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Parasegmental organization of the spider embryo implies that the parasegment is an evolutionary conserved entity in arthropod embryogenesis

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SUMMARY

Spiders belong to the chelicerates, which is a basal arthropod group. To shed more light on the evolution of the segmentation process, orthologs of the *Drosophila* segment polarity genes *engrailed*, *wingless/Wnt* and *cubitus interruptus* have been recovered from the spider *Cupiennius salei*. The spider has two *engrailed* genes. The expression of *Cs-engrailed-1* is reminiscent of *engrailed* expression in insects and crustaceans, suggesting that this gene is regulated in a similar way. This is different for the second spider *engrailed* gene, *Cs-engrailed-2*, which is expressed at the posterior cap of the embryo from which stripes split off, suggesting a different mode of regulation. Nevertheless, the *Cs-engrailed-2* stripes eventually define the same border as the *Cs-engrailed-1* stripes. The spider *wingless/Wnt* genes are expressed in different patterns from their orthologs in insects and crustaceans. The *Cs-wingless* gene is expressed in iterated stripes just anterior to the *engrailed* stripes, but is not expressed in the most ventral region of the germ band. However, *Cs-Wnt5-1* appears to act in this ventral region. *Cs-wingless* and *Cs-Wnt5-1* together seem to perform the role of insect *wingless*. Although there are differences, the *wingless/Wnt*-expressing cells and *en*-expressing cells seem to define an important boundary that is conserved among arthropods. This boundary may match the parasegmental compartment boundary and is even visible morphologically in the spider embryo. An additional piece of evidence for a parasegmental organization comes from the expression domains of the Hox genes that are confined to the boundaries, as molecularly defined by the *engrailed* and *wingless/Wnt* genes. Parasegments, therefore, are presumably important functional units and conserved entities in arthropod development and form an ancestral character of arthropods. The lack of by *engrailed* and *wingless*/*Wnt*-defined boundaries in other segmented phyla does not support a common origin of segmentation.

Key words: Evolution, Engrailed, Wingless, Wnt, Cubitus interruptus, Boundary, Segmentation, Spider

INTRODUCTION

The arthropod body consists of metameric units that become manifest as the segments at the germband stage (Anderson, 1973; Scholtz, 1997). The molecular mechanisms that underlie the segmentation process have been best studied in the insect *Drosophila*, where segmentation genes act in a hierarchic cascade; as a result, the metameric embryo is formed. A remarkable feature of *Drosophila* segmentation is that the fundamental developmental units are not the segments, but the parasegments that are defined by functional compartment boundaries (Martinez-Arias and Lawrence, 1985; Lawrence, 1988; Patel, 1994). The crustacean body is also initially built from units that resemble the parasegmental modules in insects (Patel et al., 1989a; Patel et al., 1989b; Patel, 1994; Dohle and Scholtz, 1988; Scholtz, 1997).

The subdivision of the anteroposterior body axis in the insect *Drosophila* results from the successive action of the maternal, gap, pair rule and segment polarity genes (Ingham, 1988; St Johnston and Nüsslein-Volhard, 1992; Pankratz and Jäckle, 1993). The pair rule genes delimit the parasegments, the initial metameric units in *Drosophila*, and define the domains that will express the segment polarity genes, such as *engrailed* and *wingless* (Lawrence et al., 1987; DiNardo and O'Farrell, 1987; Ingham, 1988; DiNardo et al., 1988; Baker, 1988). The *engrailed* gene encodes a homeobox-containing protein that is involved in establishing and maintaining the parasegmental boundaries in the *Drosophila* embryo. The anterior domain of the parasegment that expresses *engrailed* corresponds to the future posterior part of the segment in *Drosophila* as well as in other insects (Rogers and Kaufman, 1996; Schmidt-Ott et al., 1994; Patel et al., 1989a; Patel, 1994). *Drosophila* embryos in which *engrailed* is expressed uniformly are unsegmented (Lawrence et al., 1996). In addition, embryos that lack both *wingless* and *engrailed* function are unsegmented. The alternation of cells that express *engrailed* and non-expressing cells is essential for segmentation, and determines how these cells respond to morphogens (Lawrence et al., 1996).

In malacostracan crustaceans, Engrailed is expressed in the newly forming segments in the most anterior row of four rows of cells that form a genealogical unit (Patel et al., 1989a; Patel, 1994; Scholtz et al., 1994; Scholtz, 1995; Scholtz and Dohle, 1996). The row of Engrailed-expressing cells eventually ends up in the posterior region of each segment. The anterior part of the segment is formed from the posterior cells of the more anterior genealogical unit, which do not express Engrailed. These genealogical units correspond to units like the insect parasegments (Dohle and Scholtz, 1988; Patel, 1994).

The origin of segmentation in other arthropod groups like the chelicerates, a basal arthropod taxon, is still obscure. The chelicerates include the spiders, mites, scorpions and horseshoe crabs. Previous work suggests a role for the orthologs of the *Drosophila* pair rule genes *hairy*, *even-skipped* and *runt* in spider segmentation (Damen et al., 2000). These spider pair rule gene orthologs are expressed in a dynamic way in a domain at the posterior end of the embryo, from which stripes form. However, the exact mechanism that underlies chelicerate segmentation is still unclear. As the chelicerates form a basal arthropod group, characters in common between chelicerates and other arthropod taxa can be assumed as ancestral arthropod traits. The analysis of the segmentation process in chelicerates, therefore, may provide us with information on the basic embryonic molecular architecture of arthropods.

To obtain more insights into the evolution of developmental mechanisms that underlie the segmentation process in the arthropods, segment-polarity genes were studied in the spider *Cupiennius salei* (Chelicerata). Although there are differences, the expression of the spider *engrailed* genes, the *wingless/Wnt* genes and the *cubitus interruptus* gene imply that parasegmental boundaries are highly conserved within the arthropod clade.

MATERIALS AND METHODS

Embryos

Embryos of the Central American wandering spider *Cupiennius salei* Keys. (Chelicerata, Aranida, Ctenidae) were used (Damen et al., 1998; Damen and Tautz, 1998). Spiders were obtained from a colony bred by Ernst-August Seyfarth in Frankfurt am Main (Germany) or from our newly established colony in Cologne.

Cloning of genes from spider

Fragments for spider genes were obtained by RT-PCR as described before (Damen et al., 2000). The oligo nucleotide primers used in the initial PCR for *engrailed* were *en fw1* (TGGCCMGCMTGGG-TNTWYTGYAC) and *en bw-4* (TTRTAMARNCCYTSNGCCAT). In a nested PCR, the primers *en fw-2* (GAMGAMAARMGNCCN-MGNAC) and *en bw-3* (RTTYTGRAACCADATYTTDATYTG) were used. For *wingless*, the primers *wg-fw-1* (ATHGARWSNTGYACNT-GYGAYTA) and *wg-bw* (ACYTWRCARCACCANTGRAANGT-RCA) were used in the initial PCR, and *wg-fw-2* (TGGGAR-TGGGGNGGNTGYWSNGA) and *wg-bw* were used in a nested PCR. For *cubitus interruptus* (*ci*) the oligonucleotide primers *ci-fw* (GARCANAAYTGYCAYTGG) and *ci-bw-1* (CCRTGNACNGTYT-TNACRTG) were used in the initial PCR, and *ci-fw* and *ci-bw-2* (GGRTCNGTRTANCKYTTNG) in a nested PCR. The resulting PCR products were cloned and sequenced.

The obtained *en* PCR fragment was used to screen the embryonic *C. salei* cDNA library (Damen et al., 1998). One full-length clone (*Cs-en-1*) and three 5′ and/or 3′ truncated clones were isolated. Another *engrailed* cDNA clone, *Cs-en-2*, was recovered by screening the embryonic *C. salei* cDNA library under low stringency conditions with a probe for the homeodomain of *Cs-abd-A* (from position 410-615) (Damen et al., 1998). After an overnight hybridization at 52°C, the filters were washed twice with 2*SSC/0.1% SDS at 52°C for 15 minutes each. Several homeobox-containing genes were obtained (Damen et al., 1998) (W. G. M. D., unpublished), among them three cDNAs for *Cs-en-2*. The longest *Cs-en-2* cDNA clone was sequenced.

The complete coding region of *Cs-wg* and *Cs-Wnt5-1* were obtained by RACE-PCR (Marathon cDNA amplification kit, CLONTECH).

