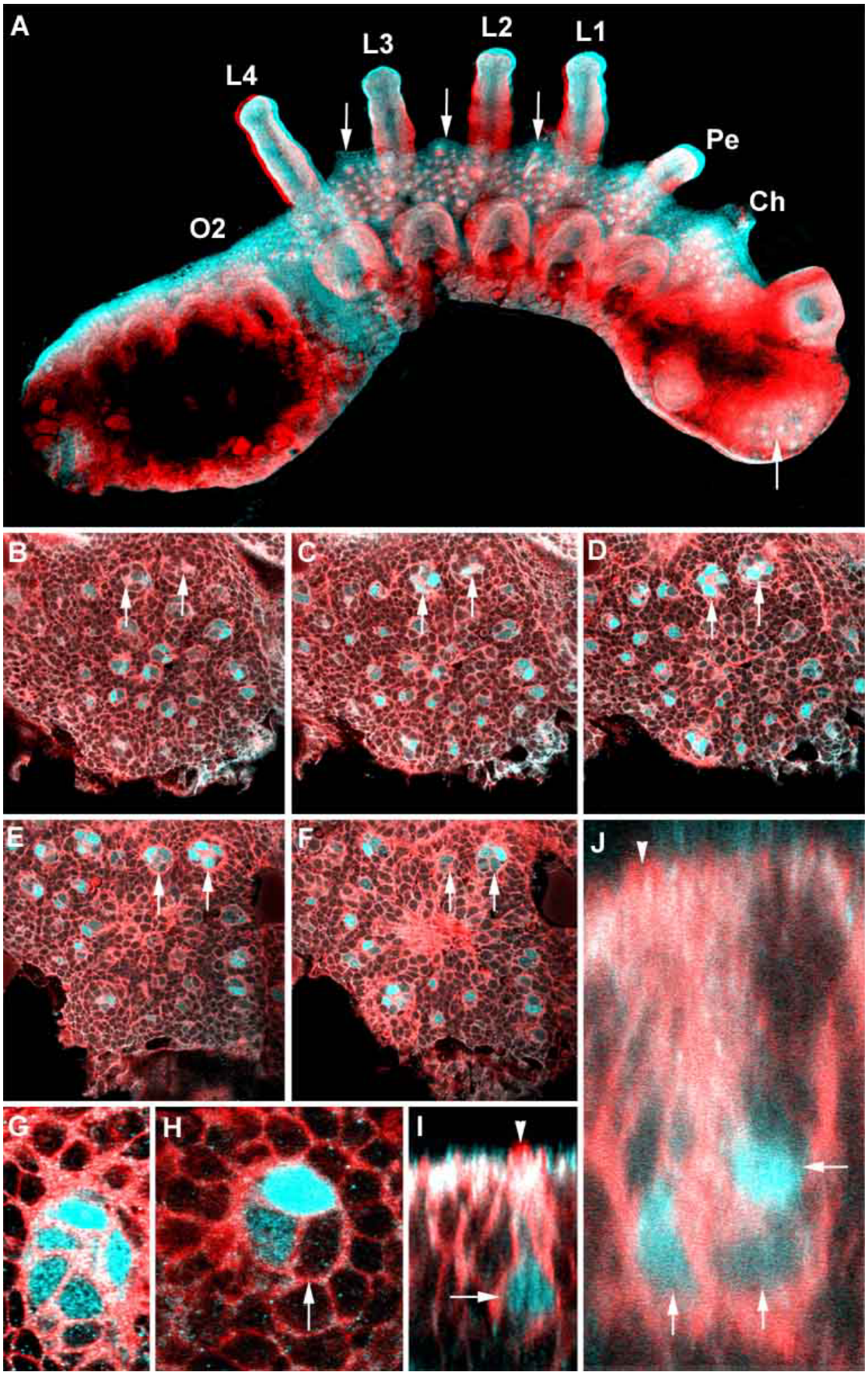
2.2. Fluorescent in situ hybridisations

Experiments with different methods of fluorescent in situ hybridisations were unsucessful. In a few cases of stainings with HNPP/Fast red (see material and methods) specific signals were visible (Fig. 9G) but these results were only poorly reproducible. Furthermore even in these experiments it was only possible to visualize specific signals clearly for a few cells within every cell cluster. There was always only a weak contrast between the background and the specific signal which made it impossible to distinguish between these for most cells (Fig. 9G). These unsucessful experiments of fluorescent in situ hybridisations contributed mainly to the decision to develop polyclonal antibodies for fluorescent staining experiments.

2.3.1. Expression of Snail in invaginating cells

In embryos where inversion has started, after about 200 hours after egg laying, 30 to 32 invagination sites can be detected in every prosomal segment (Stollewerk, et al., 2001; Seitz, 1966). Snail is expressed along the entire developing CNS (Fig. 10A, arrows). Snail is also expressed in the cephalic lobe. Optical sections from apical to basal show that Snail expressing cells are localised at invagination sites (Fig. 10B-D, arrows). In many invagination sites of the neuroectoderm the expression of Snail can be detected at different depths (Fig. 10E,F, arrows). Optical sections reveal that between 2 and 7 cells per invagination site express Snail (Figs. 10G,H 12A-J). Within one invagination site the expression levels of Snail are variable and there are also cells which do not have detectable levels of Snail protein (Fig. 10H, arrow). In basal optical sections cells from an invagination site can be identified because phalloidin-rhodamine stainings are stronger than those found in neighbouring cells (Fig. 10E, arrows). Furthermore these cells have a larger diameter basally than cells of the surounding tissues (Fig. 10H). Snail expressing cells can be further characterized by transverse optical sections. These optical sections show that Snail is expressed in invaginating cells (Fig. 10I,J). Snail was not found in completly invaginated cells, or in cells of the neuroectoderm which have not yet started invagination. This suggests that the expression of Snail starts after the beginning of invagination and ends before cells are completely invaginated. The protein of Snail is accumulating in the basal cell nuclei of invaginating cells (Fig. 11A,B, arrow heads). In the optical sections strong expression levels of Snail are always colocalized with DNA. Stainings with Yoyo-1 also show that the nuclei of Snail expressing cells and surounding tissues occupy a big part of the inner cell volume. The DNA in these cells is not condensed (Fig. 11C, arrow head). So it cannot be excluded that Snail protein is also present in the cytoplasm.

Fig. 10 Localisation of Snail expressing cells. Confocal micrographs were take from embryos about 200 hours after egg laying. Snail is cyan green. Actin is red. (A) The basal side of the right half of the germ band from an embryo. (B-D) Series of optical sections from apical to basal between the third and the fourth walking leg. Medial is at the bottom. (E-F) Two optical sections between the second and the third walking leg. The section of (F) is more basally than the section of (E). (G) Snail expressing cells of a lateral invagination site and a medial invagination site (H) in the same hemisegment of the third walking leg. (I,J) Transverse optical sections of Snail expressing cells. Apical is at the top. (A) Snail is expressed basally along the entire developing CNS (arrows). Snail is also expressed in the cephalic lobe. Expression of Snail corresponds to the region of the developing CNS. Relative positions of Snail patches and apical Phalloidin-rhodamine actin-rich dots in the neuroectoderm are overlapping. Note that the optical layer for Snail is more basal than the optical layer for Rhodamin-Phalloidin, which causes a weak optical shift. (B) In the most apical optical section the actin dots on the surface of the neuroectoderm are visualized. (C,D) Snail expressing cells are localized at these invagination sites as shown by the more basal optical sections (arrows). (E,F) Two different basal optical sections show that Snail expressing cells are localised in distinct depth (arrows). (G) Six Snail expressing cells are visible. (H) Two Snail expressing cell are visible. Expression levels are distinct. In one cell of this invagination site Snail expression is not detectable (arrow). Note that the cells of the invagination site have a larger dimeter and stronger rhodamine-phalloidin stainings than the surrouding tissues. (I) Bottle-shaped single invaginating cell expressing Snail (arrow) with a cytoplasmatic extension to the apical surface (arrow head). (J) A group of three Snail expressing invaginating cells (arrows) and the cytoplasmatic extensions to the apical surface (arrow head). *Ch*, cheliceral segment; *Pe*, pedipalpal segment; *L1* to *L4*, corresponding to prosomal segments 3 to 6; *O2*, opisthosomal segment 2.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L4
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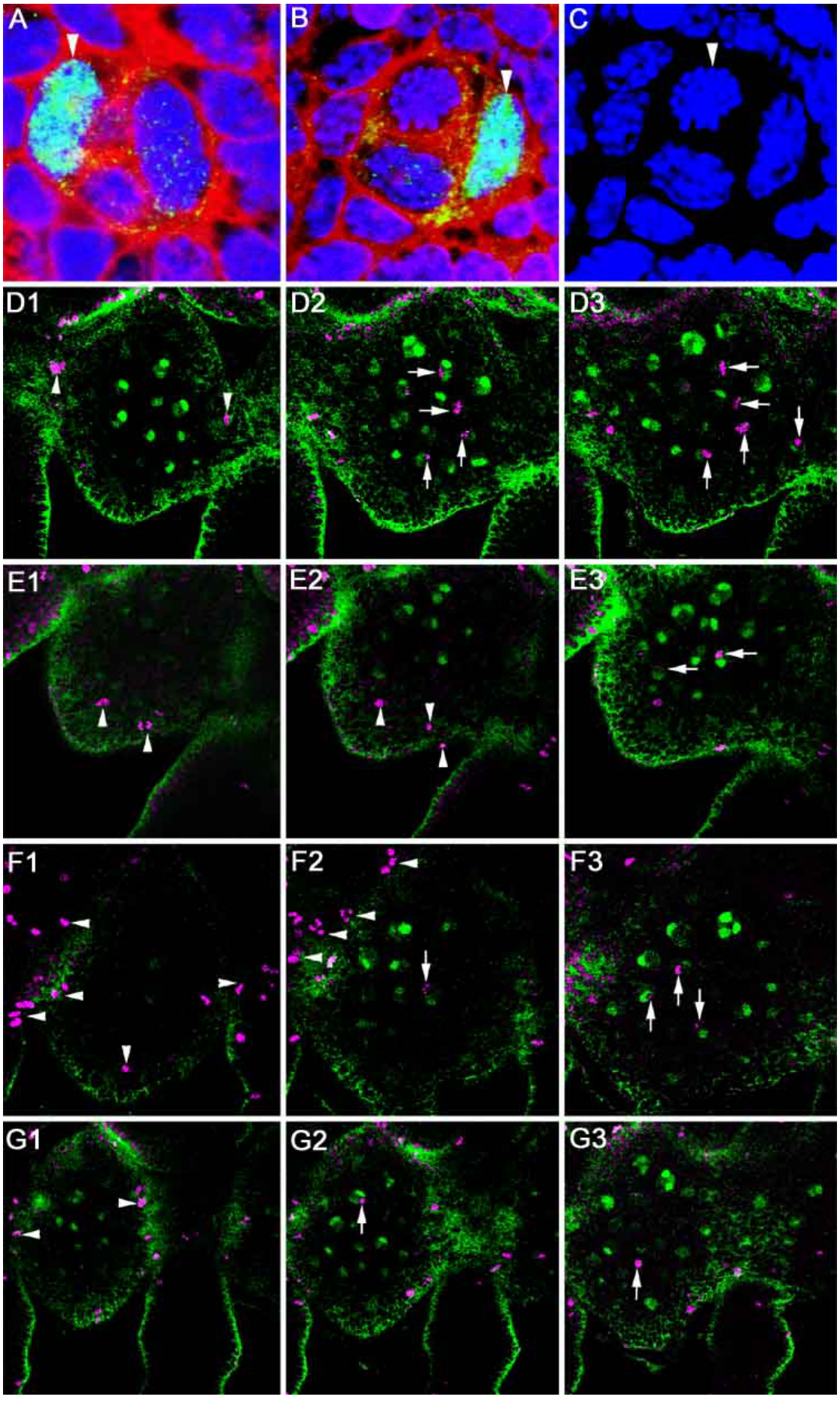
2.3.2. Snail protein is absent in mitotically active cells

Previous analyses showed that mitotic divisions in the neuroectoderm occur mainly in the most apical cell layer (Stollewerk et al., 2001). To test if Snail expressing cells undergo mitotic divisions double staining experiments were performed with the antibody against Snail and an antibody against phosphorylated Histone 3. Phosphorylated Histone 3 is a marker for all stages of mitosis (Cheung et al., 2000). Snail expressing cells which are mitotically active were not found (Fig. 11D1-G3). This is most obvious in cases where mitotically active cells are found at an invagination site next to Snail positive cells which are mitotically inactive (Fig. 11D2,D3,E3,F3,G2,G3, arrows). Most mitotically active cells are found in the apical cell layer of the neuroectoderm (Fig. 11D1,E1,E2F1,F2,G1, arrow heads).

Fig. 11 Localisation of Snail protein. Snail protein is not found in mitotically active cells. Confocal micrographs were take from embryos about 200 hours after egg laying. Snail is green. Actin is red. DNA is blue. Phosphorylated Histon 3 is shown in violet. (A,B) Two invagination sites are shown. (C) The same optical section as in (B) but only the layer of the DNA is shown. (D-G) Series of optical sections. The same capitals belong to identical positions. The lowest number (1) is the most apical and the highest number (3) the most basal optical section. The medial side is at the bottom of the pictures. (D1-3) In the hemisegment between the pedipalp and the first walking leg. (E1-3) In the hemisegment between the first and the second walking leg. (F1-3) In the hemisegment between the second and the third walking leg. (G1-3) In the hemisegment between the third and the fourth walking leg. (A) In a particular invagination site one cell is expressing Snail (arrow head). Snail is colocalized with the DNA. (B) Snail protein is localised basally like the DNA and occupies a large part of the cell volume (arrow head). (C) The DNA of this cell is not condensed (arrow head). (D1-G3) As the neuroectoderm is arched the lateral borders of the pictures correspond to tissues which are apically. Most mitotically active cells are found apically were Snail expressing cells are not detected (D1,E1,2,F1,2,G1, arrow heads). Snail expressing cells are found in the most basal tissues. The mitotically active cells in these basal tissues are not expressing Snail (D2,3,E3,F2,3,G2,3 arrows).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G3

2.3.3. History of one invagination site

Results have shown that numbers of Snail expressing cells are variable in different invagination sites of one segment (Fig. 10G,H). There exists a stereotypically repeated patteren of invagination sites in each segment (Stollewerk et al., 2001). The numbers of cells which express Snail in a single invagination site could also be variable in different segments. To test this hypothesis a single invagination site was selected. The numbers of cells which express Snail in this single invagination site were compared in different segments.

The „Big Lateral Cluster“ (BLC) is the most lateral cluster (Fig. 12N). Due to its unique position in the most lateral part of every segment in the neuroectoderm, the BLC can be identified in nearly all segments of a defined developmental stage. There are 4 cells which express Snail in the BLC of the pedipalpus segment (Fig. 12A,B).

This number increases to 6 or 7 in the first and second, and 7 or 8 cells in the third and fourth walking legs (Fig. 12C,D,E,F). The number of Snail expressing cells in the BLC decreases to 4 or 5 in the first and 2 or 3 cells in the second and third segment of the opisthosoma (Fig. 12G,H,I,J). In the seventh segment it is not possible to identify the BLC unambigously.

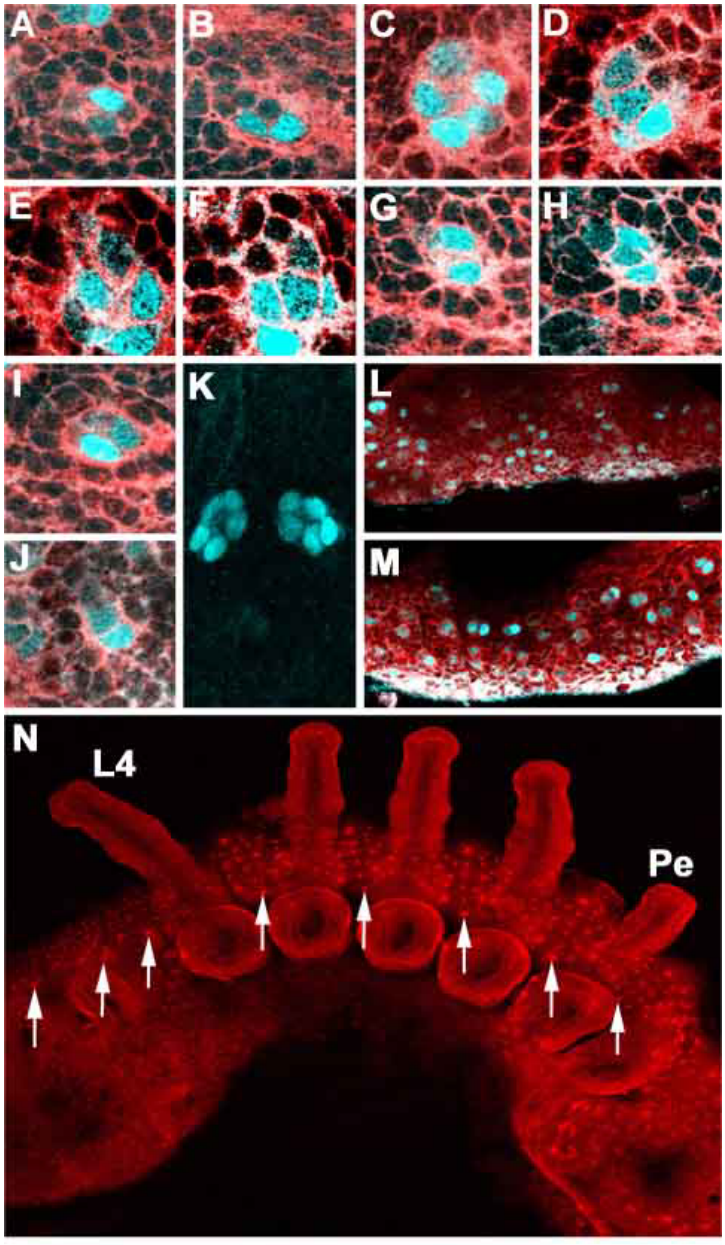
The example of the BLC shows that numbers of invaginating cells are different at an identical invagination site of different segments at the same developmental stage. For this experiment embryos about 200 hours after egg laying were used. On the one hand this could reflect differences in developmental time of segments, because they are generated sequentially at the posterior pole of the germ layer. This causes time delays between different segments. On the other hand the observed segmental variations of Snail expressing cells could also be due to segment specific differences in the numbers of invaginating cells.

A somewhat later developmental stage about 220-230 hours after egg laying was analyzed.

In the segments of the prosoma it is difficult to identify the BLC because positions of lateral invagination sites have changed relative to each other. Furthermore the invagination sites start to disappear along the medial region of the neuroectoderm in this developmental stage (Fig. 12L,M). However, in the prosoma there are still two lateral clusters which have between 5 to 6 cells expressing Snail as in previous developmental stages (Fig. 12K). In the segments of the opisthosoma there are only invagination sites with maximaly 3 cells expressing Snail (Fig. 12L,M).

Fig. 12 Expression of Snail in an identical invagination site of different segments. Snail is cyan green. Actin is red. (A-J and N) All confocal micrographs were taken from an identical developmental stage after about 200 hours after egg laying. (K, L and M) Pictures from a later developmental stage after about 230 hours after egg laying. (A-J) The big lateral cluster (BLC) is shown in different segments with cells expressing Snail. (K) Two lateral Snail expressing cell clusters. (L, M) Medial side is on the bottom of both pictures (L) Opisthosoma segments 1-4. (M) Opisthosoma segments 3-5. (N) Right half of the germ band of an embryo. More apical (A, C, E, and G) and more basal (B, D, F and H) pairwise sections of the identical BLC are shown. (A, B) In the BLC of the pedipalp segment 4 Snail expressing cells are detected. (C, D) In the BLC of the first walking leg 6 Snail expressing cells are present. (E, F) BLC of the third walking leg. 7 cells express Snail. (G, H) First segment of the opisthosoma. 4 or 5 cells express Snail. (I) Second opisthosomal segment. 2 or 3 cells express Snail. (J) Fifth opisthosomal segment. Only 2 Snail expressing cells can be detected. (K) In both lateral clusters 5 cells which express Snail are visible. Positions of invagination sites have changed and so the BLC cannot be identified unambiguously. (L and M) In the segments 1-5 of the opistosoma only invagination sites with maximally 3 cells expressing Snail can be detected. (N) Due to its most lateral position in the anterior part of the segments, the BLC (arrows) can be identified easily. Pe, pedipalpal segment; L4, walking leg 4 corresponding to the prosomal segment 6.