The sequences for the different genes were determined from both strands on an ABI-377XL automated sequencer (Applied Biosystems), using Big Dye dye-terminators (Perkin Elmer). The nucleotide data are available under Accession Numbers AJ007437 (*Cs-en-1*), AJ315944 (*Cs-en-2*), AJ315945 (*Cs-wg*), AJ315946 (*Cs-Wnt5-1*) and AJ315947 (*Cs-ci*)

In a test for *wg* genes in the spider, the following primers were used: initial PCR, *wg-fwn1* (CAYAAYAAYGARGCNGG) and *wg-bwn* (CATNARRTCRCANCCRTC); nested PCR, *wg-fwn2* (GARTGYA-ARTGYCAYGG) and *wg-bwn*.

Phylogenetic analysis

Sequences were aligned using ClustalX (Thompson et al., 1994) and the BLOSUM matrix, a gap penalty of 20 and a gap extension of 0.2. Phylogenetic analysis was carried out using PUZZLE (Strimmer and von Haeseler, 1996) as implemented in PAUP 4.0b6 (Swofford, 2001).

In situ hybridization

Whole-mount in situ hybridization was performed essentially as described previously for *Drosophila* (Tautz and Pfeifle, 1989; Klingler and Gergen, 1993) with the modifications for spider embryos (Damen and Tautz, 1998; Damen and Tautz, 1999).

RESULTS

The *engrailed*, *wingless/Wnt* and *cubitus interruptus* genes of the spider *Cupiennius salei*

cDNAs for two different *engrailed* genes were recovered from *Cupiennius salei*. *Cs-en-1* was found by RT-PCR and subsequent screening of the embryonic cDNA library (Damen et al., 1998), *Cs-en-2* by low stringency screening of the same library with a *Cs-abd-A* homeodomain probe.

The 3416 bp *Cs-en-1* cDNA contains an open reading frame (ORF) of 732 bp (position 133-864) and 2552 bp of 3′ UTR sequence with a polyadenylation signal and a short poly-A tail at its 3′ terminus. The deduced Cs-EN-1 protein is 244 amino acids long and includes an Engrailed-type homeodomain that is 67-82% identical to other Engrailed homeodomains (Fig. 1A). In addition, the Engrailed-specific-domains EH1-EH5 (Joyner and Hanks, 1991; Duboule, 1994) are recognized in the Cs-EN-1 sequence (Fig. 1B). A remarkable feature of Cs-EN-1 is the linker of 23 amino acid between EH2 and EH3, which is not found in other Engrailed sequences, where EH2 and EH3 are immediately adjacent to each other, except for a number of arthropod Engrailed/Invected proteins, which contain a two amino acids insertion (always Arg-Ser), and the amphioxus Engrailed protein, which contains a four amino acid insertion between EH2 and EH3 (Fig. 1B). The resemblance of the Cs-EN-1 homeodomain to the different Engrailed homeodomains and the presence of the Engrailed-specific-domains unambiguously show that *Cs-en-1* is a spider *engrailed* ortholog.

The 1342 bp *Cs-en-2* cDNA contains an ORF from position 1-470. The deduced and likely incomplete 156 amino acid

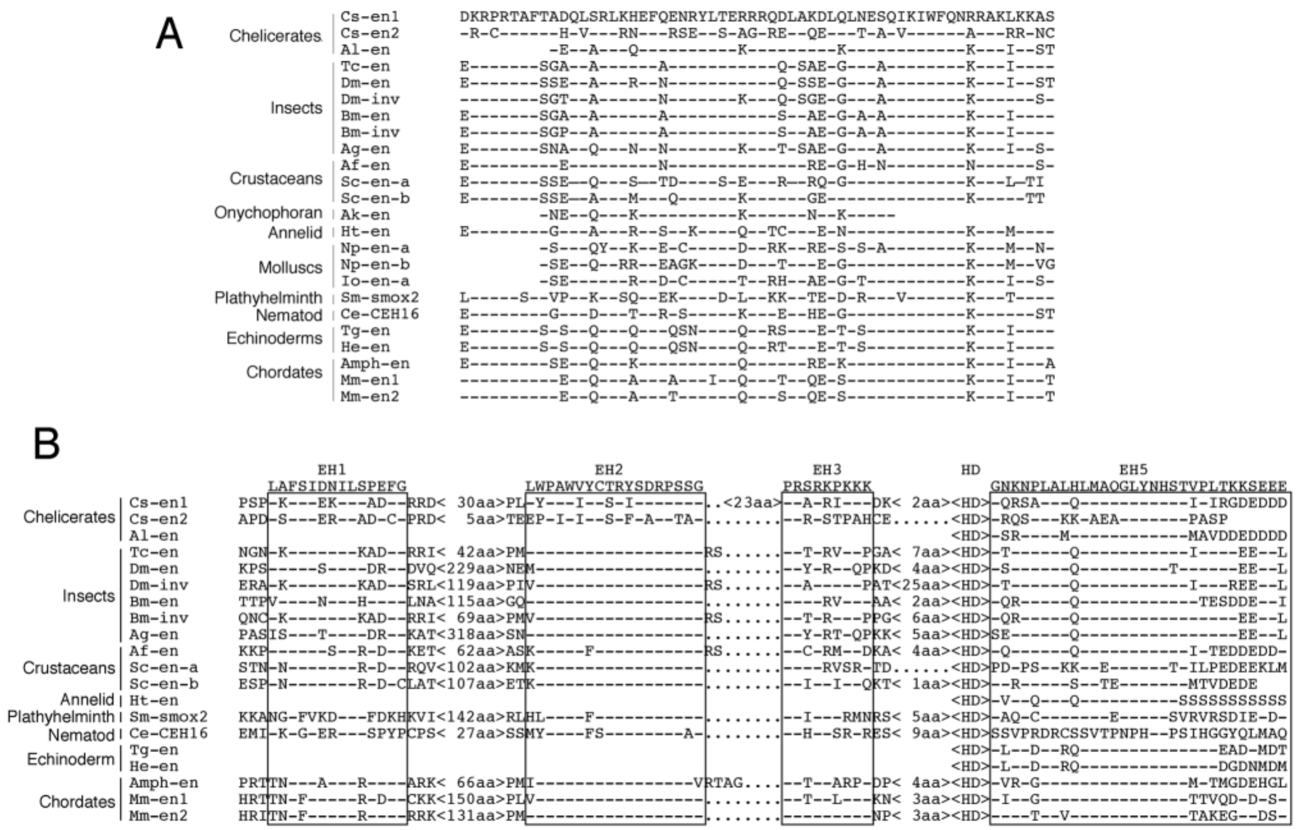

Fig. 1. Alignment of Engrailed homeodomains and Engrailed-specific domains. (A) The homeodomains are aligned to the Cs-EN-1 homeodomain. (B) Sequences of the Engrailed-specific domains are aligned to the consensus as defined by Duboule (Duboule, 1994). Engrailed-specific domains EH-1, EH-2, EH-3 and EH-5 are boxed. EH-4 corresponds to the homeodomain (HD), dashes indicate identical amino acids, dots indicate gaps. The spacing between the different domains are given in number of amino acids. Cs, *Cupiennius salei* (Chelicerata, AJ007437 and AJ315944); Al, *Archegozetes longisetosus* (Chelicerata, AF071404); Tc, *Tribolium castaneum* (Insecta, S73225); Dm, *Drosophila melanogaster* (Insecta, P02836 and P05527); Bm, *Bombyx mori* (Insecta, P27609 and P27610); Ag, *Anopheles gambiae* (Insecta, O02491); Af, *Artemia franciscana* (Crustacea, Q05640); Sc, *Saculina carcina* (Crustacea, AAC63993 and AAG40576); Ak, *Acanthokara kaputensis* (Onychophora, CAA71745); Ht, *Helobdella triserialis* (Annelida, P23397); Np, *Nautilus pompilius* (Mollusca, U23431 and U21857); Io, *Ilyanassa obsoleta* (Mollusca, S65924); Sm, *Schistosoma mansoni* (Platyhelminthes, S33640); Ce, *Caenorhabditis elegans* (Nematoda, P34326); Tg, *Tripneustes gratilla* (Echinodermata, P09532); He, *Heliocidaris erythrogramma* (Echinodermata, U58775); Amph, *Branchiostoma floridae* (Chordata, Cephalochordata, U82487); Mm, *Mus musculus* (Chordata, Vertebrata, P09065 and P09066).

protein contains an Engrailed-type homeodomain. However, this homeodomain is only 52-60% identical to other Engrailed homeodomains. The Engrailed-specific domains are also recognized in the Cs-EN-2 sequence (Fig. 1B). A remarkable point is the derived sequence of the highly conserved EH2 domain in both Cs-EN-1 and Cs-EN-2 (Fig. 1); nevertheless, the Cs-EN-2 homeodomain shows most similarities to Engrailed homeodomains in a BLAST search (Altschul et al., 1997). This similarity to other Engrailed homeodomains, the presence of the Engrailed-specific domains and many aspects of its expression (see later) suggest that *Cs-en-2* is also an *engrailed* ortholog in the spider.

The spider ortholog of the *wingless*/*Wnt1* gene was recovered by RT PCR and subsequent RACE PCR. The 3707 bp *Cs-wg* sequence contains an 1122 nucleotide ORF (position 16-1137). The likely full-length deduced protein encodes a 374 amino acid protein that clearly represents an ortholog of the Wingless/WNT1 class of WNT proteins, as becomes evident in a phylogenetic analysis (Fig. 2).

In addition to *Cs-wg*, four other members of the *Wnt* gene family were found in the spider: *Cs-Wnt5-1* and *Cs-Wnt5-2*, two orthologs of the vertebrate *Wnt5* gene (*DWnt3/5* in *Drosophila*); and *Cs-Wnt7-1* and *Cs-Wnt7-2*, two orthologs of the vertebrate *Wnt7* gene (*DWnt2* in *Drosophila*). The *Cs-Wnt-5-1* gene appears to have an interesting expression pattern with respect to segmentation. Therefore, a 2148 nucleotide sequence for *Cs-Wnt5-1* was recovered by RACE-PCR containing an 1143 nucleotide ORF (position 265-1407). The deduced Cs-WNT5-1 protein is a 381 amino acid protein. Cs-WNT5-1 clusters with vertebrate WNT5 and *Drosophila* DWNT3/5 in a phylogenetic analysis (Fig. 2).

A spider ortholog of the *Drosophila cubitus interruptus* (*ci*) gene was isolated by RT-PCR. The PCR product representing the spider *Cs-ci* gene is 391 base pairs long. The 130 amino acid Cs-CI protein fragment deduced from this sequence corresponds to amino acids 446-579 of the *Drosophila* CI protein. The Cs-CI fragment is 86% identical to the corresponding domain in the *Drosophila* CI protein, and up to 84% identical to the corresponding domain in vertebrate GLI proteins.

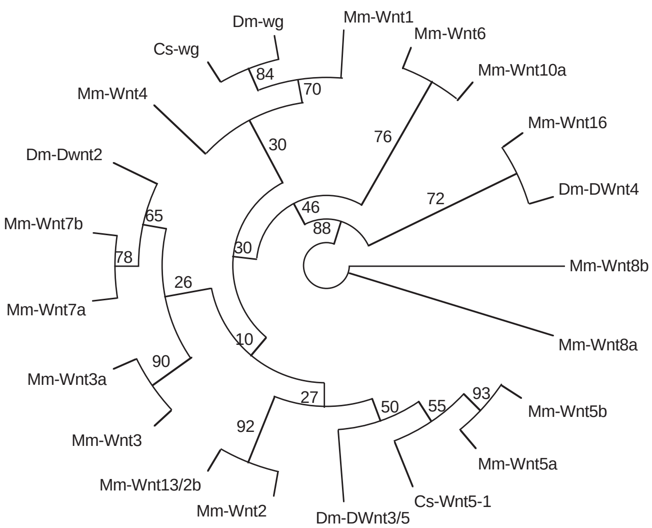

Fig. 2. Phylogram of Wingless/WNT sequences of mouse (Mm), *Drosophila melanogaster* (Dm) and *Cupiennius salei* (Cs). Phylogenetic analysis was done using PUZZLE (Strimmer and von Haeseler, 1996), as implemented in PAUP 4.0b6 (Swofford, 2001). Accession Numbers are as follows. Mm: Wnt1 (P04426), Wnt2 (P21552), Wnt3 (AAB38109), Wnt3a (P27467), Wnt4 (P22724), Wnt5a (AAA40567), Wnt5b (P22726), Wnt6 (AAA40569), Wnt7a (AAA40570), Wnt7b (AAA40571), Wnt8a (Q64527), Wnt8b (NP035850), Wnt10a (P70701), Wnt16 (AAD49352), Wnt13/2b (AAC25397). Dm: Wg (P09615), DWnt2 (S24559), DWnt3/5 (P28466), DWnt4 (P40589). Cs: Wg (AJ315945), Wnt5-1 (AJ315946).

engrailed in the spider embryo

Some aspects of the expression of *Cs-en-1* in spider embryos have been described previously (Damen et al., 1998), where its expression was used as a segmental marker in the spider embryo. The current paper describes all aspects of *Cs-en-1* expression, and, in addition, the embryonic expression of the *Cs-en-2* gene, as well as that of *wingless*, *Wnt5-1* and *cubitus interruptus*.

To allow a better understanding of the expression patterns in the spider, a short introduction is given to some morphological features. Chelicerates have two tagmata: a prosoma and an opisthosoma. The prosoma is the cephalothorax and bears six pairs of appendages in the spider: the cheliceres, the pedipalps and four pair of walking legs. The opisthosoma is the ‘abdomen’ of chelicerates. In spiders, opisthosomal limb buds (appendage anlagen) form on the second to fifth opisthosomal segment. These opisthosomal limb buds will form the respiratory organs (book lungs, first pair of buds) and the spinnerets (third and fourth pair) (Foelix, 1996).

Segmental *Cs-en-1* expression

In very early germ band stage embryos, *Cs-en-1* is expressed in five clear stripes, representing the pedipalp and the four walking leg segments; additionally, a very weak stripe is seen where the chelicere segment is forming (Fig. 3A). This chelicere segment is the anterior-most appendage-bearing segment and forms a little later than the other prosomal segments (Seitz, 1966). Somewhat later, this cheliceral *Cs-en-1* stripe is stained as strongly as the other prosomal stripes; in addition, the *Cs-en-1* stripes widen (Fig. 3B-F). Newly formed *Cs-en-1* stripes become successively visible posterior to the last prosomal segment, demarcating the first segments of the opisthosoma (Fig. 3D,F). Initially, these opisthosomal stripes seem to be somewhat narrower compared with the stripes in the prosomal segments. However, these stripes soon widen. Additional *Cs-en-1* stripes form successively at the posterior end of the embryo. At the stage when two opisthosomal stripes are visible, two spots of *Cs-en-1* expression become visible in the head region, anterior to the cheliceral *Cs-en-1* stripe (Fig. 3C). Later, these spots transform into small stripes. These spots probably demarcate the ocular segment that corresponds to the ocular (or pre-antennal) segment in insects and crustaceans, as recognized previously (Damen et al., 1998).

As soon as appendages form (Fig. 3E,F), *Cs-en-1* is primarily expressed in the ventral part of the embryo, which becomes even more prominent at later stages. Additionally, there is *Cs-en-1* expression in the posterior part of the appendages themselves. No *Cs-en-1* expression is visible dorsal to the prosomal appendages, except to the cheliceres. Only weak *Cs-en-1* expression is visible at the dorsal region of the opisthosomal segments (Fig. 3G,H,J).

At the so-called inversion stage, which results in dorsal closure, there are up to twelve *Cs-en-1* stripes detectable in the opisthosoma. The most posterior opistosomal segments appear especially to be very small; the *engrailed* stripe in the twelfth segment is only visible after DAPI counterstaining (Fig. 3K). Somewhat later, a pro-larval stage has been reached and the ring-like expression of *Cs-en-1* becomes obvious at the posterior end (Fig. 3L). This ring-like structure resembles the ring structure in a number of insects and may correspond to the proctodeum expression (Schmidt-Ott et al., 1994). From the inversion stage onwards, weak *Cs-en-1* expression is visible anterior to the labrum (Fig. 3I).

Segmental expression of *Cs-en-2*

The expression of *Cs-en-2*, the second *engrailed* gene in *Cupiennius*, deviates from that of *Cs-en-1*. The expression of *Cs-en-2* becomes apparent in a double stripe fashion somewhat later than *Cs-en-1* expression (Fig. 4A,B). The opisthosomal *Cs-en-2* stripes seem to split off from a larger domain of expression at the very posterior end of the embryo (Fig. 4C,E). These newly formed *Cs-en-2* stripes are also doublets; however, the cells between this doublet stripe express low levels of *engrailed* as becomes apparent after elongated staining (not shown). As soon as the limb buds appear as a landmark (Fig. 4D,E), it becomes evident that the anterior stripe of each double stripe marks the same anterior boundary as does *Cs-en-1*. However, *Cs-en-2* is not expressed in the posteriormost part of the appendage, whereas *Cs-en-1* is expressed in the complete posterior portion of the appendages (Fig. 4H). The posterior stripe of each doublet is located just posterior to the appendages, obeying a similar posterior border as *Cs-en-1*, although it is not possible to determine whether these posterior borders are identical, owing to the lack of a positional marker here.