Fig. 13 Expression of Snail in the PNS. Confocal micrographs were taken from embryos about 200 hours after egg laying. A part of the coxa of the third and the fourth walking leg is shown. Snail is cyan green. Actin is red. (A) A group of four cells is expressing Snail in the coxa of the third walking leg. Note that the expression of Snail in the fourth cell (arrow) is not seen in the optical section of (B, arrow). (B) The cells have cytoplasmatic extensions to a cup-shaped structure (arrow head) at the surface of the coxa. The neighbouring tissue belongs to the coxa of walking leg 4. *L3* and *L4*; Walking legs 3 and 4 corresponding to prosomal segments 5 and 6.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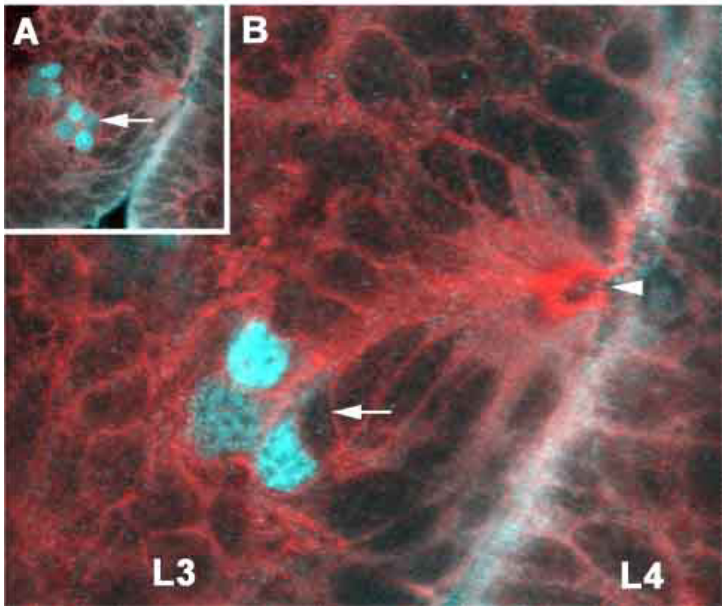

A
B
L3
L4

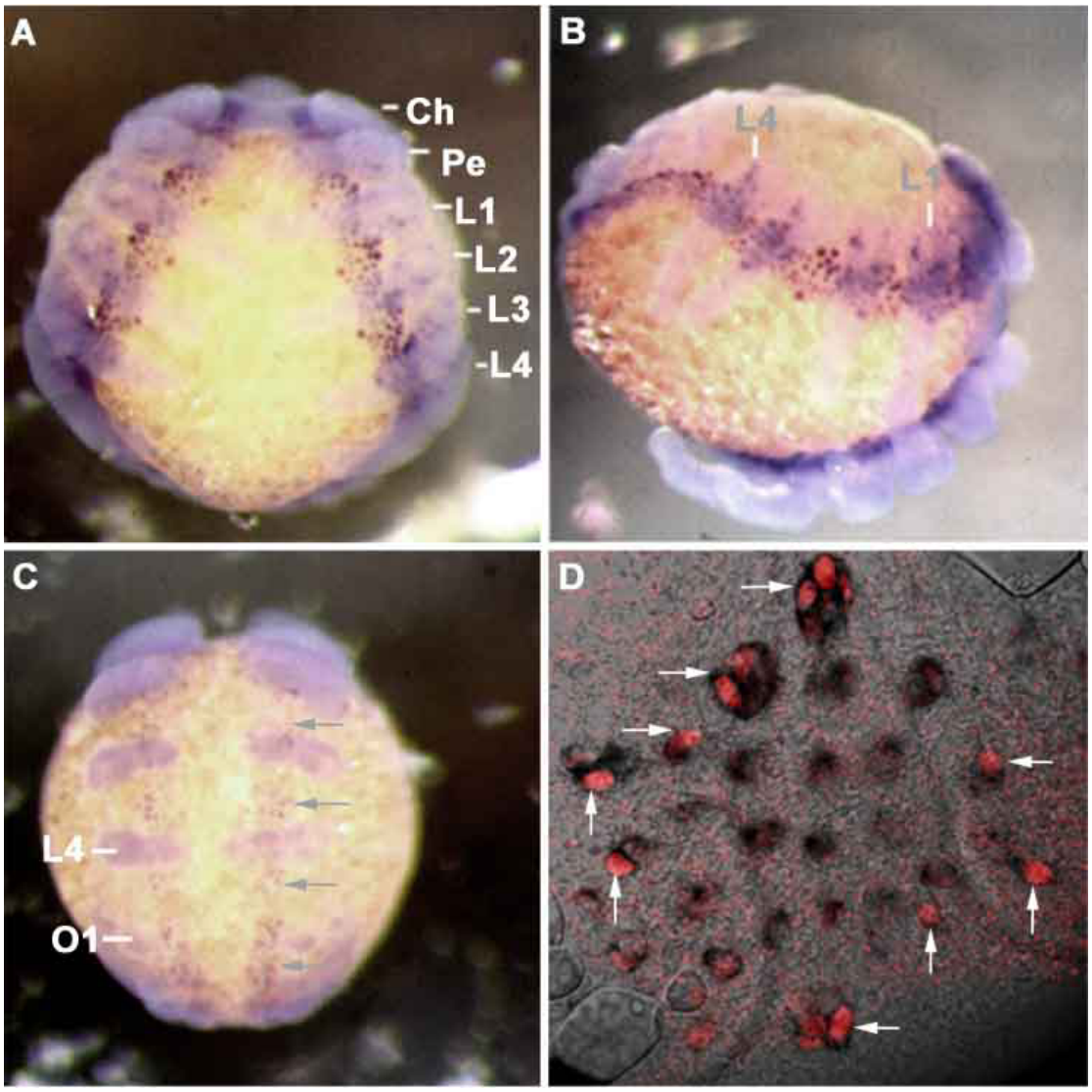
2.3.4. Transcription of *snail*

Transcription of *snail* at weak levels was found in embryos about 160-180 hours after egg laying (Fig. 14C). In later developmental stages *snail* is expressed at high levels along the entire developing CNS (Fig.14 A,B). In situ hybridisation with subsequent protein staining shows that transcription of *snail* and the expression of the protein occur at the same time (Fig. 14D, arrows).

2.3.5. Snail is expressed in the peripheral nervous system (PNS)

Spiders receive a large part of their sensory input by sensory hairs on the pedipalps and walking legs (Barth, 2001). Snail is expressed in the PNS of the walking legs and pedipalps. There are groups of 4 cells in different leg podomeres which show Snail expression (Fig. 13A, arrow). These cells have actin rich extensions which reach to a cup-shaped structure at the epidermal surface (Fig. 13B, arrow). Around such extensions, there are closely associated cells which differ in shape and size from the surounding tissue (Fig. 13B, arrow heads). Such a cup-shaped structure serves as a basis for the insertion of one mechanosensitive hair, a so called „Trichobothrium“ (Barth, 2001). In *Cuppiennius salei* 4 sensory cells are innervated by one mechanosensitive hair (Barth, 2001; Anton, 1991). Their axons, which extend to the basal part of the sensory hair, are enveloped by sheath cells. Number and position of Snail expressing cells suggest that these are the sensory cells of a „Trichobothrium“.

Fig. 14 In situ hybridisations show expression of *snail* in embryos about 220 hours (A,B and D), and 160-180 hours (C) after egg laying. (A) Frontal view shows expression of *snail* along the entire developing CNS. (B) Lateral view with the head on the right hand site. (C) Weak expression of *snail* at the positions of early forming invagination sites. (D) In situ hybridised embryo was stained with the antibody to detect protein expression of Snail (red). The transmision microscope image shows that the protein expression occurs in the same cells that are positive for RNA (black). The picture was taken between the third and the fourth walking leg. *Ch*, cheliceral segment; *Pe*, pedipalpal segment; *L1* to *L4*, corresponding to prosomal segments 3 to 6; *O1*, opisthosomal segment 1.


A
Ch
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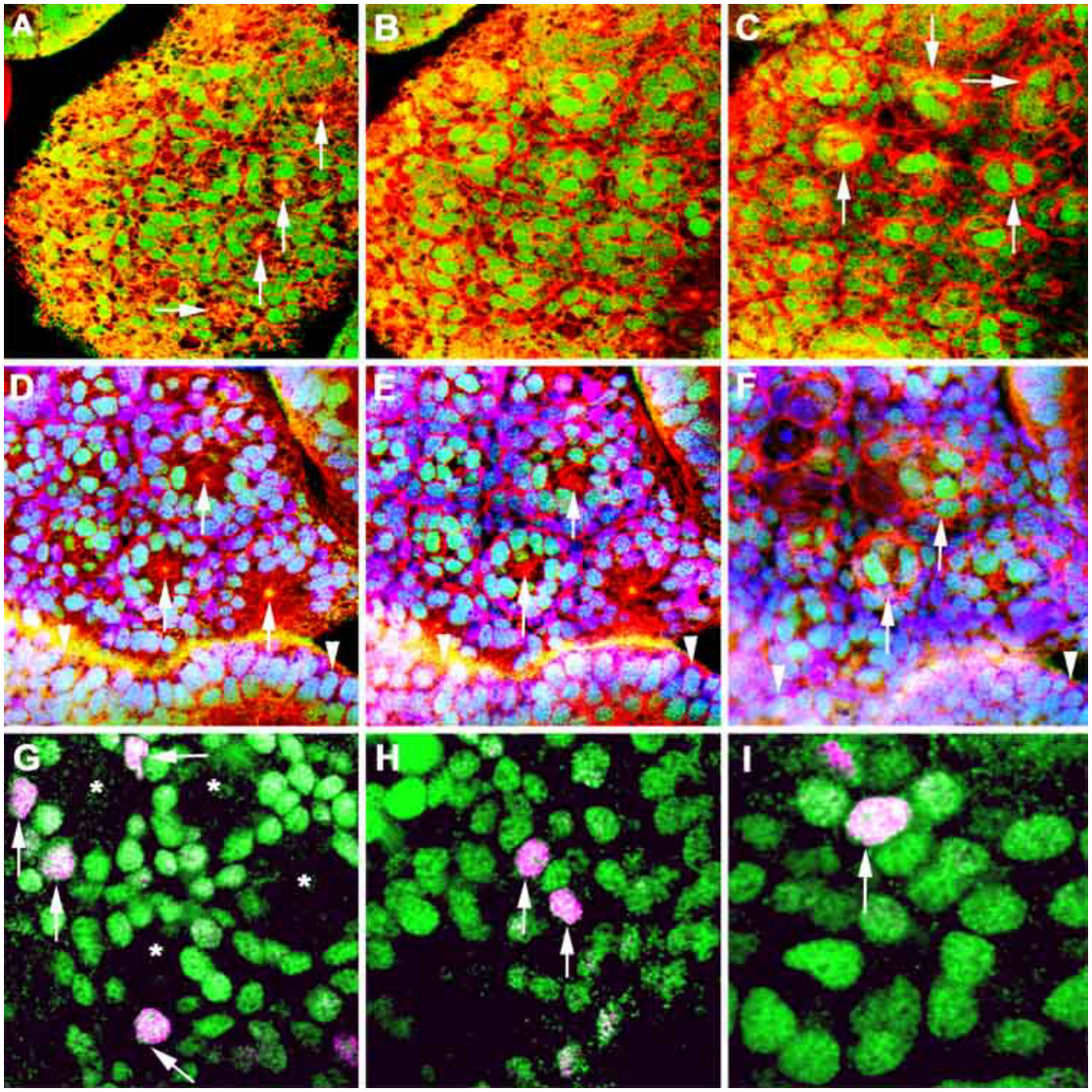
L3
L4
B
L4
L1
C
L4
O1
D

2.4. Expression of Krüppel-1

In embryos about 200 hours after egg laying Krüppel-1 is expressed in cells which are localized around the invagination sites of the neuroectoderm (Fig. 15A, arrows). Different optical sections from apical to basal reveal that Krüppel-1 is also expressed in most cells of layers which are more basally (Fig. 15B) and in the most basal cell layer (Fig. 15C). The expression levels are variable (Fig. 15A-F). Krüppel-1 is also expressed in neighbouring tissues like the legs (Fig. 15D-F, arrow heads). Despite the expression of Krüppel-1 in many cells, basal optical sections reveal that the protein of Krüppel-1 accumulates at higher levels in some cells of invagination sites than in the surounding tissue (Fig. 16C,F, arrows). Epression levels of Krüppel-1 cells at the invagination sites are variable. (Fig. 15C,F). Krüppel-1 accumulates in cell nuclei as revealed in stainings of DNA and the protein (Fig. 15D-F). The protein of Krüppel-1 is also detected in mitotically active cells (Fig. 16G-I). These mitotically active Krüppel-1 expressing cells are always in prophase but never in a later stage of mitosis (Fig. 15G-I).

Fig. 15 Localisation of Krüppel-1 expressing cells and distribution of the protein. Confocal micrographs were taken of embryos about 200 hours after egg laying. Krüppel-1 is green. DNA is shown in blue. Actin is red. Phosphorylated Histon 3 is violet. (A-C) Series of optical sections in the hemisegments between the third and the fourth walking leg. (A) is the most apical and (C) the most basal optical section. (D-F) Series of optical sections in the hemisegments between the second and the third walking leg. (D) is the most apical and (F) the most basal optical section. (G) Optical section of the apical surface and more basally (H,I). (A) Krüppel-1 expressing cells are localised in the neuroectoderm around invagination sites (arrows). (B) More basally also most cells express Krüppel-1. (C) Krüppel-1 is localised in cells of invagination sites (arrows). (D,E) In apical optical sections at invagination sites and basally (F) Krüppel-1 accumulates in cell nuclei. (D-F) Krüppel-1 is also found in the tissue of the leg (arrow heads). (G) Krüppel-1 expressing cells which are mitotically active are localised around invagination sites (asteriks). They are in prophase. (H,I) Krüppel-1 expressing cells which are mitotically active are also found basally. They are in prophase.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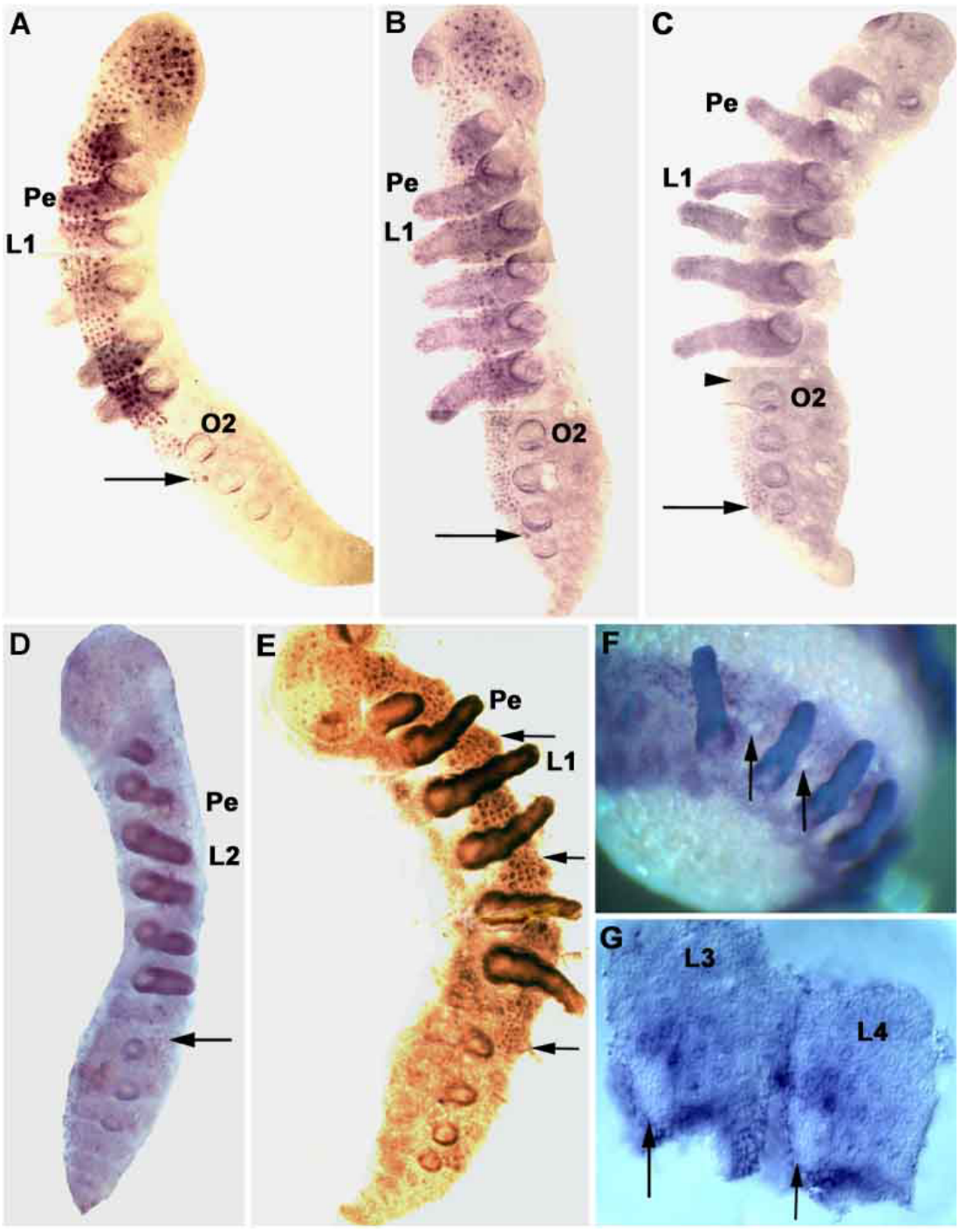
Expression levels show a heterogeneous spatial distribution and change dynamicaly over time (Fig. 16). In situ hybridisations of embryos about 180 hours after egg laying show high transcription levels of *Krüppel-1* in the cells of the invagination sites (Fig. 16A). This expression is then extended to some invagination sites in the more posterior segments of the opisthosoma (Fig. 16B; arrow).

Somewhat later it vanishes in the more anterior segments (Fig. 16C, arrow head). High transcription levels in the cells of the invagination sites are transient. The process is continuous and followed by the reduction of strong *Krüppel-1* expression to few invagination sites of the opisthosomal segments 4 and onwards (Fig. 16C; arrow).

While this strong expression in the invagination sites is vanishing *Krüppel-1* is still expressed at weaker levels along the entire neuroectoderm (Fig. 16F,G). Only in a small lateral area the expression of *Krüppel-1* disappears (Fig. 16F,G; arrows). Comparisons of *in situ* hybridisations and protein stainings of embryos from the same batch revealed that the transcription levels of *Krüppel-1* in cells of the invagination sites are strong whereas protein levels in the same developmental stage are still weak (Fig. 16A,D). In a developmental stage a few hours older, the translation of Krüppel-1 clearly reaches high levels at the same time as the transcription of *Krüppel-1* in the invagination sites is weak (Fig. 16C,E). The high protein levels of Krüppel-1 in the invagination sites are also transient like the high transcription levels. The pattern of Krüppel-1 translation resembles the pattern of transcription but there is a visible time delay between the appearance of high RNA and high protein levels for Krüppel-1. For Snail no such time delay is observed. High levels of transcription colocalize with corresponding levels of translation (Fig. 14D, arrows).

Fig. 16 Time delay between transcription and translation of Krüppel-1 in the invagination sites. (A, D) One half of the germ layer of embryos about 180 hours after egg laying. The germ layers shown are from embryos of the identical batch. (C, E) One half of the germ layer of embryos about 200 hours after egg laying. The both germ layers shown are from embryos of the identical batch. (A, B, C, F and G) In situ hybridisations. (D and E) protein expression. (A) The RNA of Krüppel-1 is expressed in invagination sites of the cephalic lobe, in the neuroectoderm of the prosoma and the first 2-3 segments of the opisthosoma (arrow shows invagination site between opisthosoma segment 2 and 3), when at the same time (D) Protein expression levels of Krüppel-1 are still very weak. (B) Later the expression of *Krüppel-1* is extended to the opisthosomal segments 4-5 (arrow). At the same time the expression is vanishing in the more anterior segments. (C) Strong expression of *Krüppel-1* in the invagination sites is reduced to segments 4 and 5 of the opisthosoma (arrows) and has vanished in more anterior segments (arrowhead). (E) In the same developmental stage strong protein expression of Krüppel-1 is visible in the invagination sites of the cephalic lobe and in the neuroectoderm of the prosoma and the opisthosoma. (F) Expression of *Krüppel-1* is not completely reduced. It remains active and only vanishes completely in a lateral area (arrows) of every segment. (G) Walking legs 3 and 4 were dissected from the tissue to visualize the lateral area (arrows) where *Krüppel-1* is not expressed any more. *Ch*, cheliceral segment; *Pe*, pedipalpal segment; *L1* to *L4*, corresponding to prosomal segments 3 to 6; *O2*, opisthosomal segment 2.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2.5. Sequence of *prospero*

Initial RT-amplification yielded only one type of fragment with similarity to *Drosophila* prospero. The complete sequence for *prospero* was then obtained by 5` and 3` RACE amplification of cDNA ends. To amplify the 5`end, a further RT-amplification made with prospero specific primers was necessary. Amplification of cDNA with nested primers resulted in one single product. However, as there is no in-frame upstream stop codon, it cannot be excluded that the first ATG codon, which is found at nucleotide position 142-144, is not the real start codon. This implies that the amino terminal part of the protein could be missing (see complete sequence in the supplement).

Phylogenetic analyses places *Cupiennius* Prospero within the other known Prospero protein sequences. While the vertebrate Prox 1 sequences share high similarity to each other, *Cupiennius* Prospero is more closely related to *Drosophila* Prospero (Fig. 17).

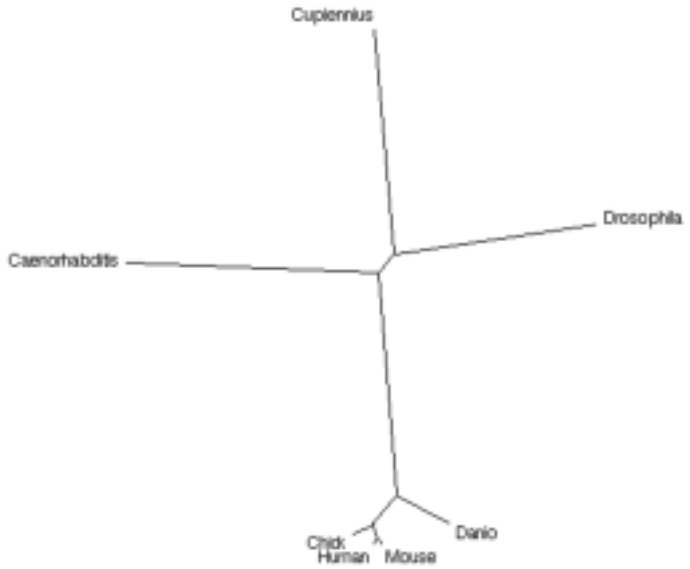

Fig. 17 Phylogram of the sequences most closely related to *Cupiennius* Prospero. The tree was made for all species with the complete amino acid sequences of the corresponding Prospero homologs. All nodes of the neighbour joining unrooted tree have bootstrap values of 100%. GenBank accession nos. for the sequences used: *Drosophila*, P29617; *Caenorhabditis*, P34522; *Danio*, AAK40357; *Chick*, JC5495; *Mouse*, P29617; *Human*, XP001994.

Within the homeobox, in the turn between helix 2 and 3 (Chu-Lagraff et al., 1991; Bürglin, 1994), *Cupiennius* Prospero, *Drosophila* Prospero and the Prospero related protein CEH-26 from *Caenorhabditis elegans* share an insertion of three identical amino acids (Lys; Asn; Asn; Fig. 18, amino acids 126-128.).

For *Drosophila* Prospero an alternative splice form exists. In this alternative splice form 29 absent amino acids are missing (Chu-Lagraff et al., 1991; Fig. 18, Amino acids 60-88 of the *Drosophila* Prospero sequence). The last 5 amino acids missing in the alternative splice form belong to the Prospero homeobox. There exists an alternative splice form also for *Cupiennius* Prospero (Fig. 18; Amino acids 60-88 of the *Cupiennius* Prospero sequence).

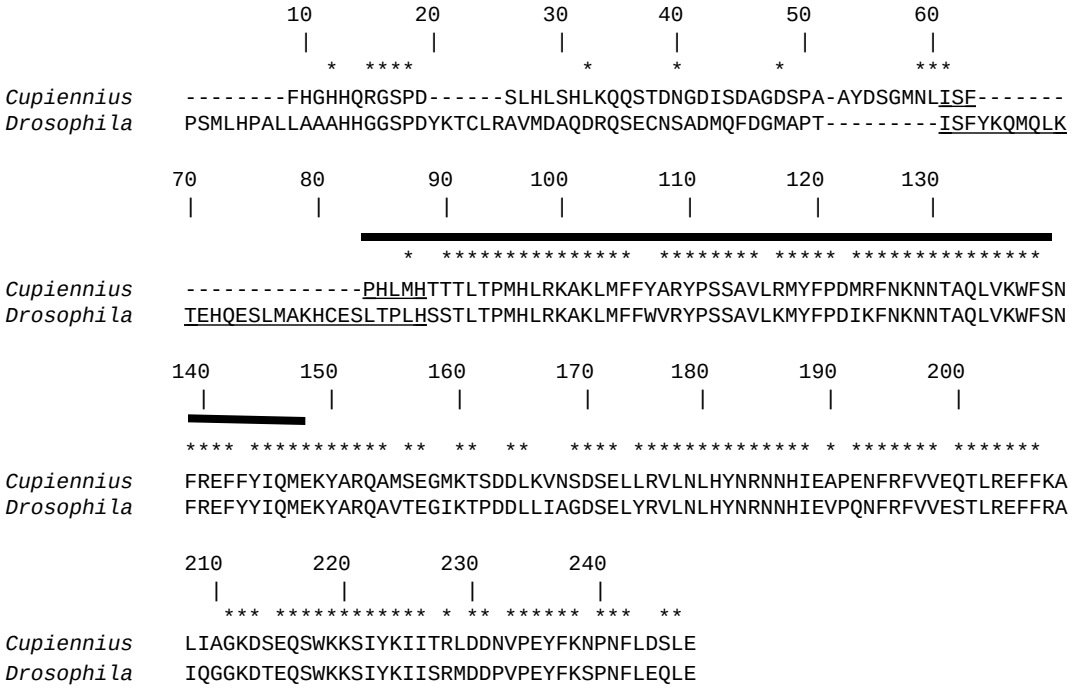

Fig. 18 Alignment of the Prospero homeodomain (Bürglin, 1994; Marked by the black bar) and the carboxyterminal Prospero domain of *Cupiennius* and *Drosophila*. The alignment was performed with the clustal program from „gene jockey“ (see material and methods). (“-“) Gaps, which were made by the alignement. (“*“) The stars mark identical amino acid positions. Underlined amino acids correspond to the missing sequences of the alternative splice forms for *Drosophila* and *Cuppiennius* Prospero (see text). Amino acids 674-884 of *Cupiennius* Prospero (see supplement), and 1166-1403 (GenBank accession no. P29617) of *Drosophila* Prospero are aligned.

In the spider sequence 8 amino acids are missing. In both Prospero homologs absent amino acid sequences start with an isoleucine, serine, phenylalanine and end with a histidine. This argues in favor of an evolutionary conservation of this alternative splice site in both species.