Similar to *Cs-en-1*, *Cs-en-2* is expressed in a ring-like structure at the posterior end of the embryo (Fig. 4I), as well as anteriorly to the labrum (not shown). In contrast to *Cs-en-1*, *Cs-en-2* is expressed in the prospective stomodeum in early germ bands (Fig. 4F); at later stages, this form a ring in the foregut.

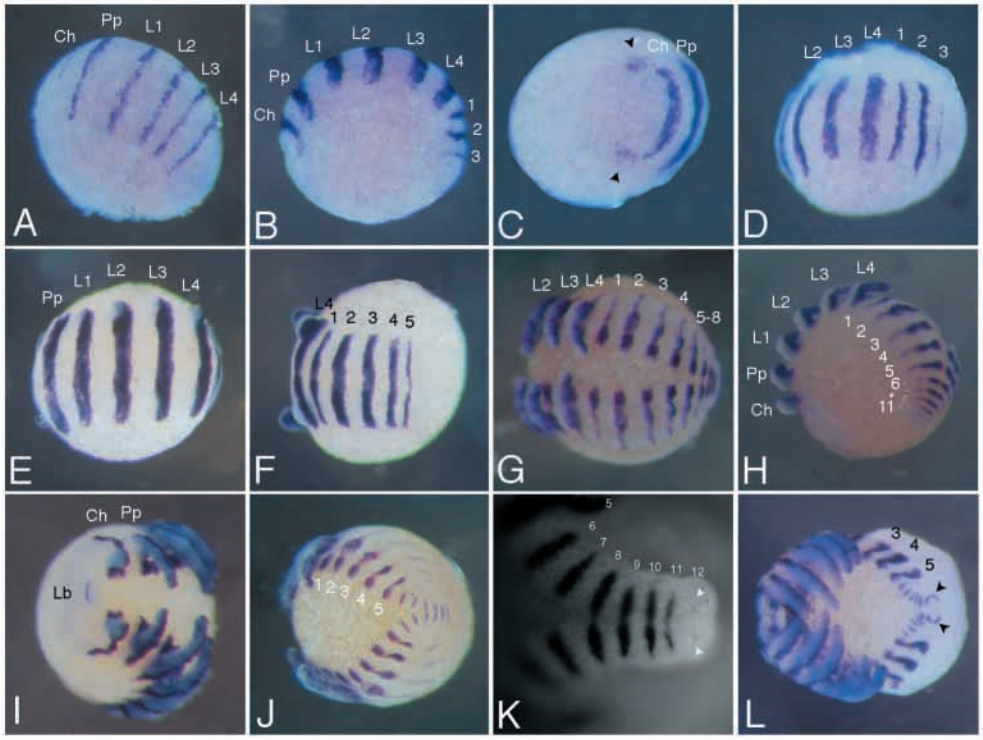

Fig. 3. Expression of *Cs-en-1* in embryos of the spider *Cupiennius salei*. (A) Young germ band stage, five clear stripes of *Cs-en-1* expression visible [Pp and legs (L)] and a weak stripe for the Cheliceres. No opisthosomal segments formed yet. (B-D) Lateral, anterior and posterior views, respectively, of the same embryo. Three opisthosomal segments are formed, marked by three stripes of *Cs-en-1*. Arrowheads in C indicate the ocular spots of *Cs-en-1* expression. (E,F) Different views of the same embryo. Five opisthosomal segments have formed. Limb buds straddle the anterior border of *Cs-en-1* expression. (G,H) Different views of the same embryo. Up to eleven *Cs-en-1* stripes are visible in the opisthosoma. The posterior part of the appendages expresses *Cs-en-1*. In addition, note that the abdominal limb buds on opisthosomal segment 2-5 straddle the anterior border of *Cs-en-1* expression. The expression of *Cs-en-1* is weaker dorsal to these opisthosomal limb buds, compared with its expression in the limb buds themselves and in the neuroectoderm ventral to them. The furrow that forms is the result of the inversion that leads to longitudinal splitting of the germ band and to dorsal closure. The two halves of the germband are connected in the head region and at the most posterior end. This is even more evident in the embryos shown in J,L. (I-K) More advanced stage of development, *Cs-en-1* expression anterior to the labrum. Twelve stripes of *Cs-en-1* expression detectable in the opisthosoma, the 12th one only as two weak spots after DAPI staining (K, arrowhead). (L) Stage after dorsal closure. Arrowheads mark the ring of *Cs-en-1* expression at the posterior end that probably represents the hind gut. Anterior is towards the left in all embryos. Ch, cheliceres; Pp, pedipalps; L1-L4, walking leg 1-4; Lb, labrum; Opisthosomal segments are indicated by 1-12.

Appendages straddle the anterior boundary of *engrailed* expression

Both *engrailed* genes are expressed in the limb buds and the appendages that form from them. The appendages on the prosomal segments (Fig. 5B, Fig. 3E,F, Fig. 4D,F), as well as the opisthosomal limb buds (second to fifth opisthosomal segment), straddle the anterior boundary of *Cs-en-1* and *Cs-en-2* expression (Fig. 3G, Fig. 4G), suggesting that this boundary is an important developmental boundary.

wingless and *Wnt* class segment polarity genes

Another remarkable aspect of the *engrailed* expression in the spider is the observation that the anterior border of the *engrailed* stripes sharpens earlier compared with the posterior border of each stripe (Fig. 5A). This may be the result of the action of *wg/Wnt* genes. In *Drosophila*, *wg*-expressing cells reside directly anteriorly to *en*-expressing cells, and a sharp parasegment boundary is formed between *wg*- and *en*-expressing cells, which are mutually exclusive. The *wg* and *en* genes function to maintain the polarity of the segments in insects (Martinez-Arias et al., 1988; Van den Heuvel et al., 1989; Nagy and Carroll, 1994; Oppenheimer et al., 1999).

The expression of *en* is activated by the action of the pair rule genes in *Drosophila*. In a second phase, *en* expression becomes autocatalytic, but is also influenced by *wg.* Later in *Drosophila* development, *en* expression becomes independent of *wg* (Heemskerk et al., 1991). The role of *wg* seems to be conserved in insects and crustaceans (Nagy and Carroll, 1994; Nulsen and Nagy, 1999; Oppenheimer et al., 1999). To test whether the signaling between *en*- and *wg*-expressing cells is present in the spider, members of the *wg*/*Wnt* gene family from the spider have been analyzed.

Expression of *Cs-wg* in the spider embryo

The *Cs-wg* gene is expressed in a segmentally iterated pattern in the spider embryo (Fig. 6). Expression is first detected after *Cs-en-1* and *Cs-en-2* expression can be detected. In the prosomal segments, *Cs-wg* is initially expressed only in a stripe in the anteroventral region of the appendages (Fig. 6A,E). The posterior expression border lies in the middle of the appendages, just anterior to the anterior border of *engrailed* expression. Unfortunately, it is not yet possible to double stain for these genes in the spider embryo to verify that the expression domains for *en* and *wg* in the spider are touching each other, as is the case in *Drosophila*. Nevertheless, the position of the anterior *en* and the posterior *wg* expression border just in the middle of the appendages strongly suggests that these expression domains are adjacent to each other.

Later, a spot of *Cs-wg* becomes visible dorsal to the prosomal appendages (Fig. 6D). In the opisthosoma, *Cs-wg* is expressed in small stripes at the dorsal side of the newly formed segments (Fig. 6B). These stripes expand later (Fig. 6C,F,H) and are just dorsal to the opisthosomal limb buds, but do not expand completely to the dorsal side of the germ band