Prospero has at least 2 more sites of alternative splicing: The amino acids corresponding to the nucleotide sequences 829-843 and 1597-1629 (see complete sequence of Prospero) can be missing.

```
                  10        20        30        40        50        60
                   |         |         |         |         |         |
                   * * **  **   ****  **  * * *      **           **  * **
Cupiennius     ----------DYEWLAESLKAKLSTSLSQVVDAVVSRCVQRKAALSKVSPPESSDPP---KDPTLLSQM
Drosophila     NSSMMRMSGTDLEGLADVLKSEITTSLSALVDTIVTRFVHQRRLFSKQADSVTAAAEQLNKDLLLASQI

                  70        80        90       100       110       120       130
                   |         |         |         |         |         |         |
                  ******** ** **  *             *  *                 *
Cupiennius     LDRKSPRTGKVIDR---GTRVNGHGLCGLRTSPYPPD-IGTAPKPSFFFPLKPPTSVAAATAAFLYGSP
Drosophila     LDRKSPRT-KVADRPQNGPTPATQSAAAMFQAPKTPQGMNPVAAAALYNSMTGPFCLPPDQQQ--QQQT

                 140       150       160       170       180       190       200
                   |         |         |         |         |         |         |
                     *       *    *  ** ** *****************  *              ***
Cupiennius     PQMPQSYSSPAHSTPTPQDAPEQTEAMSLVVTPKKKRHKVTDTRLHQRQ--------------GGPLC
Drosophila     AQQQQSAQQQQQSSQQTQQQLEQNEALSLVVTPKKKRHKVTDTRITPRTVSRILAQDGVVPPTGGPPS
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Fig. 19 Alignment of the asymmetric localisation domain (underlined sequence) and the nuclear localisation signal (in bold type) of *Cupiennius* and *Drosophila* Prospero. The alignment was performed with the clustal program from „gene jockey“ (see material and methods). (“-“) Gaps, which were made by the alignment. (“*“) The stars mark identical amino acid positions. Amino acids 410-584 of *Cupiennius* Prospero (see supplement), and 824-1027 of *Drosophila* Prospero (GenBank accession no. P29617) are aligned.

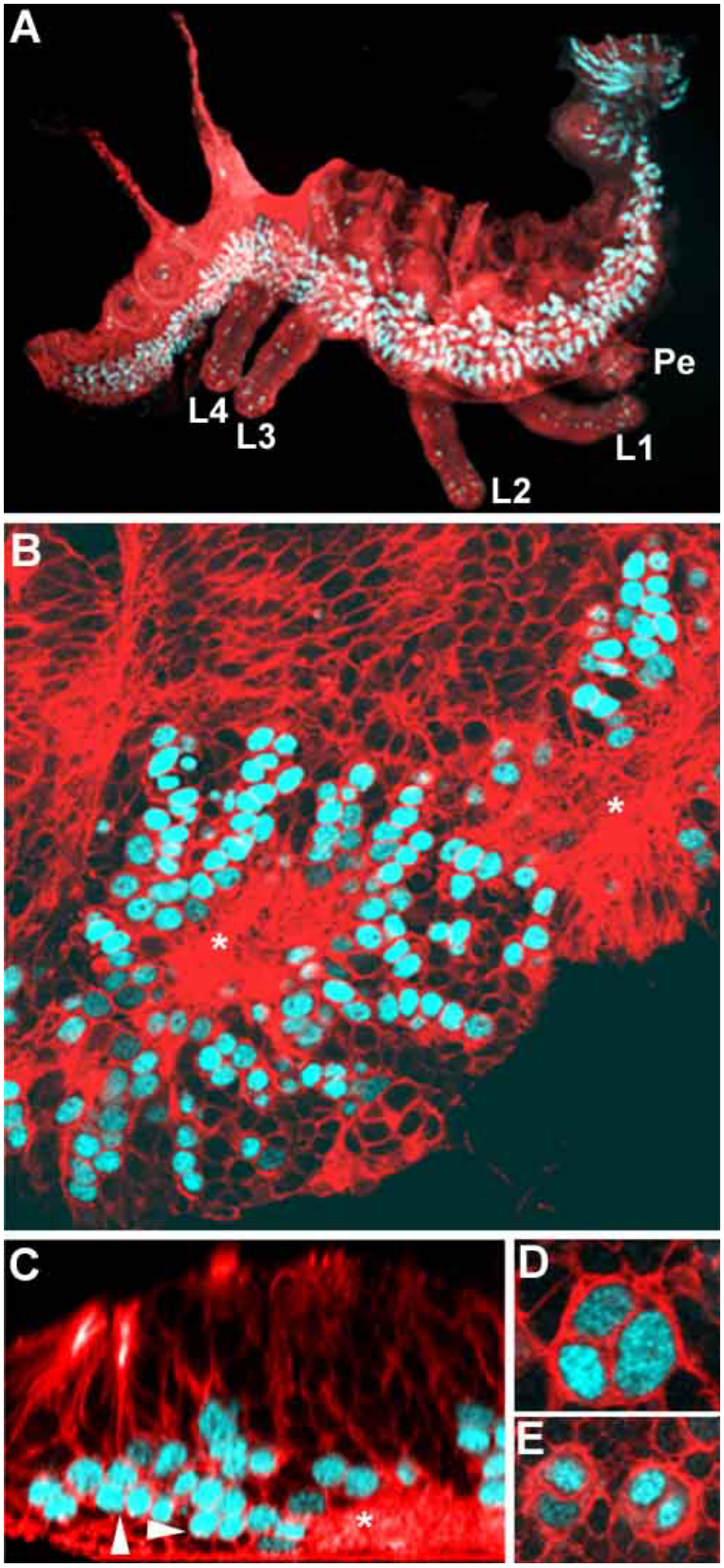
On the basis of alignment analysis *Cupiennius* Prospero only shared a maximal identity of 47% with the asymmetric localisation domain (Fig. 19; Underlined amino acids 49-78) of *Drosophila* Prospero (Hirata et al., 1995). A nuclear localisation signal (Fig. 19; Amino acids 49-78 in bold type) is evolutionary conserved between the Prospero sequences of both species. (Picard and Yamamoto, 1987 Hirata et al., 1995; Demidenko et al., 2001)

2.6.1. Expression pattern of Prospero

To visualize the expression pattern of Prospero expressing cells, a polyclonal antibody against Prospero was used. Stainings with Cy-coupled secondary antibodies were combined with phalloidin-rhodamine stainings.

In *Cupiennius salei* embryos about 220 hours after egg laying, Prospero is expressed along the entire developing CNS of the prosoma and the opisthosoma. Prospero is also expressed in the cephalic lobe and the PNS (Fig. 20A). Basal optical sections show that Prospero expressing cells are organized around the developing neuropil (Fig. 20B). Prospero is expressed in cells which detached completely from the apical surface (Fig. 20C arrow heads). These cells completed invagination. Prospero is also expressed in invaginating cells as shown by the strong phalloidin-rhodamine stainings of invagination sites. (Fig. 20D,E).

Fig. 20 (A-C) Confocal micrographs were taken from spider embryos about 220 hours after egg laying. (D-E) Confocal micrographs were taken from an embryo about 200 hours after egg laying. (A-E) Prospero is cyan green and actin is shown in red. (A) Flat preparation of the right half of an embryo. Visible is the basal side of the germ layer. (B) Basal optical section in two hemisegments through the developing neuropil between the second and the third walking leg. Medial is at the bottom. (C) Transverse optical section. Apical is at the top. (D,E) Two magnifications are shown. (A) Prospero is expressed along the entire developing CNS of the prosoma and the opisthosoma. Prospero is also expressed in the cephalic lobe and the PNS. (B) Prospero expressing cells are localised basally around the developing neuropil (asteriks). (C) Prospero is expressed in cells that have completed invagination (arrow heads). Basally the developing neuropil (asteriks) is visible. (D) and (E). Prospero is also expressed in invaginating cells as visualized by the strong phalloidine-rhodamine stainings of the actin rich membranes of the invagination sites. *Pe*, pedipalpal segment; *L1* to *L4* walking legs 1 to 4, corresponding to prosomal segments 3 to 6.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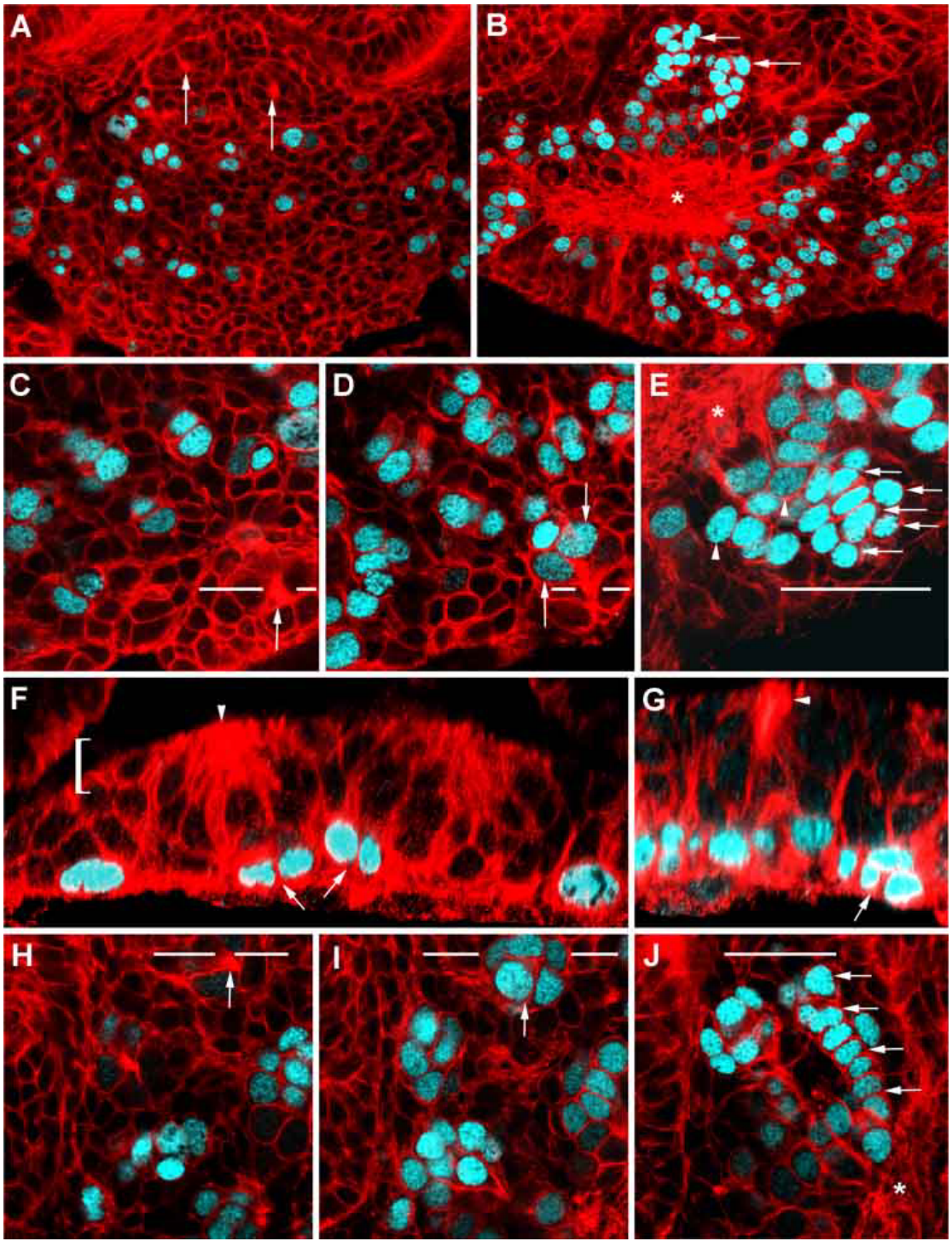
2.6.2. Localisation of Prospero expressing cells

Different optical sections show that cells which express Prospero extend basally from the invagination sites to the developing CNS (Fig. 21A,B, arrows). Transverse optical sections show that Prospero expressing cells are not found at the apical surface of the neuroectoderm (Fig. 21F, bracket). Many Prospero expressing cells belong to the most basal cell layer of the developing CNS (Fig. 21G, arrow). Two invagination sites which are medial (Fig. 21C,D,E) and lateral (Fig. 21H,I,J) of the developing neuropil show comparable localisations of Prospero expressing cells:

Series of sections from apical to basal of these invagination sites reveal that cells which express Prospero have distinct positions depending on the cell layer: There are Prospero expressing cells which are beneath the apical actin rich dots of the invagination sites (Fig. 21C,D and 21H,I, arrows). The Prospero expressing cells which are located more basally are closer to the developing neuropil (Fig. 21E arrows). Such Prospero expressing cells can be organized in a row which extends from the invagination site to the developing neuropil (Fig. 21J, arrows).

Many Prospero expressing cells can be identified as neurons by their axonal outgrowth extending into the neuropil and their cell bodys close to it (Fig. 21E arow heads). These neurons express Prospero at different levels. Some neurons show high levels of Prospero expression but many cells also show weaker levels. Most cells which are most basally but not differentiated into neurons express prospero at high levels (Fig. 21E, arrows).

Fig. 21 (A-J) Localisation of Prospero expressing cells. Confocal micrographs of tissues were taken from spider embryos about 220 hours after egg laying. Prospero is cyan green. Actin is red. (A,B) Two optical sections of a spider embryo were taken at the same position between the third and the fourth walking leg. Medial is at the bottom and lateral at the top. (A) The optical section is more apical. (B) The optical section is more basal. (C-E) Series of optical sections from apical to basal at an identical position of one invagination site. The medial side is at the bottom of the picture. The invagination site is medial to the neuropil. The white bar marks the height of the actin dot at the apical surface of the invagination site. (F,G) Two transverse optical sections. Apical is at the top. (H-J) Series of optical sections from apical to basal at an identical position of one hemisegment. Medial is at the bottom. A invagination site lateral of the neuropil in the fourth walking leg segment is visualized. The white bar marks the height of the actin dot at the apical surface of the invagination site. (C,H) The most apical optical sections. (D,I) More basal optical sections. (E,J) The most basal optical sections. (A) Two lateral invagination sites are visible (arrows). (B) The basal optical section shows that Prospero expressing cells extend from their basal position at the invagination site to the neuropil (asteriks). (C) The actin dot at the apical surface of the neuroectoderm is visible (arrow). (D) Two Prospero expressing cells at the invagination site are visible (arrows). (E) The most basal Prospero expressing cells are visible. These cells (arrows) are shifted towards the neuropil (asteriks) in comparison to the more apical Prospero expressing cells in (E, arrows). The most basal cells (arrows) express Prospero at high levels. Nerve cells (arrow heads) express Prospero at different levels. (F) There are no Prospero expressing cells in the apical layer of the neuroectoderm (bracket), but basally (arrows). The actin dot of an invagination site is visible at the apical surface (arrow head). (G) Many Prospero expressing cells belong to the most basal cell layer (arrow). The actin dot of an invagination site is visible at the apical surface (arrow head). (H) The actin dot at the apical surface of the neuroectoderm is visible (arrow). (I) Prospero expressing cells at the invagination site are visible (arrow). (J) More Prospero expressing cells are visible basally. These cells (arrows) are shifted towards the neuropil (asteriks) in comparison to the more apical Prospero expressing cells in (I, arrows).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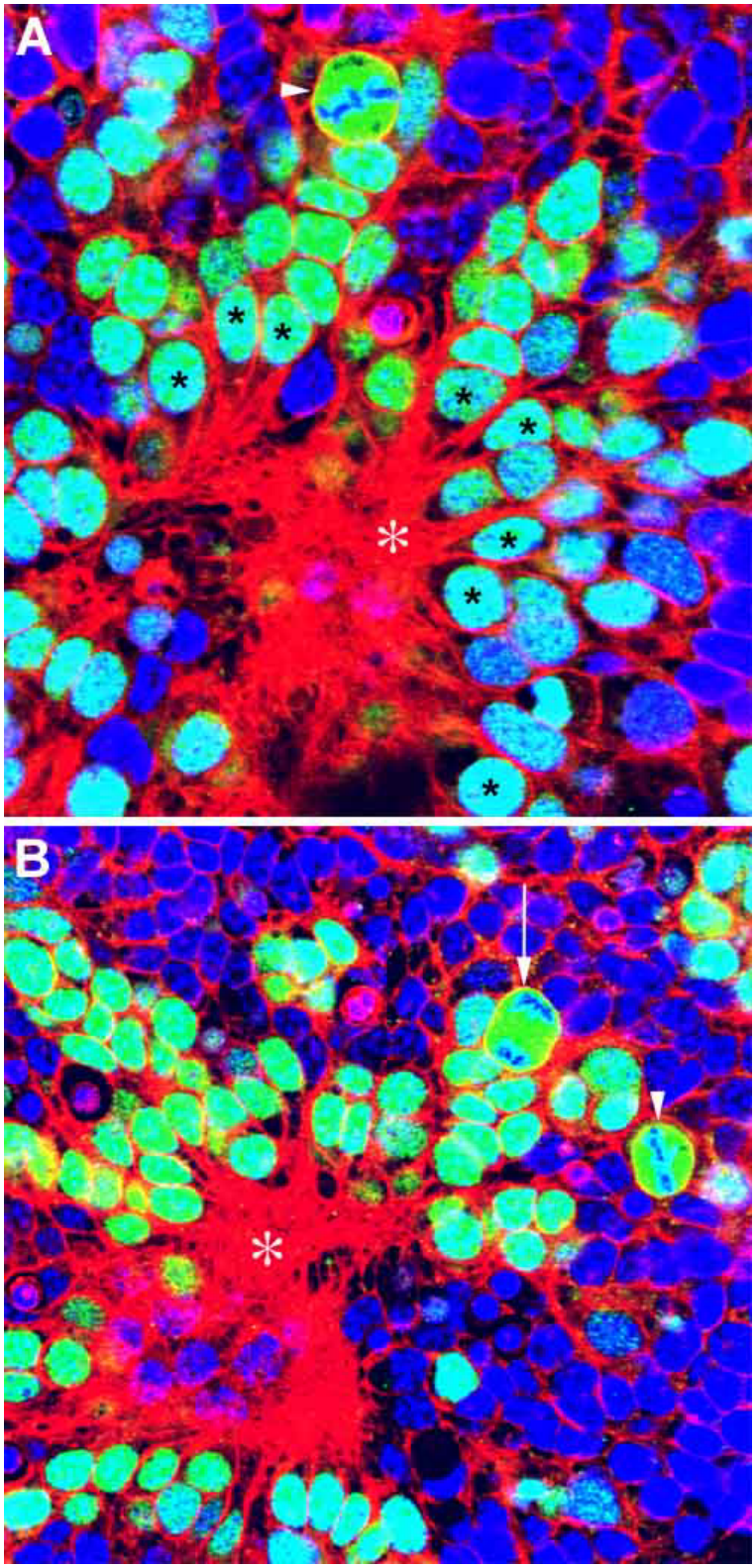
2.6.3. Localisation of Prospero protein

Prospero accumulates in cell nuclei as revealed in stainings of DNA and the protein (Fig. 22). In neurons Prospero is localised exclusively in cell nuclei (Fig. 22A; black asterisks). In cells which are mitotically active Prospero is found in the cytoplasm (Fig. 22A,B, arrow heads, arrow). In the two basal optical section one cell is in anaphase (Fig. 22B, arrow) and the other two are in metaphase (Fig. 22A,B, arrow heads). All three cells are not close to the neuropil. Prospero expressing cells which are mitotically active and directly at the neuropil were not found (see also Fig. 23D1,D2,E4,F3, and G3).

Furthermore stainings of DNA show that in mitotically active cells the protein of Prospero is equally segregated into both daughter cells.

Fig. 22 (A,B) Localisation of Prospero protein in cells. Confocal micrographs of tissues were taken from spider embryos about 220 hours after egg laying. Prospero is green. DNA is blue. Actin is red. (A) A basal optical section shows nerve cells (black asteriks) which extend their neuronal projections into the neuropil (white asteriks). Prospero protein is colocalized with the DNA but not found at the basis of these neuronal projections. Note that cell nuclei ocuppy a large diameter in all cells. A mitotically active cell in metaphase is expressing Prospero at high levels (arrow head). This cell is not directly at the neuropil. (B) A basal optical section shows a mitotically active cell in anaphase (arrow) and another one in metaphase (arrow head). Both cells express Prospero at high levels and are not directly localised at the neuropil (asteriks).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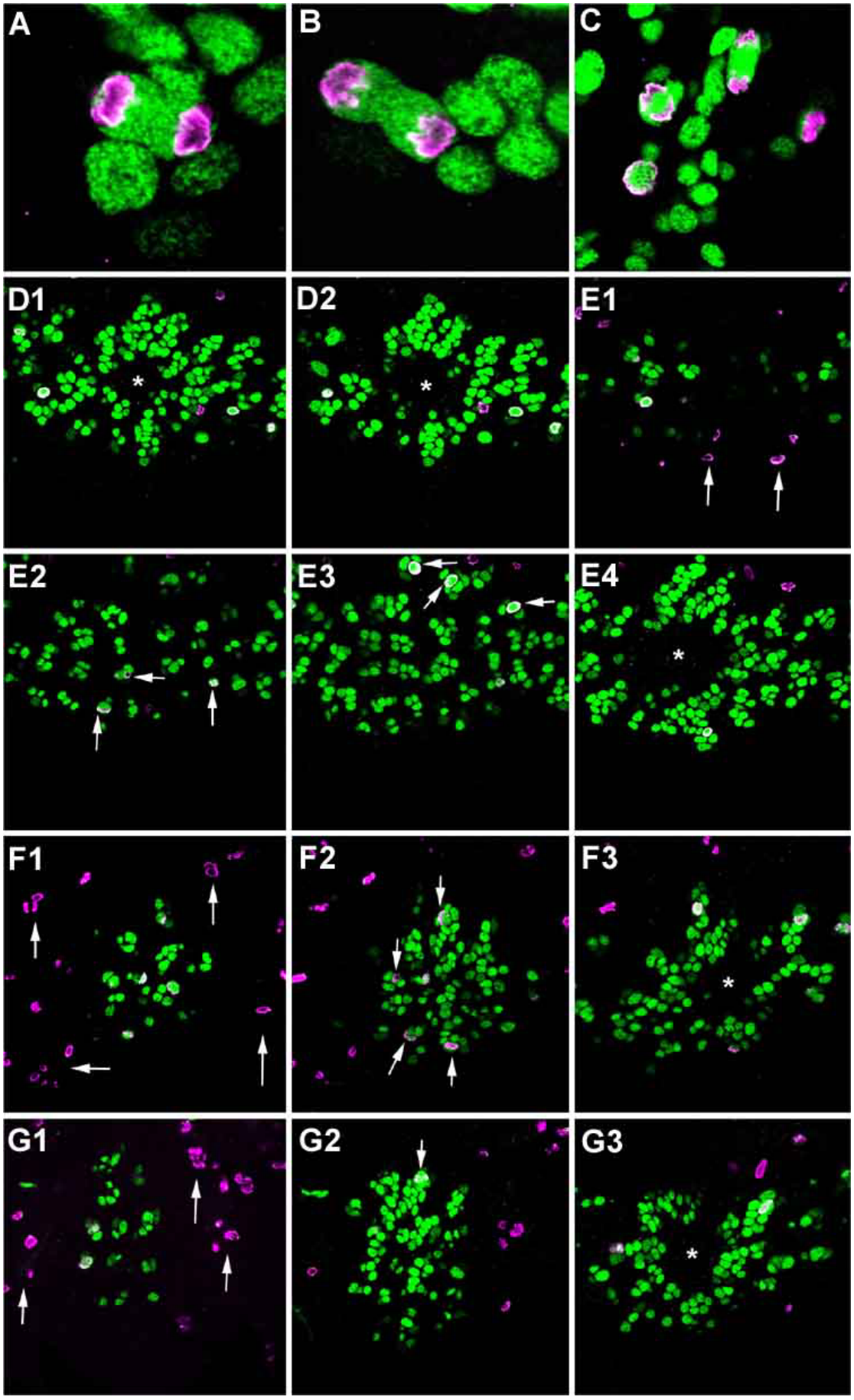
2.6.4. Symmetric segregation of Prospero protein during mitosis

To analyse the segregation of Prospero during mitosis in more detail double stainings with an antibody against phosphorylated Histone 3, which specifically stains the condensed chromosomes of mitotically active cells, were performed (Fig. 23). These experiments were performed with embryos about 220 hours (Fig. 23A-E4) and about 210 hours (Fig. 23F1-G3) after egg laying. The results are identical. There is no evidence for asymmetric segregation of Prospero during mitosis (Fig. 23A,B,C). Instead, stainings revealed that Prospero protein is detectable during different stages of mitosis and always segregates symmetrically into both daughter cells. Series of optical sections from apical to basal show that only few prospero expressing cells are mitotically active (Fig. 23D1-G3). The germ layer is not plane but arched (Fig. 20F). In the first pictures of three series the center of the optical sections correspond to basal tissue layers with Prospero expressing cells (Fig. 23E1,F1,G1). In these pictures the most mitotically active cells are found at the borders which correspond to apical cell layers (Fig. 23E1,F1,G1, arrows). Only few Prospero expressing cells are mitotically active (Fig. 23E2,E3,F2,G2, arrows). Furthermore the most basal optical sections show that there are no mitotically active Prospero expressing cells in close touch with the developing neuropils (Fig. 23D1,D2,E4,F3,G3, asteriks). The neuropils are surounded by Prospero expressing cells which are mitotically inactive.

These Prospero expressing cells form a row from the invagination site to the neuropil (asteriks).

Fig. 23 Localisation of Prospero in mitotically active cells and localisation of these cells themselfes. Prospero is green. Phosphorylated Histon 3 is violet. (D1-G3) Lateral is at the top. Series of optical sections: The lowest number (1) coresponds to the most apical and the highest number (3 or 4) to the most basal optical section of the series. The letters (D, E, F, or G) correspond each to one series of optical sections at an identical position of the developing CNS. (A-E4) Confocal micrographs of tissues were taken from embryos about 220 hours after egg laying. (F1-G3) Confocal micrographs of tissues were taken from embryos about 210 hours after egg laying. (A-C) Three magnifications of Prospero expressing cells which are mitotically active. (D1,2) Two optical sections in a hemisegment of the fourth walking leg. (E1-E4 and F1-F3) Two series of optical sections in one hemisegment of the first walking leg. (G1-G3) Series of optical sections in one hemisegment of the fourth walking leg. (A) A mitotically active cell in anaphase is segregating Prospero equally to both daughter cells. (B)) A mitotically active cell in telophase is segregating Prospero equally to both daughter cells. (C) Different mitotically active stages of Prospero expressing cells are visible. (E1,F1,G1) In the most apical optical sections of these series many mitotically active cells are visualized which do not express Prospero (arrows). (E2,E3,F2,G2) Only few Prospero expressing cells are mitotically active. (D1,D2,E4,F3,G3) There are no mitotically active cells directly at the neuropil (asteriks).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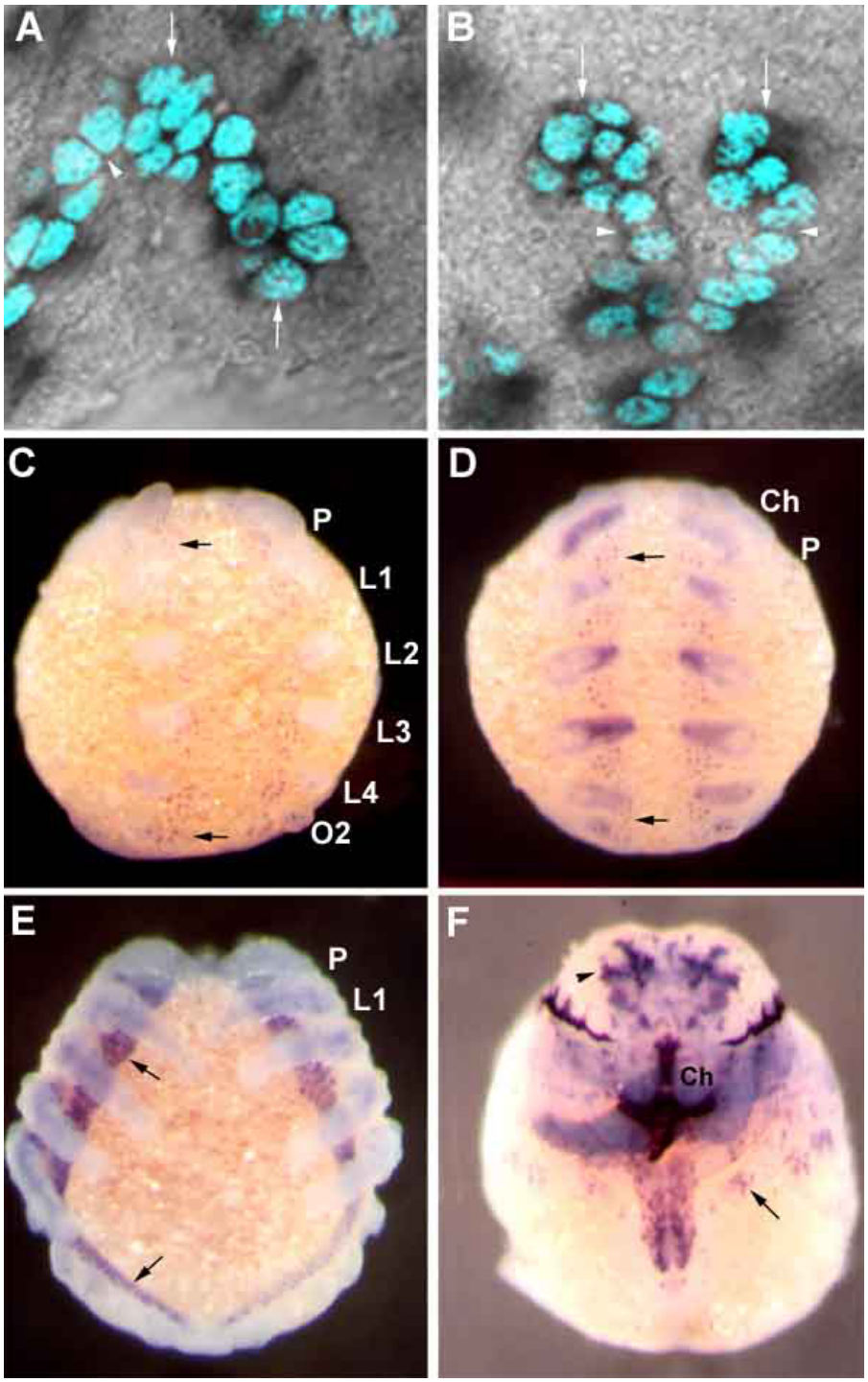
2.6.5. Transcription of *prospero*

In situ hybridisations show that high levels of *prospero* transcription are present at the invagination sites (Fig. 24A,B arrows). Many cells which express Prospero protein at high levels but are not directly at the invagination sites show only weak levels of *prospero* transcription (Fig. 24D, E, arrow heads). First expression of *prospero* is visible in the prosoma and the first opisthosomal segment at about 140 hours after egg laying (Fig. 24C). About 150 hours after egg laying 10-15 *prospero* expressing cell clusters can be detected in all prosomal hemisements (Fig. 24D). Strongest expression levels along the entire developing CNS in the prosoma and the opisthosoma are reached about 220 hours after egg laying (Fig. 24E). After about 240 hours *prospero* is still expressed in the prosomal CNS, the cephalic lobe and at high levels in the PNS (Fig. 24F).

Fig. 24 (A-F) Expression of Prospero RNA. (A,B) Confocal micrographs of optical sections were taken from embryos about 220 hours after egg laying in a hemisegment of the third walking leg. Prospero protein is cyan green. The RNA is black. Optical layers to visualize the RNA expression were taken with transmission microscopie. (A) A invagination site which is localized medial to the neuropil is shown. (B) Two invagination sites which are localized lateral to the neuropil are shown. (C-F) Different developmental stages are shown. Expression of *prospero* was visualized by in situ hybridisation. (A,B) Highest expression levels of Prospero RNA are found basally at invagination sites (arrows). Some cells which are not directly at the invagination site do not have detectable levels of Prospero RNA (arrow heads). (C) Expression of *prospero* in an embryo about 140 hours after egg laying (D) Expression of *prospero* in an embryo about 150 hours after egg laying (E) Expression of *prospero* in an embryo about 220 hours after egg laying (F) Expression of *prospero* in an embryo about 240 hours after egg laying. *Ch*, cheliceral segment; *Pe*, pedipalpal segment; *L1* to *L4* walking legs 1 to 4, corresponding to prosomal segments 3 to 6.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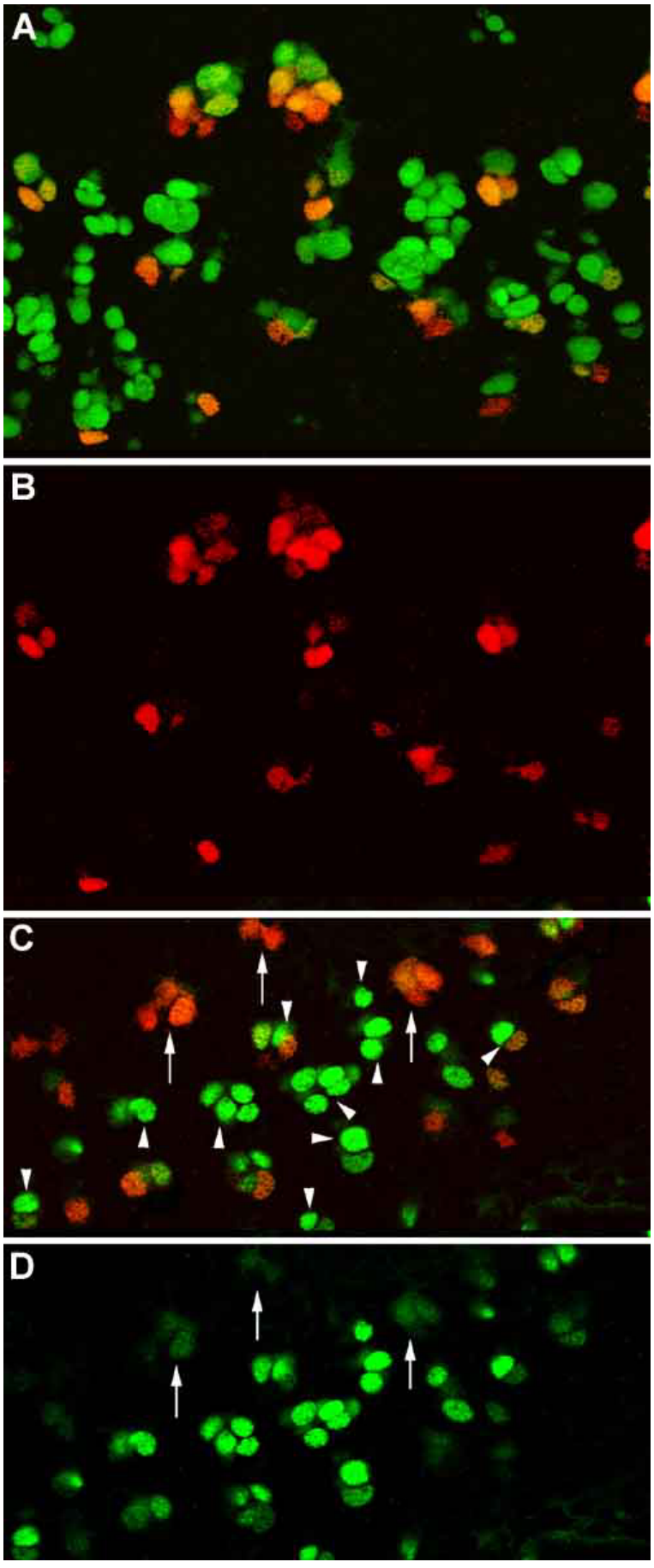
2.7. Double stainings of Prospero and Snail

Double stainings in embryos about 200 hours after egg laying reveal that more cells express Prospero than Snail (Fig. 25A). Most cells which express Snail also express Prospero (Fig. 25A,B). In many cells with detectable levels of Snail expression the corresponding levels of Prospero are absent or weak (Fig. 25C,D arrows). Cells which express Prospero strongly, never express Snail at the same time (Fig. 25C, arrow heads) but there are also cells which are double stained and express both proteins at weak levels.

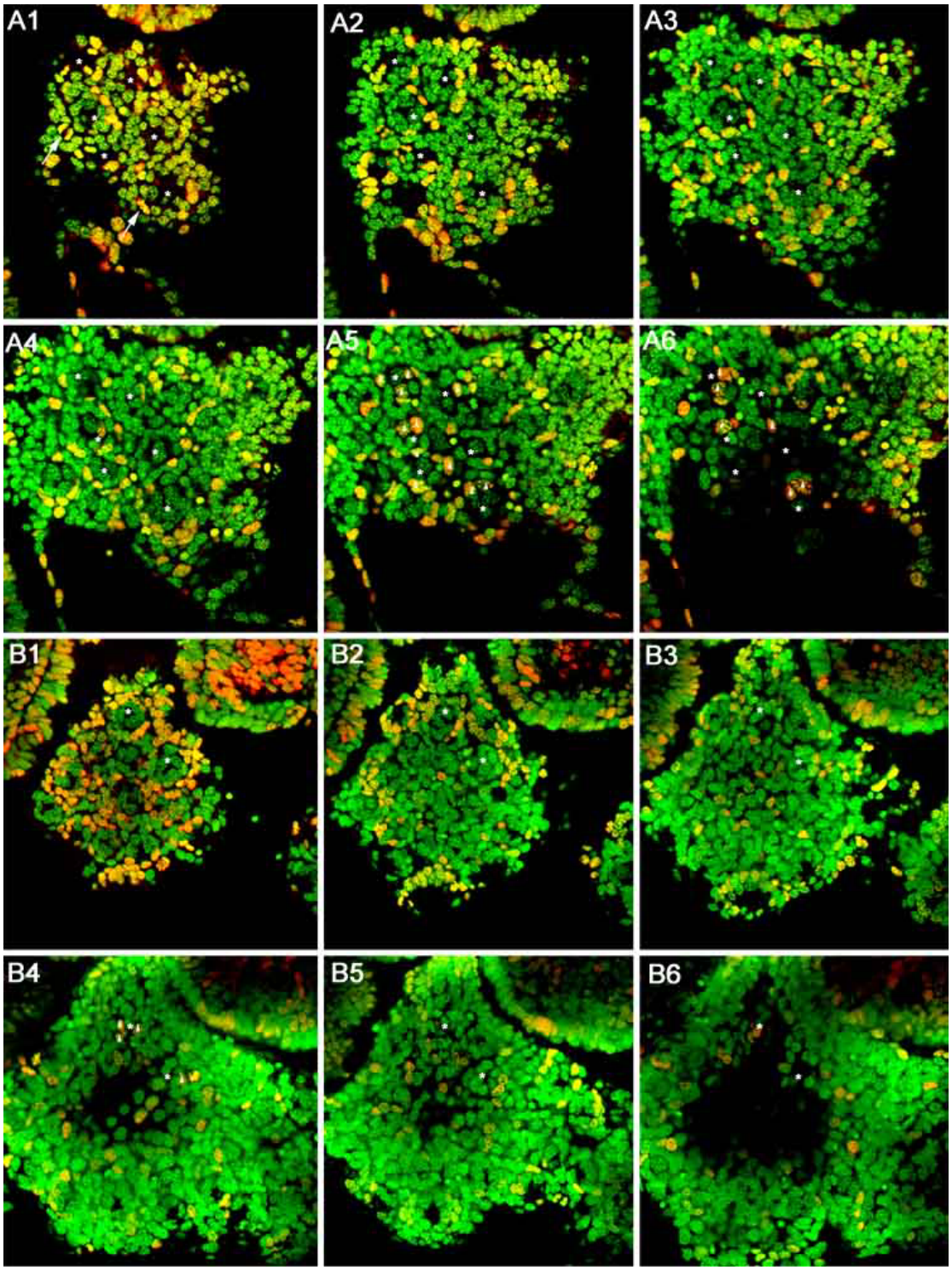
Fig. 25 (A-D) Double staining experiment. Confocal micrographs of tissues were taken from embryos about 200 hours after egg laying. Prospero is green. Snail is red. Lateral is at the top. (A,B) Two identical optical sections in two hemisegments between the third and the fourth walking leg. (C,D) Two identical optical sections in two hemisegments between the second and the third walking leg. (A) More cells express Prospero than Snail. Most cells which express Snail also express Prospero. (B) As a control the optical layer of Snail is shown. The Snail expressing cells are red and not orange or yellow like the most Snail expressing cells in (A). (C) Cells which express Prospero at high levels do not express Snail (arrow heads). (D) Only cells which express Prospero at weak levels (arrows) also express Snail (C, arrows).


A
B
C
D

2.8. Labeling experiments with BrdU

The substance „5-bromo-2`-deoxyuridine“ (BrdU) is incorporated during synthesis of DNA instead of the base thymidin. So it is a marker of cells which have been mitotically active. For the experiment of BrdU injection embryos about 190 hours after egg laying were used. After injection of BrdU embryos were incubated 90 minutes before fixation (see also material and methods). At the apical surface the invagination sites can be recognized as areas free of cell nuclei (Fig. 26,A1 asteriks). In the most apical cell layer all cells are labeled with BrdU (Fig. 26A1) as indicated by the yellow color. Furthermore basally at the invagination sites (asteriks) there are also cells which are labeled (Fig. 26,A5,A6, triangles). The large diameter of these cells which are labeled basally is a typical characteristic of invaginating cells. Another series of optical sections is starting with a more basal cell layer where two invagination sites can be identified unambigously (Fig. 26B1, asteriks). Many cells have incorporated BrdU. The other optical sections were taken from tissues which are localized more basally (Fig. 26B2-B6). Basally are only few cells which are labeled with BrdU. Optical sections of another embryo which was incubated only for 90 minutes after injection with BrdU are shown. Many cells which are localized around the invagination sites (Fig. C1, asteriks) incorporated BrdU. Optical sections which were taken of tissues more basally show that numbers of cells which incorporated BrdU are decreasing (Fig. 26C2-C6). In the performed experiments there are cells which are labeled strongly whereas others show only weak labeling. This may be due to different numbers of mitotic divisions of these cells. Maybe that after incubation times between 1 and 3 hours the cells which are labeled more strong than the others performed more than one mitotic division. Optical sections of an embryo which was incubated for 17 hours after BrdU injection are shown. Apically BrdU labeled cells (Fig. 26D1, arrows) are localized around two invagination sites (asteriks). Basally only few cells incorporated BrdU (Fig. 26D2-D6). The fact that there are apically also many cells which are not labeled may be due to a overall decrease in the concentration of BrdU after several hours. Like in the other performed experiments the relation between cells which are labeled basally and apically is the same: In all experiments performed apically are clearly more cells labelled than basally.

Fig. 26 Labeling experiments with BrdU. BrdU injections were performed with embryos about 180 hours after egg laying. DNA is green. BrdU is red. As the labeled cells are double stained they are yellow or orange. Confocal micrographs were taken of embryos incubated for 90 minutes (A1-B6), 180 minutes (C1-C6) and 17 hours (D1-D6) at 28°C. The same letters correspond to optical sections at identical positions.The lowest number (1) is the most apical and the highest number (6) the most basal optical section. The medial side is at the bottom. (A1-A6) Optical sections were taken of one cheliceral hemisegment. (B1-B6) Optical sections were taken of the hemisegments between the third and the fourth walking leg. (C1-C6) Optical sections were taken of the hemisegments between the third and the fourth walking leg. (D1-D6) Optical sections were taken between the cheliceral and the pedipalp hemisegments. (A1) Apically all cells are labeled. The invagination sites are free of cell nuclei (asteriks). (A2-A4) Basally the numbers of labeled cells are decreasing. (A5,A6) At the positions of the invagination sites (asteriks) labeled cells are visible (triangles). These cells have larger cell nuclei than the cells of surrounding tissues. (B1) Two invagination sites are visible (asteriks). In the most apical optical section many cells are labeled (B2-B6) Basal optical sections show only few labeled cells. Some labeld cells have large cell nuclei (triangles) at the invagination sites (asteriks). (C1) Many cells are labeled. Invagination sites are visible (asteriks). (C2-C4) In tissues which are localized more basally the number of labeled cells decreases. (C5,C6) In the most basal tissues localized in the center of these pictures there are only few labeled cells. (D1) Labeled cells (arrows) are found around two invagination sites (astertiks). (D2-D6) In tissues which are localized more basally the number of labeled cells decreases.


A1
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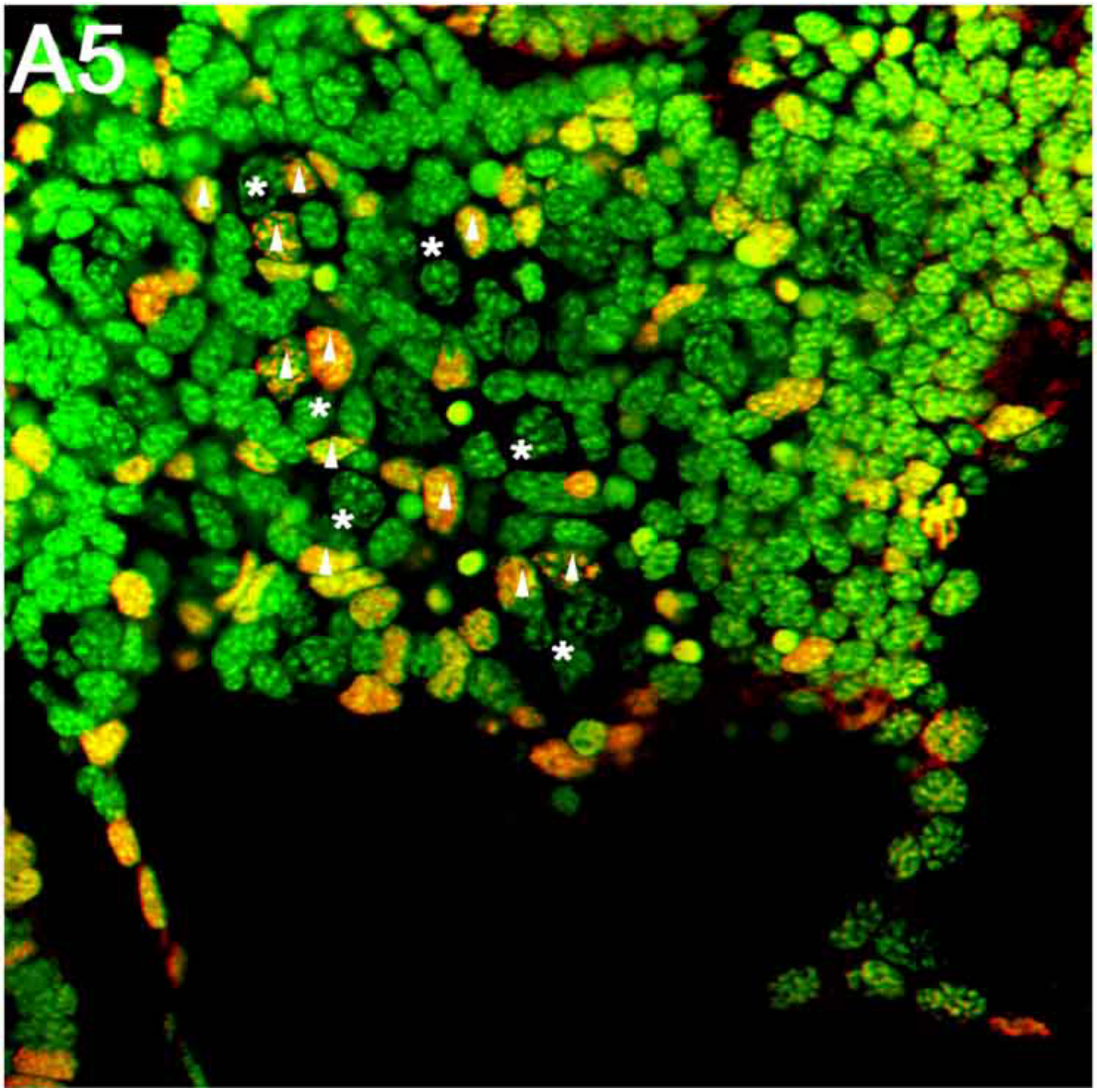
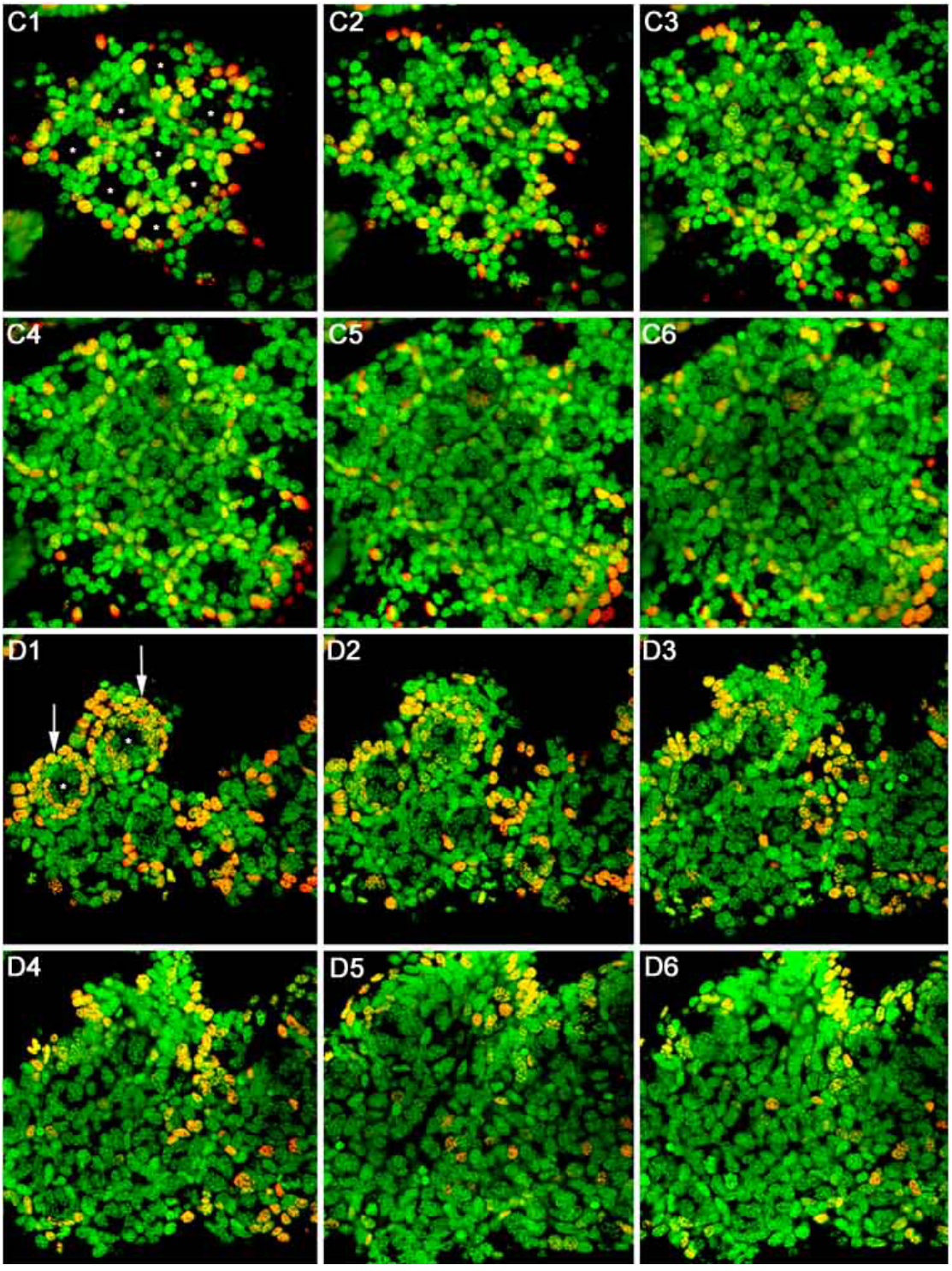

A5


C1
C2
C3
C4
C5
C6
D1
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D3
D4
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D6

Discussion

The present work shows that *prospero*, *snail* and *Krüppel* spider homologues of the respective *Drosophila* genes are expressed in the developing CNS of *Cupiennius salei*. Although this expression occurs in both species there are essential differences in the generation of the CNS between the spider *Cupiennius salei* and *Drosophila*. These differences are particularly clearly reflected in the distinct expression pattern and protein distribution of Prospero.

1. Direct recruitment instead of stem cells: the important difference between spider and insect neurogenesis

Staining experiments with antibodies against phosphorylated Histon 3 and BrdU labeling experiments show that most mitotic divisions occur in the most apical cell layer. In the basal cell layer only few cells are mitotically active. There are also no local concentrations of mitotically active cells beneath the neuroectoderm as revealed by the BrdU labelling experiment 3 hours after incubation. This would be expected if stem cells would be present in a cell layer beneath the neuroectoderm. Instead these cells which have been mitotically active are not concentrated at determined positions but distributed basally. There are also some BrdU labelled invaginating cells. Invaginating cells have cytoplasmatic extensions to the apical surface. So, they are not mitotically active. As invaginating cells are not mitotically active, the labelled invaginating cells must have incorporated the BrdU at the apical surface of the neuroectoderm. These results indicate that in *Cupiennius* there are no stem cells like neuroblasts in insects or GMC´s. Instead the experiments argue in favor of a direct recruitment of cells at the apical surface (Stollewerk et al., 2001).

In *Drosophila* the neuroblasts retain a stem cell fate during several rounds of mitotic divisions and produce GMC´s which only divide once (Doe and Goodman, 1985). Prospero is expressed in all neuroblasts (Hirata et al., 1995; Spana and Doe, 1995; Matsuzaki et al., 1998). The protein segregates from the cytoplasm into the cell nuclei of GMC`s after asymmetric division of neuroblasts (Vaessin et al., 1991; Hirata et al., 1995; Spana and Doe, 1995; Matsuzaki et al., 1998; Srinivasan et al., 1998).

Prospero is also expressed in GMC`s and it remains expressed transiently in neurons (Doe and Goodman, 1985; Doe et al., 1991; Broadus and Doe, 1995; Spana and Doe, 1995).

It was shown that *Drosophila* Prospero is a critical regulator of the transition from mitotically active cells to differentiated neurons and that it terminates cell proliferation (Li and Vaessin, 2000). The cell fate distinction between neuroblasts and GMC`s is directly coupled with the asymmetric segregation of determinants like Prospero.

Experiments have shown that in *Cupiennius* Prospero expressing cells which are mitotically active segregate the protein symmetrically into both daughter cells. There is no evidence for asymmetric segregation of Prospero into the daughter cells during mitosis in *Cupiennius*.

In *Cupiennius* the asymmetric segregation of Prospero is not necessary as there are no cells like the neuroblasts which keep a stem cell fate after several rounds of mitotic division. Instead a more simple mode of direct cell recruitement and differentiation into nerve cells is present.

In vertebrates asymmetric localization of Prox-1 was also not found. The present results suggest that the asymmetric segregation of Prospero in insects evolved as a specialized form in the arthropod clade which posesses neuroblasts. Thus it is a derived character and not shared by a basal arthropod group such as the chelicerates. In this context it would be interesting to study the expression of the prospero gene in crustacean species which are considered to have neuroblasts (Dohle and Scholz, 1988; Scholtz, 1992; Gerberding, 1997;).

Most, if not all cells which completed invagination but have not grown axons yet show the highest levels of Prospero expression in the spider. Probably most of these cells differentiate directly into neurons (see below). In contrast to the spider, highest expression levels of *Drosphila* Prospero are found in neuroblasts which are mitotically active.

2. Snail, Prospero and Krüppel-1 expression during spider neurogenesis reveals similarities between *Cupiennius* and insects.

The results show that in *Cupiennius* most invaginating cells express Snail. Expression levels are variable. Snail expression was not found in cells of the neuroectoderm which have not yet started invagination. Furthermore, double staining experiments with phosphorylated Histon 3 have shown that Snail expressing cells are not mitotically active.

This fits to the finding that Snail is expressed in invaginating cells. So results suggest that the expression of Snail starts after the beginning of invagination and ends before the cells are completely invaginated.

Prospero is expressed in cells at distinct states of differentiation. The protein is expressed in invaginating cells. Highest expression levels of Prospero are found in cells which completed invagination but do not have axons. Finally, Prospero is expressed in most nerve cells at distinct levels.

Double stainings show that strong expression levels of Snail and Prospero are in complementary cell populations. Snail is expressed in invaginating cells which show only weak expression of Prospero. In cells which express Prospero strongly Snail was not found. So the expression of Snail starts earlier than the expression of Prospero. The experiments suggest that the expression of Prospero then continues until the cells differentiate into neurons. Snail and Prospero are expressed at different states of differentiation. This argues in favor of a stepwise differentiation process of invaginating cells.

In *Drosophila* Snail is expressed in all neuroblasts and there exists also a limited expression of Snail RNA in some GMC´s (Ashraf and Ip, 2001; Cai, et al., 2001; Ashraf, et al., 1999; Ip, et al., 1994). Snail is localized in the nuclei of the neuroblasts whereas Prospero moves only into the nuclei of the GMC`s. So, in *Drosophila* and *Cupiennius* Snail proteins accumulate in the nuclei of cells before the Prospero proteins do that. Furthermore in both arthropod groups during mitotic divisions of cells which are localized basally Prospero homologues are segregated symmetrically into the daughter cells. Prospero homologues are also expressed in the neurons of *Drosophila* and *Cupiennius*.

In *Cupiennius* Krüppel-1 is expressed at variable levels in the neuroectoderm but also in the basal cell layers of the developing CNS. Krüppel-1 is expressed in most cells of these layers. The expression levels are variable. High levels of Krüppel-1 translation and transcription at the invagination sites are transient and show a dynamic change from the anterior to the more posterior segments. Furthermore there is a time delay between the transcription and translation of Krüppel-1 at high levels in the invagination sites.

In *Drosophila* Krüppel is also expressed in many cells along the entire neuroectoderm, most neuroblasts, a large fraction of GMC´s and their progeny (Romani, et al., 1996). There exist spatial differences between the distribution of the Krüppel transcript and the protein in the developing CNS of *Drosophila* (Gaul et al., 1987). The results provide evidence for posttranscriptional control of Krüppel (Gaul et al., 1987).

In *Cupiennius* a spatial difference between transcription and translation of Krüppel-1 was not observed. Instead there exists a temporal difference between transcription and translation of Krüppel-1.

In spider embryos about 200 hours after egg laying Krüppel-1 is expressed in most cells of the neuroectoderm. In the same developmental stage Snail is expressed only in invaginating cells. Similarly, in *Drosophila* stage 8 embryos Krüppel is expressed in many cells of the neuroectoderm (Romani et al., 1996). The Snail transcript accumulates in neuroblast precusors and in segregated neuroblasts of stage 8 embryos (Alberga et al., 1991). So, whereas the Krüppel homologues are expressed in many cells of the neuroectoderm in both arthropod groups the expression of the Snail homologues is reduced to cells which give raise to basal nerve cells.

There exists a similar sucession of the expression of Snail and Prospero homologues in *Drosophila* and *Cupiennius*. Furthermore Snail and Krüppel homologues are expressed in comparable tissues of the neuroectoderm of both species. The results show that in the spider *Cupiennius*, as in *Drosophila* homologues of Prospero, Snail and Krüppel are expressed in cells of the developing CNS. This suggests that the Prospero, Snail and Krüppel spider homologues probably also play a role in the differentiation process of cells which give rise to neurons. The expression pattern of these genes in spiders and insects argues in favor of a common ancestral process of nerve cell specification in the arthropod clade.

3. The pattern of Prospero expression suggests that cells move into the direction of the developing neuropil

Prospero expressing cells are organized in rows which extend from the sites of invaginations to the developing neuropil. The most basal Prospero expressing cells at an invagination site are more close at the neuropil than Prospero expressing cells which are more apically.

These results suggest that Prospero expressing cells in embryos about 220 hours after egg laying invaginate into the direction of the developing neuropil. Then these cells move into the direction of the developing neuropil and differentiate into nerve cells. It was not shown directly that Prospero expressing cells are moving into the direction of the developing neuropil.

Alternatively, cells which never invaginated could start to express Prospero. This hypothesis is not favored because it does not explain the organized pattern of Prospero expressing cells around the neuropil.

Prospero double stainings with phosphorylated Histon 3 did not identify Prospero expressing cells which are mitotically active directly at the neuropil. However, this indicates that most or all cells close to the neuropil are differentiated.

4. How many Prospero expressing cells are mitotically active ?

Prospero expressing cells which are mitotically active can be directly beneath the invagination sites. They can also be between the invagination sites and the developing neuropil but they are neither found directly at the neuropil nor can they be invaginating cells. These mitotically active cells express Prospero at high levels. The BrdU labeling experiments have shown that basally only few cells are mitotically active. However, it cannot be excluded totally that every cell which detached completely from the neuroectoderm is undergoing mitosis one time. Low concentrations of BrdU or death of embryonic tissue after longer incubation times can lead to the „false negative“ impression that basally only few cells were mitotically active.

However, results so far suggest that basally probably only a small group of Prospero expressing cells is undergoing mitosis. BrdU labelling coupled with Prospero staining experiments will provide further insights to this question. If there exists only a small population of Prospero expressing cells which is mitotically active then it is not known if these cells give rise to a distinct specialized cell population, different to those cells that do not undergo mitosis.

5. How are final nerve cell numbers defined in the spider ?

Results show that in a spider embryo about 200 hours after egg laying the numbers of invaginating cells at a defined invagination site vary in different segments. In a later developmental stage about 230 hours after egg laying the invagination sites in segments of the opisthosoma do not reach the same high numbers of invaginating cells as the BLC in the prosomal segments at a developmental stage of after about 200 hours after egg laying.

Final nerve cell numbers could be mainly defined by the direct recruitment of cells in the neuroectoderm without further mitotic divisions.

So, one hypothesis is that there exist segment specific differences in the numbers of invaginating cells at an identical invagination site. The other hypothesis is that the observed differences only reflect time delays in the development of different segments along the anterior-potserior axis. To find out which hypothesis is true, it will be necessary to compare the numbers of invaginating cells at determined invagination sites of more different developmental stages.

In insects the pattern of neuroblast delamination is almost identical between the thoracic and abdominal segments (Truman et al., 1998; Doe and Goodman, 1985). The final number of nerve cells are mainly defined by the number of mitotic divisions which the neuroblasts undergoe to produce GMC`s (Truman et al., 1998).

6. The expression of Snail in the developing PNS

Snail is expressed in cells which are associated with mechanosensitive hairs. Position and number of these cells suggest that they are the sensory cells of a „Trichobothrium“. Some authors argue that this mechanosensitive structure is believed to have evolved several times independently in different arthropod groups (Westheide und Rieger, 1996). So the „Trichobothria“ of spiders, myriapods and insects are considered to have a polyphyletic origin (Westheide und Rieger, 1996).

In *Drosphila* Snail is also expressed in the PNS (Ip, et al., 1994). Expression of Snail was found in sensory mother cells. These give rise to cells of the mechanosensitive hairs.

The expression of Snail could reflect a common ancestral origin of „Trichobothria“ in the PNS of both arthropod groups instead of a polyphyletic origin. So the expression of Snail in cells which are associated with mechanosensitive structures argues in favor of a monophylectic origin of „Trichobothria“ in spiders and insects. On the other hand, as mechanosensitive structures perhaps evolved several times independently in different arthropod groups, Snail could also have been recruted twice in spiders and insects to specify cell fates of the PNS.

It will be interesting to find out whether other molecular markers than Snail are expressed in the „Trichobothria“ of both species. Expression of spider Prospero in cells which are associated with a „Trichobothrium“ was not yet discovered so far, but cannot be excluded.

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Cupiennius *Prospero*

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                9          18          27          36          45          54
5'  AAA GGG AAA TCG TCT GCC GCC AAG ACG AAA AGG GTC CGT CAG CGC GTG GAT GCG
     K   G   K   S   S   A   A   K   T   K   R   V   R   Q   R   V   D   A

               63          72          81          90          99         108
    GGG GAG CCT CGG AAC AGT TAC GCC AGC ATC CCC AAC TTC AGT TCC AGA CCC GCG
     G   E   P   R   N   S   Y   A   S   I   P   N   F   S   S   R   P   A

              117         126         135         144         153         162
    GGG TTC GGC GTG AAC AAC GGC TTC GCC AGC AAG ATG CTC TGC GAC CTC ATA GGT
     G   F   G   V   N   N   G   F   A   S   K   M   L   C   D   L   I   G

              171         180         189         198         207         216
    TCG GGA CTT AGG CCC AAG GAG GAG TTG CTG CTG GAC AGC GGC GAT CGC AAC GGA
     S   G   L   R   P   K   E   E   L   L   L   D   S   G   D   R   N   G

              225         234         243         252         261         270
    GCG TCG CGG ATG TAC ATG AAC GGG GGC GAC CTG GTG TTC GGG AGG GGT CTC GAG
     A   S   R   M   Y   M   N   G   G   D   L   V   F   G   R   G   L   E

              279         288         297         306         315         324
    AGC CCC GAG AGG TGC AGC AGC GCG GAG AGC TCG AAC AGC CAT TTG CTG CGC GAC
     S   P   E   R   C   S   S   A   E   S   S   N   S   H   L   L   R   D

              333         342         351         360         369         378
    ATT CTG CAG CAG GGG AGG GCC GCT TCC GGC GGT CAC GCT CCA AAG AGC CCC GCC
     I   L   Q   Q   G   R   A   A   S   G   G   H   A   P   K   S   P   A

              387         396         405         414         423         432
    AGG CAC GAC GAA GAT TCG AAC GAG AGC AAG GCC AGC ATC AGA ATG TCG GCG GCA
     R   H   D   E   D   S   N   E   S   K   A   S   I   R   M   S   A   A

              441         450         459         468         477         486
    GTC GGC TCG GCA GCA GCG GAC CGC AGT CCG GGA TCG TCC CGC GCC AGC AAC AGT
     V   G   S   A   A   A   D   R   S   P   G   S   S   R   A   S   N   S

              495         504         513         522         531         540
    CCC CCG CTC GAG ACG CTG TGG CAA CAG GAG GCC CCC TCC GCC CCC CTC AGT GCC
     P   P   L   E   T   L   W   Q   Q   E   A   P   S   A   P   L   S   A

              549         558         567         576         585         594
    CCG CCT TCC TCC TCC ACG GCC GAA GTG AAG CGG GCT CGC GTG GAG ACC ATA GTC
     P   P   S   S   S   T   A   E   V   K   R   A   R   V   E   T   I   V

              603         612         621         630         639         648
    AAC AAC ATG CTG CAG GGG CCT AGG AAC AGC GGT GTC AGT TCC GGG ATC AGC GAG
     N   N   M   L   Q   G   P   R   N   S   G   V   S   S   G   I   S   E

              657         666         675         684         693         702
    GGC CAG GCG CCC GTC AAC GGT TGC AAG AAG AGG AAG CTC TAC CAG CCC CAG CAG
     G   Q   A   P   V   N   G   C   K   K   R   K   L   Y   Q   P   Q   Q

              711         720         729         738         747         756
    CAC GAG ACC TCG TCC AAG AAC CCC GTG AAC GGA GAC TCG GAG CTC TAC GAG GAA
     H   E   T   S   S   K   N   P   V   N   G   D   S   E   L   Y   E   E
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       765        774        783        792        801        810
GAC GAG GAT TTC AGC GAC AAC GGG GGC AGC CCG GTG GCG AAG CGC CGG AAC ATC
 D   E   D   F   S   D   N   G   G   S   P   V   A   K   R   R   N   I

       819        828        837        846        855        864
AGC GGC GGC ACC CAG AAC AAT GAC CTG TTC TCC TTC AAA CAG CAG ATC AGG CAG
 S   G   G   T   Q   N   N   D   L   F   S   F   K   Q   Q   I   R   Q

       873        882        891        900        909        918
GTG CAG CAG CAA CTC GTG GCG CTG CAG CAA CAG TAC ATG GAG ATG GTT GTC GGT
 V   Q   Q   Q   L   V   A   L   Q   Q   Q   Y   M   E   M   V   V   G

       927        936        945        954        963        972
GAC AGC ACT GAC GAC GAT ATC TCC CGA GTG AAT TCC ACC ACG ACC ACA GCC AAC
 D   S   T   D   D   D   I   S   R   V   N   S   T   T   T   T   A   N

       981        990        999       1008       1017       1026
AAC AAC GGA AAC GAT CGA CCG AGG AGT GCC GAG GAA CTG TCC GAC TGC GAA GAC
 N   N   G   N   D   R   P   R   S   A   E   E   L   S   D   C   E   D

      1035       1044       1053       1062       1071       1080
ATG AAG CCT CGA ATC ATG GAA CAC AAA CCG GAC AGC ATG AGG ACA AAT AAA TCG
 M   K   P   R   I   M   E   H   K   P   D   S   M   R   T   N   K   S

      1089       1098       1107       1116       1125       1134
TCC TCC TTC GTG GAC TAC GAC AAC GCT AGC CTT TAT GAG GAG CAA CGT CGC CTG
 S   S   F   V   D   Y   D   N   A   S   L   Y   E   E   Q   R   R   L

      1143       1152       1161       1170       1179       1188
GTG ATT GAT GAC GGC AAA GAT ACA GCC CCA AAA TCT GAC CTC CCA GAG CTG CGA
 V   I   D   D   G   K   D   T   A   P   K   S   D   L   P   E   L   R

      1197       1206       1215       1224       1233       1242
CAG TCG TGC ATC GTG CAG CCA TCC CCG TGC CCT AAC TTG GAT TAC GAG TGG CTC
 Q   S   C   I   V   Q   P   S   P   C   P   N   L   D   Y   E   W   L

      1251       1260       1269       1278       1287       1296
GCG GAA TCT CTG AAA GCA AAG CTG TCC ACT TCC CTC TCT CAG GTG GTG GAT GCT
 A   E   S   L   K   A   K   L   S   T   S   L   S   Q   V   V   D   A

      1305       1314       1323       1332       1341       1350
GTG GTG TCC AGG TGC GTG CAG CGA AAA GCC GCC TTA TCC AAG GTG TCT CCT CCA
 V   V   S   R   C   V   Q   R   K   A   A   L   S   K   V   S   P   P

      1359       1368       1377       1386       1395       1404
GAA TCC TCG GAT CCT CCC AAG GAT CCG ACG TTG CTG TCT CAG ATG CTG GAT AGG
 E   S   S   D   P   P   K   D   P   T   L   L   S   Q   M   L   D   R

      1413       1422       1431       1440       1449       1458
AAA TCT CCC AGG ACT GGA AAA GTG ATA GAT CGC GGG ACG AGG GTC AAC GGG CAC
 K   S   P   R   T   G   K   V   I   D   R   G   T   R   V   N   G   H

      1467       1476       1485       1494       1503       1512
GGT TTG TGT GGA CTG AGG ACC AGC CCT TAC CCT CCT GAC ATC GGC ACG GCT CCG
 G   L   C   G   L   R   T   S   P   Y   P   P   D   I   G   T   A   P

      1521       1530       1539       1548       1557       1566
AAA CCT TCG TTC TTC TTC CCC TTG AAA CCC CCC ACG AGT GTT GCC GCA GCC ACG
 K   P   S   F   F   F   P   L   K   P   P   T   S   V   A   A   A   T
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      1575        1584        1593        1602        1611        1620
GCT GCG TTC CTG TAC GGC TCA CCT CCT CAA ATG CCC CAG TCC TAT TCC TCA CCC
 A   A   F   L   Y   G   S   P   P   Q   M   P   Q   S   Y   S   S   P

      1629        1638        1647        1656        1665        1674
GCC CAC TCT ACG CCC ACC CCT CAG GAT GCC CCC GAG CAG ACA GAG GCT ATG TCT
 A   H   S   T   P   T   P   Q   D   A   P   E   Q   T   E   A   M   S

      1683        1692        1701        1710        1719        1728
TTG GTA GTC ACT CCC AAA AAG AAA CGC CAC AAA GTT ACC GAC ACT CGA CTC CAC
 L   V   V   T   P   K   K   K   R   H   K   V   T   D   T   R   L   H

      1737        1746        1755        1764        1773        1782
CAG CGA CAA GGA GGC CCC CTC TGC GGT CTC AGA GAC GAC GTG AGC CCC AAG TAC
 Q   R   Q   G   G   P   L   C   G   L   R   D   D   V   S   P   K   Y

      1791        1800        1809        1818        1827        1836
TCA AGT ATG CTG GAC CCC TTA CCA CCT GTG TAC CAT CAC CCA CCA CCG CCT CTT
 S   S   M   L   D   P   L   P   P   V   Y   H   H   P   P   P   P   L

      1845        1854        1863        1872        1881        1890
GTA CCG GTC AGT CTT CCG ACC ACG GTC GCC ATT CCT AAC CCC AGC CTC AAC CAG
 V   P   V   S   L   P   T   T   V   A   I   P   N   P   S   L   N   Q

      1899        1908        1917        1926        1935        1944
TCG GAG TTA TTC CAC GGC TTT CCG TAC GAG AGA CTC TCC CAG CAC TTC GGG GCG
 S   E   L   F   H   G   F   P   Y   E   R   L   S   Q   H   F   G   A

      1953        1962        1971        1980        1989        1998
CCG CCC ATG GAG CCC CCT CCT CAG GAC GAC GAC GGG CCG CCC ATG CAG CTG GGG
 P   P   M   E   P   P   P   Q   D   D   D   G   P   P   M   Q   L   G

      2007        2016        2025        2034        2043        2052
TCC CTG CAC CCC ATG CTC CCC TTC CAC GGC CAC CAC CAA AGG GGA TCT CCG GAC
 S   L   H   P   M   L   P   F   H   G   H   H   Q   R   G   S   P   D

      2061        2070        2079        2088        2097        2106
TCC TTG CAC CTA TCC CAC CTG AAG CAG CAG TCG ACG GAC AAC GGG GAT ATT TCC
 S   L   H   L   S   H   L   K   Q   Q   S   T   D   N   G   D   I   S

      2115        2124        2133        2142        2151        2160
GAC GCG GGA GAT AGC CCA GCC GCC TAC GAC TCC GGA ATG AAT CTC ATA TCC TTT
 D   A   G   D   S   P   A   A   Y   D   S   G   M   N   L   I   S   F

      2169        2178        2187        2196        2205        2214
CCA CAC CTG ATG CAC ACC ACA ACC CTG ACG CCG ATG CAT CTC CGG AAG GCA AAG
 P   H   L   M   H   T   T   T   L   T   P   M   H   L   R   K   A   K

      2223        2232        2241        2250        2259        2268
CTC ATG TTC TTC TAC GCC CGC TAC CCC AGC TCG GCG GTA CTC AGG ATG TAC TTC
 L   M   F   F   Y   A   R   Y   P   S   S   A   V   L   R   M   Y   F

      2277        2286        2295        2304        2313        2322
CCA GAC ATG CGG TTC AAC AAG AAC AAC ACC GCA CAG CTC GTC AAG TGG TTC TCC
 P   D   M   R   F   N   K   N   N   T   A   Q   L   V   K   W   F   S

      2331        2340        2349        2358        2367        2376
AAC TTC AGG GAA TTT TTC TAC ATC CAG ATG GAG AAA TAC GCT CGC CAG GCC ATG
 N   F   R   E   F   F   Y   I   Q   M   E   K   Y   A   R   Q   A   M
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        2385        2394        2403        2412        2421        2430
AGC GAG GGC ATG AAG ACG TCA GAC GAT TTG AAA GTG AAC TCC GAC TCG GAG CTA
 S   E   G   M   K   T   S   D   D   L   K   V   N   S   D   S   E   L

        2439        2448        2457        2466        2475        2484
CTC AGA GTC CTA AAC CTA CAT TAC AAC AGG AAC AAT CAC ATT GAG GCT CCA GAG
 L   R   V   L   N   L   H   Y   N   R   N   N   H   I   E   A   P   E

        2493        2502        2511        2520        2529        2538
AAC TTC CGG TTC GTC GTG GAG CAG ACG CTT CGA GAA TTC TTC AAG GCC CTG ATA
 N   F   R   F   V   V   E   Q   T   L   R   E   F   F   K   A   L   I

        2547        2556        2565        2574        2583        2592
GCC GGT AAA GAC TCT GAA CAG TCT TGG AAG AAG TCC ATT TAC AAA ATC ATC ACC
 A   G   K   D   S   E   Q   S   W   K   K   S   I   Y   K   I   I   T

        2601        2610        2619        2628        2637        2646
CGC CTG GAC GAC AAT GTG CCC GAG TAC TTC AAA AAC CCC AAC TTC TTA GAT TCG
 R   L   D   D   N   V   P   E   Y   F   K   N   P   N   F   L   D   S

        2655
CTC GAG TGA CGGTGGTGGA ATCTCTGAAC AACACACCCC CGTGCCTTCT CTCCCAACTC AAAAT
 L   E   *

AACGTAACAC ACCCCCGTGC CTTCTCTCCC AACTCAAAAT AACGTGCAGC TGGACAGATC GGGACAACCG
AACTCGGGTT ACTGCGCCTG CGTTTGGAAG AGGTCACCCG GTGTCCTCTG GCAGTAACAC CGGGTGGTTG
ATACCTCCCT TCCGCACATC TTCGATGCAC TCTGGACGAC TCGGATTTTA CCGCCTGGCA TCCTCTGGAC
ATCGGTGCAT GAAAATTCCG GAGGGAGCTG GGCATTTGAT ACTATTGCTT TATACAATCA CTTATCGCAA
GGGCCAATAC ATACTGGTAC ATCTGGGTTG TTGGGGATAT TCATAACTCC TTACTGGAAA CCTTCACAGA
ACTCTGGTCG GGAGGCCTGG CATTCGACGC TTTTAATAAA TACTTGGAAT TTTTTTTT – 3`
```