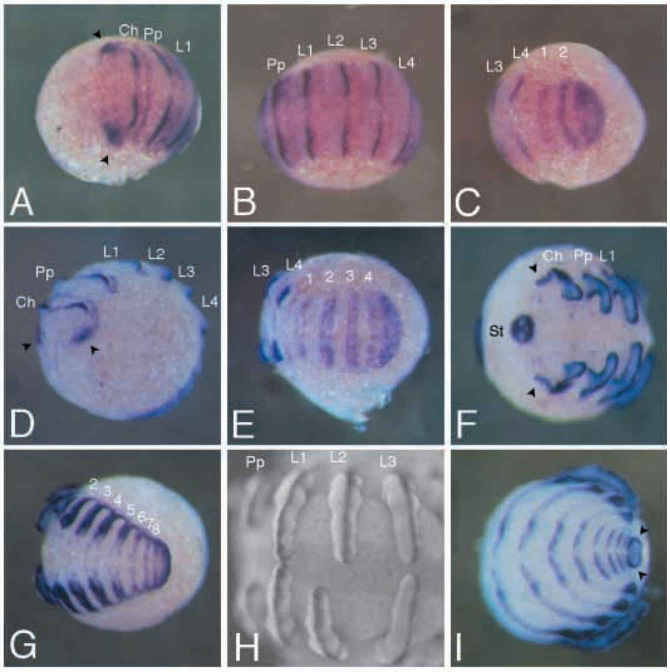

Fig. 4. Expression of *Cs-en-2* in embryos of the spider *Cupiennius salei*. (A-C) Different views of the same embryo (embryo is slightly younger than the one in Fig. 3B). Two stripes and a posterior cap of *Cs-en-2* expression are visible in the opisthosoma. The stripes in the more anterior segments are doublet stripes (most prominent for the Ch and Pp, A). Arrowheads in A indicate the ocular spots. Expression of *Cs-en-2* seems to diminish in the ventral midline, in contrast to *Cs-en-1* expression (compare with Fig. 2A-F). (D,E) Slightly older stage; four opisthosomal segments are present. Limb buds straddle the anterior border of each double stripe. Arrowheads in D indicate the ocular spots of *Cs-en-2* expression that became small stripes (F). (F,G) germ band stage at onset of inversion (comparable stage as in Fig. 3G). Strong expression of *Cs-en-2* in the stomodeum (st). Strong dorsal *Cs-en-2* expression and expression in two paired segmental spots in the neuro-ectoderm. (H) Detail of a DAPI counter-stained embryo. Expression of *Cs-en-2* has an anterior border in the appendage at the same position as *Cs-en-1*, but, by contrast, it does not cover the complete posterior part of the appendage. (I) Dorsal view of an embryo after dorsal closure (comparable stage to that in Fig. 3K). Arrowheads indicate the ring-like expression that probably represents the onset of the hind gut. Anterior is towards the left in all embryos. Ch, cheliceres; Pp, pedipalps; L1-L4, walking leg 1-4; St, stomodeum; Opisthosomal segments are indicated by 1-12.

(Fig. 6G). In addition, there is a small spot of *Cs-wg* expression just ventral to the opisthosomal limb buds, but not in the neuroectoderm more ventrally. The limb buds are good landmarks for demonstrating indirectly that the *Cs-wg* expression is just anterior to *engrailed* expression (Fig. 3G,H). Remarkably, the posterior margin of *Cs-wg* expression, just anterior to the sharp *engrailed* margin, forms a sharp border (Fig. 6C,F,G).

There is also a spot of *Cs-wg* expression posteriorly, in the abdominal limb buds on O2, O4 and O5, but not on O3 (Fig. 6F,G, arrowhead). This spot is in the *Cs-en-1* domain that covers the complete posterior part of the limb buds.

Although there is expression of *Cs-en-1* in the ventral neuroectoderm, there is no adjacent expression of *Cs-wg* here. To test whether there is a second *wg* gene in the spider that might function in this region of the embryo, RT-PCR was performed. Degenerated primers were used that lie in other domains than the ones used in the cloning of the *Wnt* genes (see Materials and Methods section) on RNA from an early germband stage spider (limb buds just forming). At this stage, segmentation takes place, and one would expect the gene involved in the segmentation process to be expressed. No additional genes were found in this PCR screen. Forty-three clones were sequenced: seven corresponded to *Cs-wg-1*, nine to *Cs-Wnt5-1*, six to *Cs-Wnt5-2*, eight to *Cs-Wnt7-1* and 13 to *Cs-Wnt7-2*. Although this does not form indisputable evidence, it is not very likely that there is a second *wg/Wnt1* gene in the spider. This is supported by the expression of the *Cs-Wnt5-1* gene, which might act in the ‘missing’ domain (see below).

In addition to the segmental expression, *Cs-wg* is expressed in two spots in the head (Fig. 6A,E), in a small stripe anterior in the labrum (Fig. 6E) and at the posterior end of the embryo (Fig. 6B,H). A comparable posterior domain is found in embryos of *Drosophila*, *Tribolium* (beetle) and *Triops* (branchiopod, crustacean) (Baker, 1988; Nagy and Carroll, 1994; Nulsen and Nagy, 1999). It has been proposed that the posterior *wg*-expressing cells could act as a source for a morphogen necessary for the function of the growth zone (Nulsen and Nagy, 1999).

Expression of *Cs-Wnt5-1* in the spider embryo

Surprisingly, the *Cs-Wnt5-1* gene shows a segmental expression in those regions of the embryo where *wg* expression is expected but where *Cs-wg* is not expressed (Fig. 7). The *Drosophila* ortholog *DWnt3/5* does not have a segmental function (Fradkin et al., 1995; Klingensmith and Nusse, 1994).

Cs-Wnt5-1 is expressed ventrally to the appendages and the opisthosomal limb buds, and also in an identical position in the segments that do not bear appendages (Fig. 7A-E). Although its posterior expression border is not as sharp as that of *Cs-wg*, the posterior border is just anterior to the position of the *engrailed* expression domains, again using the appendages as a landmark (Fig. 7E,F). The *Cs-Wnt5-1* gene, therefore, may act in this ventral region as a segment polarity gene. An in situ hybridization with both the *Cs-wg* and *Cs-Wnt5-1* probe shows that the two genes together cover the whole width of the germ band (Fig. 7H), just interrupted by the appendage anlagen, suggesting that both genes act similarly but in a different domain along the dorsoventral axis. Furthermore, *Cs-Wnt5-1* is expressed in segmentally dorsal spots in the opisthosoma (Fig. 7F,G), in rings in the appendages (Fig. 7F), in four spots in the head, in the labrum (Fig. 7A) and weakly at the very posterior end (Fig. 7C-E).

Expression of the spider *cubitus interruptus* gene

Additional evidence for the conservation of the Engrailed-Wingless/Wnt pathway comes from expression of the *cubitus interruptus* (*ci*) ortholog in the spider. Ci is a transcriptional activator for *wg* expression in *Drosophila*, and is expressed in the cells that do not express *engrailed* (Eaton and Kornberg, 1990; Motzny and Holmgren, 1995; Aza-Blanc and Kornberg, 1999). The spider *Cs-ci* gene is also expressed in the segmental

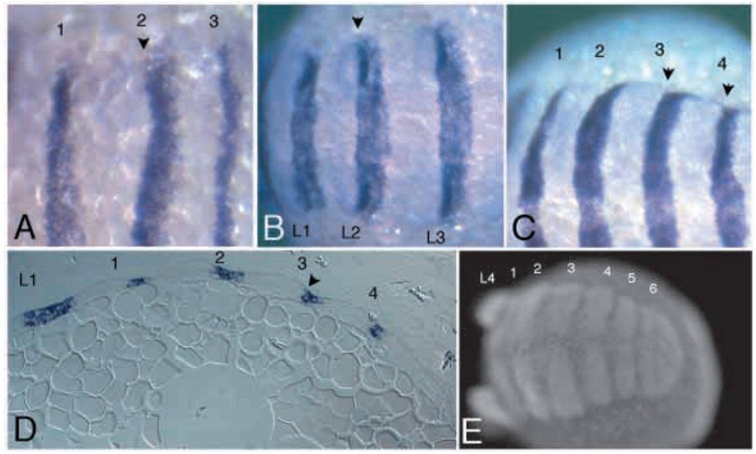

Fig. 5. Details of *Cs-en-1* expression point to a parasegmental organization of the spider embryo. (A) The opisthosomal *Cs-en-1* stripes have a sharp anterior boundary (arrowhead). The opisthosomal stripes of *Cs-en-1* expression are shown in an embryo in which three opisthosomal stripes are present. (B) Limb buds of the L1-L3 segments that straddle the boundary of *Cs-en-1* expression (arrowhead). (C) Anterior border of *Cs-en-1* expression correlates with a morphological visible groove (arrowheads). The opisthosomal stripes 1-4 in an embryo are shown in which seven opisthosomal stripes are present. (D) Longitudinal section of a *Cs-en-1* stained embryo. The grooves are visible at the domains of *en* expression (arrowhead). (E) DAPI stained embryo (no in situ hybridization). Grooves are clearly visible. Anterior is towards the left in all embryos. L1-L4, walking leg 1-4; Opisthosomal segments are indicated by 1-12.

regions that do not express *engrailed*, in a similar way to its ortholog in the fly (Fig. 8).

These data show that some of the major players that establish the parasegment boundary in *Drosophila* are also present in the spider, and that the segment-polarity network that establishes and maintains the parasegment boundary is likely to be present in the spider.