Fig. 27 The sequence of *Cupiennius* Prospero with the corresponding open reading frame and the 3`-UTR are shown. The DNA sequence shown in bold type letters was used for in frame cloning for Prospero 1 (underlined) and Prospero 2 (The whole DNA sequence written in bold type letters). The amino acid sequence written in bold type letters shows the homeodomain and the Prospero domain (Chu-Lagraff et al., 1991).

Cupiennius Even skipped

```
5`-                                        ACT CACTATAGGG CTCGAGCGGC CGCCCGGGCA
GGTATTCGGG GAAGGACCGA GTGGGGAAAA AGTGTTCGCC TCGAGACCCA ATGAGCCTGC ATTTAGTCGT
TCGCACGAAT TAAAGGAAGT GAGTTTATTC TTCTGTTAGT TCCTCAACTC AGCCGTGTAC TGGACTGTGA
            9          18          27          36          45          54
   CAG CAT TTG ACG GCG AAA GCG ATG CAA CAG GGG TTG AGG GCT CAG GAC GTT TGT
    Q   H   L   T   A   K   A   M   Q   Q   G   L   R   A   Q   D   V   C

          63          72          81          90          99         108
   TTA CTG GAG GAC GAT CTA GAG AAA CAG AAG TTC GAG ATG AGA CAG CTG CAT ACC
    L   L   E   D   D   L   E   K   Q   K   F   E   M   R   Q   L   H   T

         117         126         135         144         153         162
   GAA CAG CTT AGG CAC AGG ACG TCT TTA GAT GTC GGG AAA ACG TGC AGA ACC TCT
    E   Q   L   R   H   R   T   S   L   D   V   G   K   T   C   R   T   S

         171         180         189         198         207         216
   GAG GAG AGG ACG GGG AAA ATC ACG TCC CTC AAA GAC AGT CCC CCA GAC CTG AAA
    E   E   R   T   G   K   I   T   S   L   K   D   S   P   P   D   L   K

         225         234         243         252         261         270
   GAC GAA AAG AAC AGA AAA GAG GAC CTG AGC TCC ATC CGT CGA TAC CGC ACT GCG
    D   E   K   N   R   K   E   D   L   S   S   I   R   R   Y   R   T   A

         279         288         297         306         315         324
   TTC ACG AGG GAG CAG CTG GCG CGG CTC GAG AAG GAG TTC ATG CGG GAG AAC TAC
    F   T   R   E   Q   L   A   R   L   E   K   E   F   M   R   E   N   Y

         333         342         351         360         369         378
   GTG TCT CGG CCG AGG AGA TGC GAG CTG GCG ACC GCG CTC AAC CTG CCC GAG TCC
    V   S   R   P   R   R   C   E   L   A   T   A   L   N   L   P   E   S

         387         396         405         414         423         432
   ACC ATC AAG GTA TGG TTC CAG AAT CGG AGG ATG AAA GAC AAG CGC CAG CGC ATG
    T   I   K   V   W   F   Q   N   R   R   M   K   D   K   R   Q   R   M

         441         450         459         468         477         486
   TCC CTG CCG TGG CCG TAC GAC CCC CAC CTG GCT GCG TAC GTC ATC AAT GCA GCC
    S   L   P   W   P   Y   D   P   H   L   A   A   Y   V   I   N   A   A

         495         504         513         522         531         540
   TAC TCC GGC TAC CCT CTG CCG CCA CCT TTT GCC GGG TAC TAC GCC TCC TTC GCC
    Y   S   G   Y   P   L   P   P   P   F   A   G   Y   Y   A   S   F   A

         549         558         567         576         585         594
   GCA TCG CGT TAC CCG CCA ACG CCG ACT CCC TAC CTG GCC GCC CCC AGG CCC CAC
    A   S   R   Y   P   P   T   P   T   P   Y   L   A   A   P   R   P   H

         603         612         621         630         639         648
   ATA GCG CCC GCG CCC GCC CAG GCC ACC GCC TAC CCG AGA GGG GTC ACC GAA ACC
    I   A   P   A   P   A   Q   A   T   A   Y   P   R   G   V   T   E   T

         657         666         675         684         693         702
   CCA ACT TTC GCC ACC TTC GGG ACG CCG TGC GTG GAC CCT TGC AGG TGC CAC CTG
    P   T   F   A   T   F   G   T   P   C   V   D   P   C   R   C   H   L

         711         720         729         738         747         756
   GTG ACG TTC CCA CAG AGG CCG ATC GCG TCC ACC ACC CCG CCG AGG TCC ACC CTC
    V   T   F   P   Q   R   P   I   A   S   T   T   P   P   R   S   T   L
```

```
          765         774         783         792         801         810
     ACG CCG CCA CCC GAG ACG ACC AGG GTC GCG TCC GGC GAG ACG CAG CCC GTT CAG
      T   P   P   P   E   T   T   R   V   A   S   G   E   T   Q   P   V   Q

          819         828         837         846         855
     CAG AGG ACT CTG TTC CAG CCC TAC AAA ACG GAC ATC GAC AGG GCG TGA AAGTCCTC
      Q   R   T   L   F   Q   P   Y   K   T   D   I   D   R   A   *

        GC TCCCTTTTCT TCACTTCACT TCCGTTTTTC TCCGAGTAGG CTTCTATTAT AGGTTCGGTA
TTGACTTTCG CCTGGACGTT AGCAATGCCA CACGCTCGAG GAGATGTACT CACTGTCAAC TGAGGAACTG
CCAGCGTCCT CTCTGGAGTA GAGAGTCGAA GAGGATGCAA GCTAGGGTGC AAGTGTTGGA CGCAAAAGTT
TCATAAACCC GTTGAGCAGC TGACTCTGTT TCAGACCTAC TAAACGGACA TCGACAGAGC GTGAAAGACG
TTACTTCCTA CTCCCTTCAC TTCGTTTTCT ATGAGTAAGC TTCTATTAAA GGTTTGGTAT TGACTTTTCG
CCTGGGAGTT GGCAATGTCA CACGCTCGCG GAGATGTGTG CTGATTGTCA ACTGAGGAAC TGTCAGCGTC
CTATCTGGAG TAGGGAGTCG AAGAGGACGC AAGCTAGGGT GCACGTGTTG GACGTAAAAG TTTTAGGAGG
AACTCTTTTT CAGATCTTGT TTCTTCTGGC GACGAAGGAA TCATCGTGTC AAACTCACCG CGTCTTTGAA
CTACGGTTTT TGGTTGGTTT TCATTATTTG CGCTCGTTTT ATTGTAAGAA GTTATGTGCT GGTTCTATAT
GTGAAAATCT AAAAAAAAAA AAAAAAAAAA AAAAAAAAAA GCGGCCGCTG AATTCTAGAC CTGCCCGGGC
GGCCGCTCGA GCCCTATAGT GAGT –3`
```

Fig. 28 The sequence of *Cupiennius* Even skipped with the corresponding open reading frame, the 5`and the 3`-UTRs are shown. The DNA sequence written in bold type letters was used for in frame cloning. The amino acid sequence written in bold type letters shows the Even skipped homeobox (Damen et al., 2000).

Cupiennius *Runt 1*

```
              9          18          27          36          45          54
5'  TCG GTG AAC GGA TCT CCA AGT CCA GTT GAT CGA ACG ACC AAT CTG GAC TAT GCG
     S   V   N   G   S   P   S   P   V   D   R   T   T   N   L   D   Y   A

             63          72          81          90          99         108
    CAT GAA CGG CTG TTC ACA GAC GTA ATA GAT AAC CTC CCA AGC GAG TTA GTA AAG
     H   E   R   L   F   T   D   V   I   D   N   L   P   S   E   L   V   K

            117         126         135         144         153         162
    ACT GGA AGC CCC TGC TTT GTG TGT TCT GTT TTG CCA GGT CAC TGG CGA TCG AAC
     T   G   S   P   C   F   V   C   S   V   L   P   G   H   W   R   S   N

            171         180         189         198         207         216
    AAG ACT TTG CCA TTG CCG TTC AAA GTT ATT TGC CTT GGC GAA GTG GCC GAT GGG
     K   T   L   P   L   P   F   K   V   I   C   L   G   E   V   A   D   G

            225         234         243         252         261         270
    ACT ATG ATC ACT ATC AGG GCA GGA AAT GAC GAG AAT TTT TGC GGC GAG CTC AGA
     T   M   I   T   I   R   A   G   N   D   E   N   F   C   G   E   L   R

            279         288         297         306         315         324
    AAT GCG TCT GCT GTC ATG AAA AAT CAA GTT GCA AAA TTT AAT GAC CTC AGG TCC
     N   A   S   A   V   M   K   N   Q   V   A   K   F   N   D   L   R   S

            333         342         351         360         369         378
    GTC GGA AGG AGT GGA CGG GGT AAG AGC TTC TCG CTA ACG ATA TCC ATC AGT ACC
     V   G   R   S   G   R   G   K   S   F   S   L   T   I   S   I   S   T

            387         396         405         414         423         432
    AGT CCT CCT CAT GTG GTA ACT TAC AAC GAA GCT ATC AAA GTG ACG GTC GAT GGA
     S   P   P   H   V   V   T   Y   N   E   A   I   K   V   T   V   D   G

            441         450         459         468         477         486
    CCA CGA GAA CCT CGC AGG CAG CAA CAG CAG CTG AGA GCT TTC GCG ACT GCT TTT
     P   R   E   P   R   R   Q   Q   Q   Q   L   R   A   F   A   T   A   F

            495         504         513         522         531         540
    GGA CAC AGA CCA GCG CCG TAC CTT GAT CCG CGA TTT CCG GAT CCT CCG TGG GAA
     G   H   R   P   A   P   Y   L   D   P   R   F   P   D   P   P   W   E

            549         558         567         576         585         594
    CAC CAC ATC AGA CGA AAA ACC GCC GGG CAC TGG ACT CTA GAT CTC CCG AGG AGA
     H   H   I   R   R   K   T   A   G   H   W   T   L   D   L   P   R   R

            603         612         621         630         639         648
    ATA GGG CCT GTG CAA GAT TCC CTT CAT CTT GGA GAA GGT CAC TGG GCA CCT TAC
     I   G   P   V   Q   D   S   L   H   L   G   E   G   H   W   A   P   Y

            657         666         675         684         693         702
    GGA CAT CAC TAC TCT TAC TTA GCC TCA GCT TCT GGA CTG CAA GGG CCA GGT TTT
     G   H   H   Y   S   Y   L   A   S   A   S   G   L   Q   G   P   G   F

            711         720         729         738         747         756
    CCA CCG TAT TCC CTC GAC ACG GCT CTG AGC GGA GTT TCC TCG GCA TCT CAG GAC
     P   P   Y   S   L   D   T   A   L   S   G   V   S   S   A   S   Q   D
```

```
        765         774         783         792         801         810
TCG TGC TCG TCA TCG CCA CCT CTG CCA GAA AAT CAT CTA GTG TCG CCA CGA AGT
 S   C   S   S   S   P   P   L   P   E   N   H   L   V   S   P   R   S

        819         828         837         846         855         864
TAC GTT GCA AAA GAA AAC ATA AAA CCA AGG AGA AAA GAA TCC ATT GTA GGA CAC
 Y   V   A   K   E   N   I   K   P   R   R   K   E   S   I   V   G   H

        873
CAG ACC CAC ATG TTC 3'
--- --- --- --- ---
 Q   T   H   M   F
```

Fig. 29 The sequence of *Cupiennius* Runt 1 with the corresponding open reading frame, the 5`and the 3`-UTRs are shown.

Cupiennius Runt 2

```
                                  5`- CCGCCC TTGTTGACGT CGAACGCCGT GCCGCACGTT
TTCGTTATCG GCACCACGTT TTCGTTATCG GCACTAATAT TCTTCGCTCT GTCTCAACTC ATATAAACAC
ATGGTCCCCT GAAATTCTTT CTCACCCTAG CTGCAGACGA AAAATCCATC AGCTGACAGC
GTCTATTTAA-

           9          18          27          36          45          54
    AGG GAC TGT GGG ATC ACC GTA GTA GGC CTA GCT GTC GAC GGC GGT TTC CCG ATG
     R   D   C   G   I   T   V   V   G   L   A   V   D   G   G   F   P   M

          63          72          81          90          99         108
    CAT TTG TCG GCG GAG AGT GGG GTC AAT TCC CGC GAC CCG ATG TCG GAC TTC TTC
     H   L   S   A   E   S   G   V   N   S   R   D   P   M   S   D   F   F

         117         126         135         144         153         162
    GTA CCG TAC GAG AGG ACA ATA ACT GAA GTT TTA AAC GAG CAT CCC GGT GAA CTG
     V   P   Y   E   R   T   I   T   E   V   L   N   E   H   P   G   E   L

         171         180         189         198         207         216
    GTG AAG ACG GGA TCA CCG AAC GTC GTC TGT TCG GCC CTC CCC ACA CAC TGG AGG
     V   K   T   G   S   P   N   V   V   C   S   A   L   P   T   H   W   R

         225         234         243         252         261         270
    TCC AAT AAG ACC CTC CCG GTC GCG TTC CGA GTG GTC AGC TTG GGG GAG GTA CTG
     S   N   K   T   L   P   V   A   F   R   V   V   S   L   G   E   V   L

         279         288         297         306         315         324
    GAC GGG ACA GTT GTC ACA ATT AAA GCC GGT AAT GAT GAC AAT TAC TGC GCC GAA
     D   G   T   V   V   T   I   K   A   G   N   D   D   N   Y   C   A   E

        333         342         351         360         369         378
    CTC AGA AAT GCT ACA GCT GTG ATG AAG AAT CAG GTA GCG AAA TTC AAT GAC CTA
     L   R   N   A   T   A   V   M   K   N   Q   V   A   K   F   N   D   L

         387         396         405         414         423         432
    AGA TTC GTT GGA AGA AGT GGA AGA GGT AAA AGC TTC TCA TTA ACG ATA ACA CTG
    --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- --- ---
     R   F   V   G   R   S   G   R   G   K   S   F   S   L   T   I   T   L

         441         450
    AGC ACT TCA CCA CCC CAG 3'
    --- --- --- --- --- ---
     S   T   S   P   P   Q
```

Fig. 30 The sequence of *Cupiennius* Runt 2 with the corresponding open reading frame and the 5`UTR are shown.