Morphologically visible grooves demarcate the parasegmental boundaries

Expression of segmentation genes point to a parasegmental organization. The presumptive parasegment borders are defined by grooves and are morphologically visible in the spider embryo, as in the fly embryo (Lawrence, 1992). Metamerization becomes morphologically visible in the spider embryo as soon as grooves form, as visualized by DAPI staining in Fig. 5E. As the anterior border of *engrailed* expression (Fig. 5C,D) and the posterior border of *Cs-wg* and *Cs-Wnt5-1* expression (Fig. 6B, Fig. 7D,E) are confined to the edge of these grooves, this suggests that the grooves define a parasegmental, rather than a segmental, subdivision.

DISCUSSION

Parasegmental organization of the chelicerate and arthropod embryo

Genetic and molecular studies have shown that parasegments are fundamental units in the design of the *Drosophila* embryo (Martinez-Arias and Lawrence, 1985; Lawrence et al., 1987; Lawrence, 1988; Lawrence, 1992). In addition, molecular comparisons demonstrate that parasegments are almost certainly the fundamental units of development not only in insects, but also in crustaceans (Dohle and Scholtz, 1988; Patel, 1994). Several pieces of evidence demonstrate that the spider embryo is presumably also built from parasegmental units. The parasegment, thus, is probably an entity that is evolutionarily conserved in arthropods.

In the spider, the appendages straddle the anterior border of the *engrailed* expression domain, as in insects and crustaceans, where they match exactly to the borders of parasegmental boundaries (Patel et al., 1989a; Martinez-Arias, 1993; Scholtz, 1995; Dohle and Scholtz, 1988). Boundaries play an important role as organizers. The appendages start forming at the

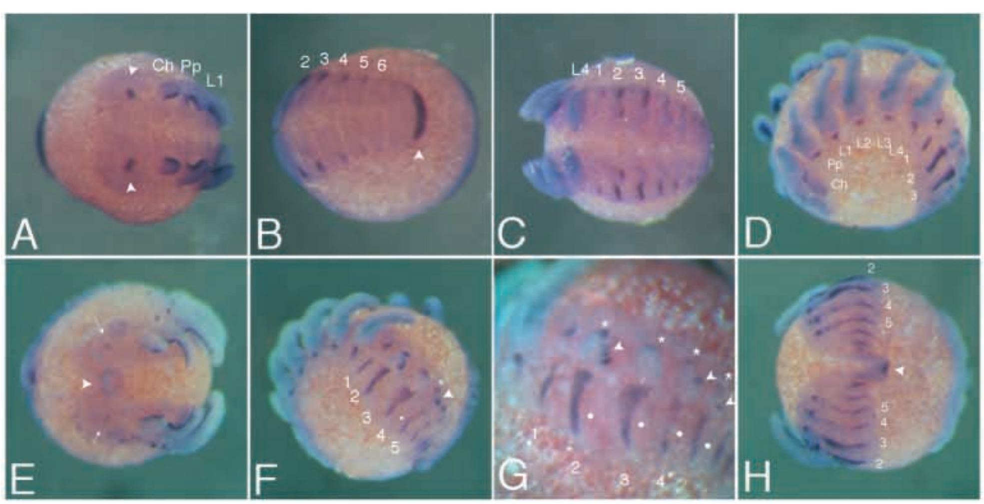

Fig. 6. Expression of *Cs-wg* in embryos of the spider *Cupiennius salei*. (A,B) Different views of the same embryo, showing the head region and the posterior region, respectively. *Cs-wg* is expressed in the ventral-anterior portion of the appendages. In B, *Cs-wg* is clearly expressed just anterior to the grooves that delimit the parasegments. (C) Slightly older embryo. *Cs-wg* expression dorsally to the opisthosomal limb buds. (D-G) Different views of one embryo, showing *Cs-wg* expression dorsal to the prosomal appendages (D), in the anterior labrum (arrowhead), the ventral-anterior region of the appendages (arrowheads) (E) and the opisthosoma (F). (G) A detail of the expression in a stripe dorsally (white circle) and a spot ventral (star) to the opisthosomal limb buds. Expression is also seen posterior to the opisthosomal limb buds of O2, O4 and O5 (arrowheads). In O2 these represent the lamellae of the book lung. (H) Posterior view of an embryo at the inversion stage. *Cs-wg* is still expressed at the very posterior end of the embryo (arrowhead). Anterior is towards the left in all embryos. Ch, cheliceres; Pp, pedipalps; L1-L4, walking leg 1-4; Lb, labrum; Opisthosomal segments are indicated by 1-12.

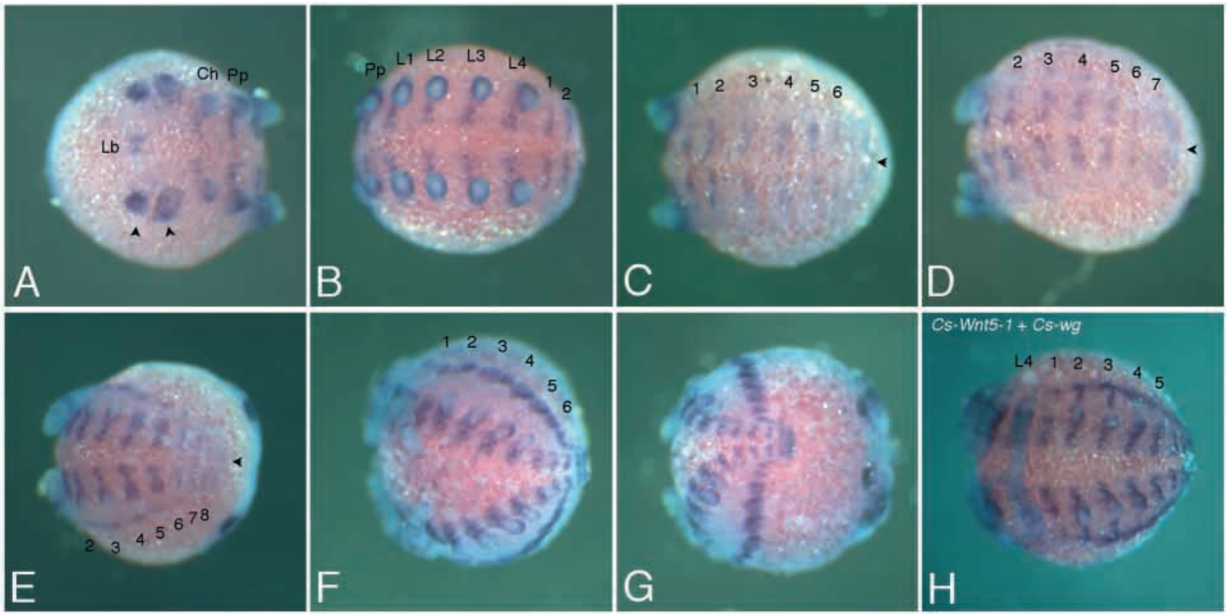

Fig. 7. Expression of *Cs-Wnt5-1* in embryos of the spider *Cupiennius salei*. (A,B) Different views of the same embryo. Expression of *Cs-Wnt5-1* is visible in segmentally iterated stripes. Furthermore, expression is seen in a ring in the appendages, four spots in the head (arrowheads) and the labrum. (C,D) Slightly older embryos with six and seven opisthosomal segments formed, respectively. The *Cs-Wnt5-1* stripes are located ventrally and do not completely extend dorsally. The arrowhead marks a weak expression domain at the posterior end of the germband. (E) Slightly older embryo. The stripes of *Cs-Wnt5-1* expression are ventral to the opisthosomal appendages; there is an incomplete ring of expression in these opisthosomal appendage anlagen. Expression becomes visible in segmental dorsal spots that almost form a continuous stripe. (F,G) An even older embryo. The dorsal spots are clearly visible now. The region dorsal to the appendage anlagen is free of *Cs-Wnt5-1* expression. (H) Expression of *Cs-wg* and *Cs-Wnt5-1* in one embryo. Together the two genes form one stripe of expression covering the complete width of the embryo, just interrupted by the opisthosomal limb anlagen. Anterior is towards the left in all embryos. Ch, cheliceres; Pp, pedipalps; L1-L4, walking leg 1-4; Lb, labrum; Opisthosomal segments are indicated by 1-12.

intersection of the anteroposterior and dorsoventral boundaries, as has been demonstrated by producing ectopic boundaries (Cohen, 1993; Cohen et al., 1993; Tabata et al., 1995; Serrano and O'Farrell, 1997; Niwa et al., 2000). At this intersection of boundaries, both Wg and DPP are produced, and their synergistic activity determines an organizer for appendage formation. It is not yet known how the dorsoventral axis is determined in the spider. Nevertheless, the formation of appendages on the border defined by *engrailed* indicates that this border specifies a functional compartment boundary for appendage formation in the spider.

An additional piece of evidence comes from the observation that the anterior border of the *engrailed* stripes sharpens earlier than the posterior border of each stripe, as in insects and crustaceans (Patel, 1994). The posterior margin of *Cs-wg* expression that is adjacent to the sharp *engrailed* expression border is a sharp border as well. This implies that in chelicerates the parasegments are also the first metameric units to be resolved.

Another important argument for the parasegmental organization of the insect embryo is that key developmental genes are expressed in such domains (Struhl, 1984; Martinez-Arias and Lawrence, 1985; Lawrence, 1988) (summarized in Fig. 9). The chelicerate posterior Hox genes (*Antennapedia*, *Ultrabithorax-2*, *abdominal A* and *Abdominal-B*) obey anterior expression borders (Damen et al., 1998; Telford and Thomas, 1998; Damen and Tautz, 1999) that correspond to boundaries defined by *engrailed*, as in insects (Struhl, 1984; Kaufman et al., 1990), and imply the existence of a functional parasegmental organization (Fig. 9).

By contrast, the anterior Hox genes (*labial*, *proboscipedia*, *Hox3*, *Deformed* and *Sex comb reduced*) are expressed in a segmental register rather than a parasegmental one in both chelicerates and insects (Kaufman et al., 1990; Damen et al., 1998; Telford and Thomas, 1998; Damen and Tautz, 1999; Abzhanov et al., 1999) (M. Schoppmeier and W. G. M. D., unpublished data). Remarkably, the anterior expression borders of most of these anterior Hox genes lie outside the region that, in *Drosophila*, is patterned as a result of the action of the pair rule genes and the *engrailed* stripes that directly depend on these pair rule genes (Fig. 9). The patterning of this anterior head region is probably controlled in a different way (Gallitano-Mendel and Finkelstein, 1997). Although pair rule gene orthologs might be involved in spider segmentation (Damen et al., 2000), it is not known yet whether these genes act in comparable regions of the embryo, as in *Drosophila*.

Evolution of the segmented body plan

There is an ongoing discussion of whether segmentation in different phyla has a common origin (Davis and Patel, 1999). The presumably conserved segment-polarity network and the organization into parasegments can be seen as an ancestral character for arthropods. In the closely related onychophorans, *engrailed* expression points to a comparable organization (Wedeen et al., 1997). However, segment polarity gene orthologs are apparently not involved in body segmentation in

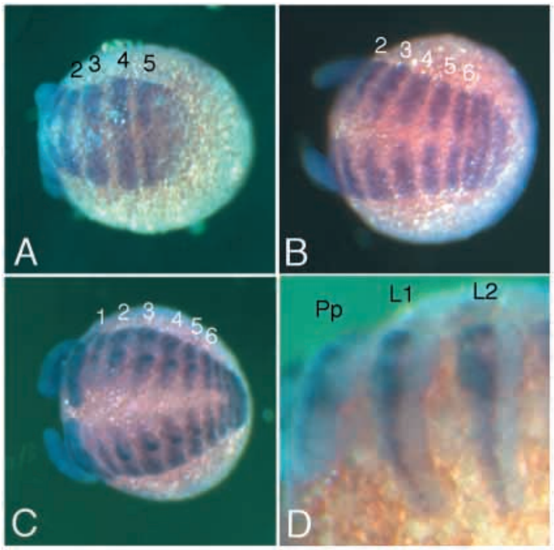

Fig. 8. Expression of *Cs-ci* in embryos of the spider *Cupiennius salei*. (A) Segmentally iterated expression of *Cs-ci* in opisthosoma of young germ band stage embryo. (B) Slightly older embryo, new stripes form posteriorly. (C,D) Expression of *Cs-ci* is in the anterior part of the opisthosomal limb anlagen (C) and appendages (D), showing that *Cs-ci* is expressed in the *engrailed* negative cells. Anterior is towards the left in all embryos. Pp, pedipalps; L1-L2, walking leg 1-2; Opisthosomal segments are indicated by 1-12.

other segmented phyla. In annelids, *engrailed* is expressed in segmentally iterated spots in the CNS and in mesodermal cells, but is probably not involved in body segmentation as in arthropods (Wedeen and Weisblat, 1991; Lans et al., 1993; Seaver and Shankland, 2001; Seaver et al., 2001). The establishment of segment polarity in leeches is independent of cell interactions along the anteroposterior axis; this is in contrast to the situation in arthropods, where anterior and posterior fates of the segments are specified by intercellular signaling between *wg*- and *en*-expressing cells. (Seaver and Shankland, 2001). Furthermore, there are no indications that the annelid embryo is constructed from units like the parasegment. In the leech, progeny of particular teloblasts overlap with respect to segmental boundaries and do not form genealogical units like in crustaceans (Weisblat and Shankland, 1985; Irvine and Martindale, 1996). Some key aspects of arthropod segmentation are thus not present in annelids. The segmentation of annelids and arthropods, therefore, seems to be brought about by different mechanisms. This is an important argument against a common origin of segmentation in annelids and arthropods.

In chordates it is also doubtful whether *engrailed* plays a role in somitogenesis. *engrailed* but not *wingless* is expressed in reiterated pattern in the somites of the cephalochordate amphioxus (Holland et al., 1997; Holland et al., 2000), which suggests that the segment polarity gene network as present in arthropods is not conserved. Furthermore, vertebrate *engrailed*

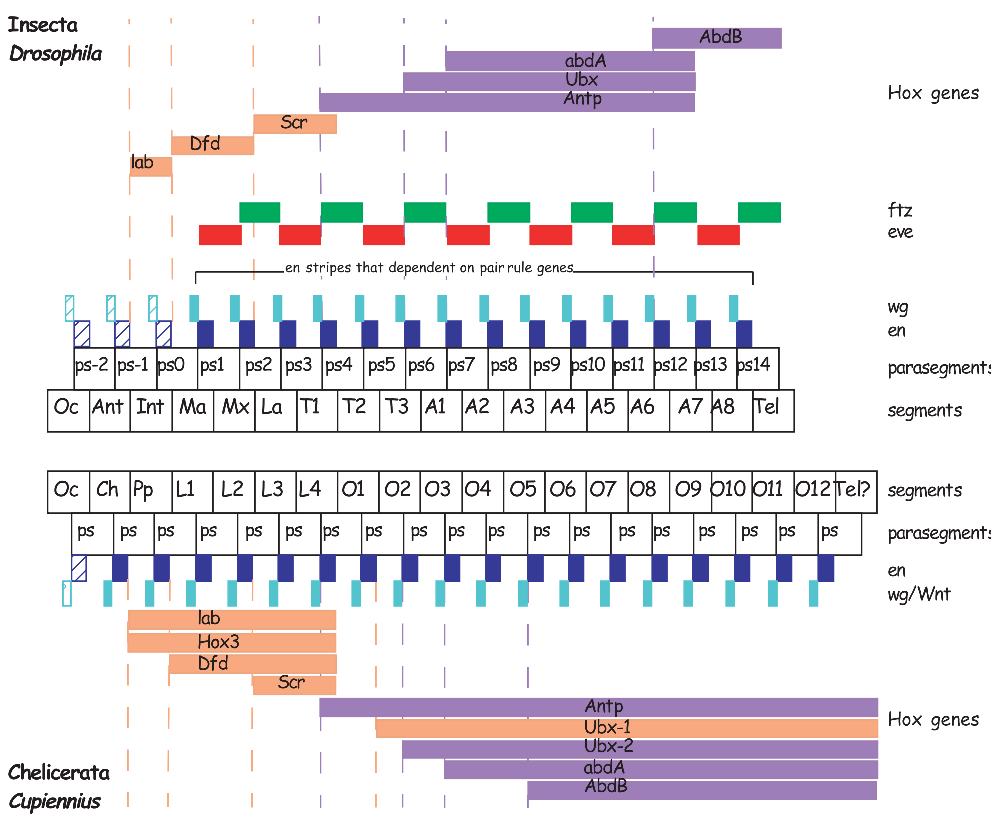

Fig. 9. Engrailed expression in the spider marks probable fundamental boundaries. The upper part shows the situation in *Drosophila*. The stripes of *en* expression mark the anterior parasegment boundaries, whereas *wg* marks the posterior parasegment boundaries. The parasegments 1-14 are dependent on the action of the pair-rule genes. The anterior expression border of the posterior Hox genes obeys a parasegmental boundary (purple), in contrast to the anterior one that obeys a segmental boundary (orange). Lower part shows the situation in the spider *Cupiennius*. The insect and chelicerate segments are homologized as proposed by Telford and Thomas (Telford and Thomas, 1998) and Damen et al. (Damen et al., 1998). Note that the *Cs-Ubx1* gene probably obeys a segmental boundary. For *Abd-B*, the domain of strong expression is used. Data are taken from Kaufman et al. (Kaufman et al., 1990), Schmidt-Ott and Technau (Schmidt-Ott and Technau, 1992), Jürgens and Hartenstein (Jürgens and Hartenstein, 1993), Damen et al. (Damen et al., 1998), Damen and Tautz (Damen and Tautz, 1998; Damen and Tautz, 1999) and M. Schoppmeier and W. G. M. D. (unpublished). Ant, antennal segment; Int, intercalary segment; La, labial segment; Ma, mandibular segment; Mx, maxilar segment; Oc, ocular segment/acron; ps, parasegment; T1-T3, thoracic segment 1-3; A1-A8, abdominal segment 1-8; Tel, telson; Ch, cheliceres; Pp, pedipalps; L1-L4, walking leg 1-4; O1-O12, opisthosomal segments 1-12; *ftz*, *fushi tarazu*; *eve*, *even-skipped*.

orthologs do not play a role in somite formation or maintenance of the somite boundaries. This points to different mode of segmentation in vertebrates and arthropods, and does not support a common origin of segmentation. However additional evidence is required to prove this.