Cupiennius Engrailed-1

```
                9          18         27         36         45         54
5'  TGC AGT TCT CAC CCC CTT TCG AAT TCC CAT AAC TCC TGA CCA CTG GGA TCT TTA
     C   S   S   H   P   L   S   N   S   H   N   S   *   P   L   G   S   L

               63         72         81         90         99        108
    GCC TTT TCC GAT GCA GCT GCG GGC TGC AGG GGG TTG GAG CAA ATA GCG ATA GTG
     A   F   S   D   A   A   A   G   C   R   G   L   E   Q   I   A   I   V

              117        126        135        144        153        162
    AAA GTT AAT CTC GAG TGC TCG ACA ATG GCT CTG GAC TTG GAG CGG CCG AGT GCC
     K   V   N   L   E   C   S   T   M   A   L   D   L   E   R   P   S   A

              171        180        189        198        207        216
    GCA GTG CCC ACA AGT TGC CGG TCA GCA TCG CCA CAA GAT CAG AGG TCA CCA CAG
     A   V   P   T   S   C   R   S   A   S   P   Q   D   Q   R   S   P   Q

              225        234        243        252        261        270
    GAC CAG CGG TCA CCC CAT CCC ACT CTA GGA CCA TCC CCT TTA AAG TTT TCC ATC
     D   Q   R   S   P   H   P   T   L   G   P   S   P   L   K   F   S   I

              279        288        297        306        315        324
    GAA AAG ATT CTG TCA GCG GAC TTT GGG CGG AGG GAC ACT CCC GTC GAA AAA GAA
     E   K   I   L   S   A   D   F   G   R   R   D   T   P   V   E   K   E

              333        342        351        360        369        378
    AAA CAA CCC TTA CCT CCT CAA AAT GAG AAC TCT TCC TCG GCC GGT GCC CAG GTG
     K   Q   P   L   P   P   Q   N   E   N   S   S   S   A   G   A   Q   V

              387        396        405        414        423        432
    ATG AAT CAG ACC ACC AAT CCA CTC CTC TAT CCA GCC TGG ATT TAT TGC TCC AGG
     M   N   Q   T   T   N   P   L   L   Y   P   A   W   I   Y   C   S   R

              441        450        459        468        477        486
    ATA TCA GAT AGA CCG TCA AGT GGT CCA CGC AGA ATC AGA TCC AAA GCA GGG AAG
     I   S   D   R   P   S   S   G   P   R   R   I   R   S   K   A   G   K

              495        504        513        522        531        540
    GGG AGC AGC AGC CAA GAC CTC TCG GAC GAC GAC CAA AGT CCC AGA GCG AGA CGG
     G   S   S   S   Q   D   L   S   D   D   D   Q   S   P   R   A   R   R

              549        558        567        576        585        594
    ATC AAG AAA AAA GAC AAG AAA CCC GAC GAC AAA CGG CCC CGT ACA GCT TTC ACT
     I   K   K   K   D   K   K   P   D   D   K   R   P   R   T   A   F   T

              603        612        621        630        639        648
    GCT GAT CAG CTG TCC CGA TTA AAA CAC GAG TTT CAA GAA AAT CGA TAT CTG ACA
     A   D   Q   L   S   R   L   K   H   E   F   Q   E   N   R   Y   L   T

              657        666        675        684        693        702
    GAG AGA AGG CGA CAA GAT TTG GCT AAA GAT CTA CAG CTT AAC GAA AGT CAA ATA
     E   R   R   R   Q   D   L   A   K   D   L   Q   L   N   E   S   Q   I

              711        720        729        738        747        756
    AAA ATC TGG TTT CAG AAC CGG CGA GCC AAG CTT AAG AAA GCC TCA GGT CAG CGG
     K   I   W   F   Q   N   R   R   A   K   L   K   K   A   S   G   Q   R
```

```
        765         774         783         792         801         810
AGC GCG CTG GCG TTG CAG CTT ATG GCA CAG GGC CTG TAC AAT CAC TCA ACG ATA
 S   A   L   A   L   Q   L   M   A   Q   G   L   Y   N   H   S   T   I

        819         828         837         846         855         864
CCC ATC AGA GGC GAC GAG GAT GAC GAT GAG AGA CCG AAA TCC TCC TCG TCT TCC
 P   I   R   G   D   E   D   D   D   E   R   P   K   S   S   S   S   S

TAA – 3`
 *
```

Fig. 31. The sequence of *Cupiennius* Engrailed-1 (provided by Wim Damen; Damen et al., 1998) with the corresponding open reading frame. The 5` UTR is shown. The DNA sequence used for in frame cloning is shown in bold type letters. The amino acid sequence shown in bold type letters corresponds to the Engrailed homeodomain.

Cupiennius Snail

```
                9          18          27          36          45          54
5'  TGA TAC AAT ATG CCT CGA GCT TTT CTT ATC AAG AAG AAA CAA CAG TGT GCG AAG
     *   Y   N   M   P   R   A   F   L   I   K   K   K   Q   Q   C   A   K

               63          72          81          90          99         108
    AAT GGT CAG TCC CTG GCC AGG ACT AAC TGG TTG GAA GAT TCG GAC AAT ATG GAT
     N   G   Q   S   L   A   R   T   N   W   L   E   D   S   D   N   M   D

              117         126         135         144         153         162
    AGT TCG AGA GAC AAC CCT CAA TTC ACG CCG CTG ACG ATC GTA GCC CCA GAT ACC
     S   S   R   D   N   P   Q   F   T   P   L   T   I   V   A   P   D   T

              171         180         189         198         207         216
    AAA GGG CCA TAT GAT TTA AGT ATG AAG CCT AAA AAT TTT GAT GAG AGC TCT AAT
     K   G   P   Y   D   L   S   M   K   P   K   N   F   D   E   S   S   N

              225         234         243         252         261         270
    GAT AGT GAG CAG AGA TTA GTT ATT TCT CCA AGA CCA ACG ATA AAT ATA AGT TCG
     D   S   E   Q   R   L   V   I   S   P   R   P   T   I   N   I   S   S

              279         288         297         306         315         324
    CAT CCT CAA GGG ACC CTA ATA GCT CCC AAG CCC ATC AAA CCA ACC CCT AAA ACA
     H   P   Q   G   T   L   I   A   P   K   P   I   K   P   T   P   K   T

              333         342         351         360         369         378
    CCA GAC GAG ATT GCA GCT TCT AGA TCT CAC TGG CAA AAA CAG ATG ATG TCT CCG
     P   D   E   I   A   A   S   R   S   H   W   Q   K   Q   M   M   S   P

              387         396         405         414         423         432
    TAC TTA CCT TTC AAC TAT CCA GTC TAT GCG TAT CCA GGA CGG CCA TCA GAA ATT
     Y   L   P   F   N   Y   P   V   Y   A   Y   P   G   R   P   S   E   I

              441         450         459         468         477         486
    TAC CCA TTT GGT AAC AAC TAT ATG AGC AGT CAA AAC TCT ATG GTA CCG CCT CCA
     Y   P   F   G   N   N   Y   M   S   S   Q   N   S   M   V   P   P   P

              495         504         513         522         531         540
    CTA GTG CCT TTA AGC TCA GCT TCT TCC AAT GTG GAT AGG TAC TCG CCA ACC AGG
     L   V   P   L   S   S   A   S   S   N   V   D   R   Y   S   P   T   R

              549         558         567         576         585         594
    GAC AGG TAC GAA GTC CCC CCA AGA AGA GCA GTG TCT CCT GTA GTT GCA ATG TCT
     D   R   Y   E   V   P   P   R   R   A   V   S   P   V   V   A   M   S

              603         612         621         630         639         648
    GGT AGC CCT TCT CCT CCA GCT GTC TTC CCA GGA TGG TAC TCT GAC GGC CAG GAT
     G   S   P   S   P   P   A   V   F   P   G   W   Y   S   D   G   Q   D

              657         666         675         684         693         702
    TCT GGC CTC GCC TCC TCC CCA AGT CCC AGT TCA GAG GAT GGA GAA GCT GCC GCG
     S   G   L   A   S   S   P   S   P   S   S   E   D   G   E   A   A   A

              711         720         729         738         747         756
    TCG AAG CCG AAC CCC ACC CGC TAT CAG TGC CCG GAT TGT AAC AAG AGT TAC TCC
     S   K   P   N   P   T   R   Y   Q   C   P   D   C   N   K   S   Y   S
```

```
        765         774         783         792         801         810
ACC TAC AGC GGA CTG TCT AAG CAT AGG CTG ATG CAC TGT GCA ACC CAA GCC AAA
 T   Y   S   G   L   S   K   H   R   L   M   H   C   A   T   Q   A   K

        819         828         837         846         855         864
AAG  TCT TTC GGA TGT AAA TAC TGC GAC AAG GTC TAC GTG TCT TTG GGA GCC CTC
 K   S   F   G   C   K   Y   C   D   K   V   Y   V   S   L   G   A   L

        873         882         891         900         909         918
AAG ATG CAC ATC AGG ACC CAC ACC CTT CCT TGC AAA TGC AAG CTC TGC GGC AAA
 K   M   H   I   R   T   H   T   L   P   C   K   C   K   L   C   G   K

        927         936         945         954         963         972
GCC TTC TCA CGT CCC TGG CTC CTG CAG GGC CAC ATC CGC ACC CAC ACT GGC GAG
 A   F   S   R   P   W   L   L   Q   G   H   I   R   T   H   T   G   E

        981         990         999        1008        1017        1026
AAA CCC TTC TCC TGT CCC CAC TGC AGC AGG GCT TTC GCC GAC AGA TCC AAC CTC
 K   P   F   S   C   P   H   C   S   R   A   F   A   D   R   S   N   L

       1035        1044        1053        1062        1071        1080
AGA GCT CAT CTC CAG ACC CAC TCC GAA GTC AAG AAG TAC AGA TGC AAG ACC TGT
 R   A   H   L   Q   T   H   S   E   V   K   K   Y   R   C   K   T   C

       1089        1098        1107        1116        1125        1134
AGC AAG ACT TTC TCC AGG ATG TCC TTG CTG CTG AAA CAC GAA GAC GGA GGC TGC
 S   K   T   F   S   R   M   S   L   L   L   K   H   E   D   G   G   C

       1143        1152        1161        1170        1179        1188
GCG GGC GCT GCC GCA AGT CAG CAA CAA CCT CCA GTC AAT ACC GCA AAC TAT GCC
 A   G   A   A   A   S   Q   Q   Q   P   P   V   N   T   A   N   Y   A

       1197
TAA AGT GTT 3'
 *   S   V
```

Fig. 32 The sequence of *Cupiennius* Snail (provided by Monika Retzlaff; Retzlaff, 1996) with the corresponding open reading frame. The DNA sequence used for in frame cloning is shown in bold type letters. Amino acids written in bold type letters correspond to the five zinc finger domains (Manzanares et al., 2001).

Cupiennius Krüppel-1

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                9          18         27         36         45         54
5'  TAG GCT GGC TGG AAA GAA ACG ATG AAG GAA GGA TCG GAG GGG GAT GGC GGC GGT
     *   A   G   W   K   E   T   M   K   E   G   S   E   G   D   G   G   G

               63         72         81         90         99        108
    AGC AGT GAT GAT GTA CCG ACT TCC AGG GAT GCC GAC AGT ACC GTC TCC AAC GGA
      S   S   D   D   V   P   T   S   R   D   A   D   S   T   V   S   N   G

              117        126        135        144        153        162
    GGA ACC CAC AGA ATT GGA GCA GCT CCG GGA GGG TTG CCG CTC GGT GGA ACG ACC
      G   T   H   R   I   G   A   A   P   G   G   L   P   L   G   G   T   T

              171        180        189        198        207        216
    CTC GCG AAC GCA TTG CTG GGC ATC ACC TTG GAC CGC GGC CCA GCA AAC GGA GGT
      L   A   N   A   L   L   G   I   T   L   D   R   G   P   A   N   G   G

              225        234        243        252        261        270
    TCA GCA GGA AAT GGC GGC AAC AGC AAC AAC AAC GTG CCC CAC GTG GAC GGT TTG
      S   A   G   N   G   G   N   S   N   N   N   V   P   H   V   D   G   L

              279        288        297        306        315        324
    TTT GGA ATT CAC TCG ACA AGC GCC GCC AAA GGC GCG AAG GGT GGC GAT AGT AAC
      F   G   I   H   S   T   S   A   A   K   G   A   K   G   G   D   S   N

              333        342        351        360        369        378
    GGT CGC GAC AAG TTA TTC GTG TGC AAC ATC TGC CAC CGG TCC TTC GGA TAC AAG
      G   R   D   K   L   F   V   C   N   I   C   H   R   S   F   G   Y   K

              387        396        405        414        423        432
    CAC GTG CTG CAG AAC CAC GAG AGG ACG CAC ACC GGA GAG AAG CCT TTC GAG TGC
      H   V   L   Q   N   H   E   R   T   H   T   G   E   K   P   F   E   C

              441        450        459        468        477        486
    AAG GAA TGC CAC AAG CGG TTC ACA CGT GAC CAC CAC CTC AAG ACA CAC ATG CGT
      K   E   C   H   K   R   F   T   R   D   H   H   L   K   T   H   M   R

              495        504        513        522        531        540
    CTG CAC ACG GGA GAG AAG CCT TAC CAC TGT TCG CAC TGC GAG CGT CAG TTT GTG
      L   H   T   G   E   K   P   Y   H   C   S   H   C   E   R   Q   F   V

              549        558        567        576        585        594
    CAG GTC GCC AAC CTG CGG AGG CAT CTC CGC GTG CAC ACG GGC GAG AGG CCC TAC
      Q   V   A   N   L   R   R   H   L   R   V   H   T   G   E   R   P   Y

              603        612        621        630        639        648
    TCC TGC ACG CTG TGT CCC TCG CGC TTC TCG GAC AGT AAC CAG CTG AAG GCG CAC
      S   C   T   L   C   P   S   R   F   S   D   S   N   Q   L   K   A   H

              657        666        675        684        693        702
    CTC CTC ATC CAC GAG GGC AAG AAG CCG TAC GAG TGT CCC AAG TGC AAC GGC CAC
      L   L   I   H   E   G   K   K   P   Y   E   C   P   K   C   N   G   H

              711        720        729        738        747        756
    TTC AGG CGG AGG CAC CAC CTC GTC CAC CAC AAG TGC CCC AGA GAC GAG GCG AAC
      F   R   R   R   H   H   L   V   H   H   K   C   P   R   D   E   A   N
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        765         774         783         792         801         810
ATC GGC AAG CCG AGG CGT GGG CGA CGG CCC AAA GCG TAC GAG GAG CTG CCC ACC
 I   G   K   P   R   R   G   R   R   P   K   A   Y   E   E   L   P   T

        819         828         837         846         855         864
TTG CTG TCG CCG GTG TTG CAG GAG CGT CTG TCG ACG CCC GTC CCC GAC CTG CCC
 L   L   S   P   V   L   Q   E   R   L   S   T   P   V   P   D   L   P

        873         882         891         900         909         918
CCG CCG CCA CCA CCA CCC GTG GCG GTC GGC CTC ACG TCG GTC ATC ACA AGA GGG
 P   P   P   P   P   P   V   A   V   G   L   T   S   V   I   T   R   G

        927         936         945         954         963         972
CCC TCG CCG CCC CTT GTT CCG CAA CCT GCA CAC ATG CCA TCC AGG AGG AAC ACC
 P   S   P   P   L   V   P   Q   P   A   H   M   P   S   R   R   N   T

        981         990         999        1008        1017        1026
CAC CCC ATG CAC CAC CCC CTG CTA GGG CTA GGT GGT TCC AAC GGG CGT TAT CAC
 H   P   M   H   H   P   L   L   G   L   G   G   S   N   G   R   Y   H

       1035        1044        1053        1062        1071        1080
CCA GAA CAG TCA GGG CCC TTG GAT ATG ACT GTG TCA TCT GCC TCG GCG CCC GTC
 P   E   Q   S   G   P   L   D   M   T   V   S   S   A   S   A   P   V

       1089        1098        1107        1116        1125        1134
TCA GTC ATC GTA CCG CTA GTA GCC TAC AAC CAC CAG CAC CAC CGC GCC GCA TGC
 S   V   I   V   P   L   V   A   Y   N   H   Q   H   H   R   A   A   C

       1143        1152        1161        1170        1179        1188
TCC GTG GAT GGT GTC CTC GAC TTG TCC AAT TCG AGG AGC GAC TCC GAG GCG GAA
 S   V   D   G   V   L   D   L   S   N   S   R   S   D   S   E   A   E

       1197        1206        1215        1224        1233        1242
CCC ATC GAG GAG GAA GTG GAC GAA GAG GAC GGC TGC GTT GAC GAG GGC ATC GAT
 P   I   E   E   E   V   D   E   E   D   G   C   V   D   E   G   I   D

       1251        1260        1269        1278        1287        1296
TCG GAT AGC GAC GAG GAG GAG GAG GAG GAG CAC CGC CTG CGC CTG CTT GCG TGC
 S   D   S   D   E   E   E   E   E   E   H   R   L   R   L   L   A   C

       1305        1314        1323        1332        1341        1350
TCA TGG AAA CAA AGG GAT GAA TTG AGA CAC CGA CCG GAG GAC CTG AGG CGG CGC
 S   W   K   Q   R   D   E   L   R   H   R   P   E   D   L   R   R   R

       1359        1368        1377        1386        1395        1404
GGA GAC CGA TAC GAC GGC AGC GAA GGC GAA AAC GGG GGA AGC AAC CAA CTA GCA
 G   D   R   Y   D   G   S   E   G   E   N   G   G   S   N   Q   L   A

       1413        1422
TTA CAG CTT ACC ACC ACA TCC TAG 3'
 L   Q   L   T   T   T   S   *
```

Fig. 33 The sequence of *Cupiennius* Krüppel-1 (Complete sequence provided by Wim Damen; Sommer et al., 1992; Retzlaff, 1996) with the corresponding open reading frame. The DNA sequence used for in frame cloning is shown in bold type letters. The amino acid sequence written in bold type letters contains the domain of the zinc fingers (Retzlaff, 1996; Rosenberg et al., 1986)

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Erklärung

"Ich versichere, daß ich die von mir vorgelegte Dissertation selbständig angefertigt, die benutzten Quellen und Hilfsmittel vollständig angegeben und die Stellen der Arbeit einschließlich Tabellen, Karten und Abbildungen, die anderen Werken im Wortlaut oder dem Sinn nach entnommen sind, in jedem Einzelfall als Entlehnung kenntlich gemacht habe; daß diese Dissertation noch keiner anderen Fakultät oder Universität zur Prüfung vorgelegen hat; daß sie abgesehen von unten angegebenen Teilpublikationen noch nicht veröffentlicht worden ist sowie, daß ich eine solche Veröffentlichung vor Abschluß des Promotionsverfahrens nicht vornehmen werde.

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Köln, den 18.04.2002 Mathias Weller

Teilpuplikationen:

Damen, W. G.M., Weller, M. and Tautz, D. (2000). Expression patterns of *hairy, even-skipped*, and *runt* in the spider *Cupiennius salei* imply that this genes were segmentation genes in a basal arthropod. *PNAS* 97, 4515-4519.

Stollewerk, A., Weller, M. and Tautz, D. (2001). Neurogenesis in the spider *Cupiennius salei*. *Development* 128, 2673-2688.

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Seit dem 22.11.1996 verheiratet mit Patricy de A. Salles. Am 27.12.1999 wurde unser Sohn Samuel Salles Weller geboren.

Sprachkenntnisse: Brasilianisches Portugiesisch, Spanisch, und Englisch. Deutsch als Muttersprache.