Duplication of *engrailed* genes

In several metazoan phyla there are representatives that contain duplicated *engrailed* genes, whereas others contain only one gene, pointing to independent duplications. Duplicated *engrailed* genes have been found in several insect groups, like the two *engrailed* paralogs *engrailed* and *invected* in *Drosophila* (Coleman et al., 1987; Hui et al., 1992; Peterson et al., 1998; Marie and Bacon, 2000), whereas in insects such as *Tribolium* and *Schistocerca*, only one *engrailed* gene has been detected (Patel et al., 1989a; Brown et al., 1994). Independent duplications of the *engrailed* gene also appear to have taken place in some crustacean lineages (Gibert et al., 1997; Gibert et al., 2000; Abzhanov and Kaufman, 2000). The same is known from other phyla, as in some molluscs (Wray et al., 1995) and chordates (Joyner and Martin, 1987; Joyner and Hanks, 1991; Holland and Williams, 1990; Holland et al., 1997).

In the spider, two *engrailed* genes have been found; however, phylogenetic analyses (not shown) do not allow conclusions on the origin of the duplication. Only one *engrailed* gene has been described for another chelicerate, the mite *Archegozetes longisetosus* (Telford and Thomas, 1998), which suggests that the duplication of *engrailed* genes in chelicerates is restricted to the spider lineage. However, the spider *Cs-en-2* gene was not found in our PCR screen with redundant primers, probably owing to sequence derivation of the *Cs-en-2* EH2 domain (see Fig. 1) to which the PCR-primers were directed. The PCR method was also used to find the mite *engrailed* ortholog (Telford and Thomas, 1998). Therefore, a second *engrailed* gene could be missed in the PCR screen for the mite, as was the case for *Cs-en-2* of the spider. Nonetheless, a duplication of the *engrailed* gene took place somewhere in the chelicerate lineage. It remains to be elucidated whether this duplication took place before or after the spiders and mites diverged.

Different regulation of the two spider *engrailed* genes

The two spider *engrailed* genes both seem to define the same boundary; nevertheless, the way they appear is very different and suggests different modes of regulation. *Cs-en-1* is expressed in a comparable way to *engrailed* in insects and crustaceans. Its expression starts in the region where expression of the spider orthologs of the *Drosophila* pair rule genes *hairy*, *even-skipped* and *runt* diminishes (Damen et al., 2000). It is not yet possible to produce double labeling in the spider; nevertheless, this correlation suggests that the pair rule gene orthologs may act upstream of the *Cs-en-1* gene in the spider, as is the case in insects where the *engrailed* expression domains are defined by the action of the pair rule genes (DiNardo and O'Farrell, 1987; DiNardo et al., 1988; Patel et al., 1994; Rohr et al., 1999).

However, both the expression of *Cs-en-2* at the most posterior end of the embryo and the doublet stripes are atypical and unique for *engrailed* genes. The way the *Cs-en-2* stripes form is not completely clear; they seem to originate from the broad posterior domain and than split to form the doublet (Fig. 4E). Nonetheless, the final anterior position of the anterior stripe of the doublet seems to be identical to the ones for *Cs-en-1* and might also be maintained by interaction with *Cs-wg*/*Cs-Wnt5-1*-expressing cells.

The broad posterior domain of *Cs-en-2* expression in the spider embryo is in a comparable domain to the spider pair rule orthologs (Damen et al., 2000), giving some indication that the *Cs-en-2* gene might act as a more upstream segmentation gene. However, in contrast to the spider pair rule gene orthologs *hairy*, *even-skipped* and *runt* (Damen et al., 2000), the expression of *Cs-en-2* is not dynamic in this posterior domain. However, *Cs-en-2* expression is only detected after *Cs-en-1* expression in the early germ band stages when the prosomal segments form. Thus, there might be a difference between the specification of the prosomal segments and the opisthosomal segments that are formed from the posterior growth zone. Further analysis of the *Cs-en-2* gene is required to answer these questions.

Dorsoventral differences in segmental *engrailed* and *wingless/Wnt* expression

During the course of development, the two spider *engrailed* genes predominantly act in different domains along the dorsoventral axis. At the onset of inversion, *Cs-en-1* is less intensively expressed at the future dorsal side, whereas expression of *Cs-en-2* is completely reduced at the future ventral side. By contrast, the duplicated insect *engrailed* genes are expressed in more or less redundant domains (Coleman et al., 1987; Peterson et al., 1998; Marie and Bacon, 2000), whereas the duplicated crustacean *engrailed* genes have different modes of expression (Gibert et al., 2000; Abzhanov and Kaufman, 2000). However, these differences are not as dramatic as the ones seen in the spider. The spider *wg/Wnt* class genes *Cs-wg* and *Cs-Wnt5-1* are also differently expressed along the dorsoventral axis of the embryo and together they appear to cover the complete dorsoventral axis.

In *Drosophila*, cells along the dorsoventral axis acquire stable *en* expression at different times and no longer need *wg* function for *en* expression (Bejsovec and Martinez Arias, 1991). This transition of *en* regulation happens first at the dorsal side of the embryo and later also at the ventral side of the embryo, and is even reflected in dorsoventral differences in activity of the *en* promoter (DiNardo et al., 1988). The different modes of regulation of the *engrailed* gene in *Drosophila* along the dorsoventral axis of the embryo might be reflected in the differential expression along the dorsoventral axis of the spider *engrailed* genes, as well as the spider *wg/Wnt5-1* genes.

Segment-polarity role for *Cs-Wnt5-1*

The *Cs-Wnt5-1* gene is probably involved in segmentation. The *Cs-Wnt5-1* expression pattern suggests that the gene acts in the ventral region of the germ band as a segment-polarity gene in a domain where the *Cs-wg* gene is not expressed. In insects (*Drosophila*, *Tribolium* and the cricket *Gryllus*) and the crustacean *Triops*, the *wg* gene seems to cover the complete width of the germ band (Baker, 1988; Nagy and Carroll, 1994; Nulsen and Nagy, 1999; Niwa et al., 2000). The *Drosophila* ortholog of *Cs-Wnt5-1*, *DWnt3/5*, does not have a function in segmentation, whereas crustacean *Wnt5* orthologs have not yet been analyzed (Fradkin et al., 1995; Klingensmith and Nusse, 1994). The spider *Cs-wg* gene and *Cs-Wnt5-1* gene thus seem together to perform the function of the single *wg* gene in insects or crustaceans. It remains open to speculation whether the segmental role of *Cs-Wnt5-1* in the spider is one that has been acquired in the chelicerate lineage and replaces the function of *wg* in this region, or one that has been lost in the lineage leading to the insects and crustaceans, and has been replaced there by *wg*.

The spider opisthosoma consist of twelve segments

Seitz (Seitz, 1966) recognized in his morphological description of the *C. salei* embryo, nine segments in the opisthosoma of the developing spider embryo. However, the 12 *engrailed* stripes as well as 12 segmentally iterated spots of both *Cs-Pax6* and *Cs-prd-1* (W. G. M. D., unpublished) in the opisthosoma of the *Cupiennius* embryo points to 12 opisthosomal segments. Twelve opisthosomal segments probably represents the ancestral state for spiders, and for chelicerates in general (Foelix, 1996; Westheide and Rieger, 1996). Mesothelae, the phylogenetically oldest spiders, still contain a segmented opisthosoma that consists of 12 metameres (Foelix, 1996). This is in contrast to more advanced spiders, like *Cupiennius*, where the segmentation of the opisthosoma is obvious only in embryos. These data thus show that, although morphologically hardly detectable, the opisthosoma of *Cupiennius* consists of 12 segments, which represents the ancestral state for spiders and chelicerates.

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