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SHORT COMMUNICATION

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A Hox class 3 orthologue from the spider *Cupiennius salei* is expressed in a Hox-gene-like fashion

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Abstract The class 3 Hox gene orthologue in insects, *zerknüllt* (*zen*), is not expressed along the anterior-posterior axis, but only in extra-embryonic tissues, suggesting that it has lost its function as a normal Hox gene. To analyse whether this loss of Hox gene function has already occurred in a basal arthropod lineage, we have isolated a Hox3 orthologue from the spider *Cupiennius salei*. In contrast to the insect *zen* sequences, which have a highly diverged homeobox, the spider Hox3 gene orthologue, *Cs-Hox3*, shows a high sequence similarity to the class 3 Hox genes of other phyla, including chordates. In situ hybridization in early embryos shows that it is expressed in a continuous region covering the pedipalp segment and the four leg-bearing segments. This expression pattern suggests a Hox-gene-like function for *Cs-Hox3*. On the other hand, the expression pattern does not strictly follow the colinearity rule, as it overlaps fully with the expression domain of the class 1 orthologue of the spider, *Cs-lab*. Still, our data suggest that the ancestor of the arthropods must have had a class 3 Hox gene with a function in anterior-posterior axis specification and that this function has been lost in the lineage leading to the insects.

Introduction

The Hox genes play a role in the control of regionalization along the anterior-posterior body axis in metazoa and mutations in Hox genes cause homeotic transformation of body parts (Lewis 1978; Krumlauf 1994). Hox genes have a wide phylogenetic distribution within the metazoa including protostomes and deuterostomes. They are usually arranged in genomic clusters and are expressed in a colinear fashion (Beeman 1987; Duboule and Dollé 1989; Graham et al. 1989; Akam 1989; Kaufman et al. 1990). The Hox gene cluster is thought to have arisen from a single gene by three rounds of serial duplications (Schubert et al. 1993; Zhang and Nei 1996). Furthermore, the whole Hox cluster in the vertebrate lineage underwent further duplications, resulting in four paralogous Hox clusters in mouse and humans. Insects have a single cluster that consists of eight Hox genes. The last common ancestor of the protostomes and deuterostomes may have had a cluster of probably six or seven Hox genes, containing orthologues for the *lab*-Hox1, *pb*-Hox2, Hox3, *Dfd*-Hox4, *Scr*-Hox5, *Antp*/*Ubx*/*abdA*-Hox6-7-8 and *AbdB*-Hox9–13 genes (Akam et al. 1994; Duboule 1994; Garcia-Fernàndez and Holland 1994; Carroll 1995; Valentine et al. 1996; Kourakis et al. 1997). The insect *zerknüllt* (*zen*) orthologues are potential candidates for a Hox3 orthologue in protostomes (Falciani et al. 1996). Though they show only poor sequence similarity, they are found at the expected position in the Hox cluster of the fly and beetle (Rushlow et al. 1987a; Kaufman et al. 1990; Falciani et al. 1996). However, they are not expressed in a Hox-gene-like fashion, but are involved in dorso-ventral specification in *Drosophila* (Rushlow et al. 1987b; Rushlow and Levine 1990) and possibly generally in extra-embryonic tissue formation (Falciani et al. 1996). Accordingly, Falciani et al. (1996) concluded that *zen* is a divergent class 3 Hox gene which has lost its Hox gene function in the insect lineage.

The existence of a putative class 3 Hox orthologue has been demonstrated in polymerase chain reaction (PCR) screens for three other protostome species, the horseshoe crab *Limulus* (Arthropoda) and the polychaetes *Ctenodrilus* and *Chaetopterus* (Annelida) (Cartwright et al. 1993; Dick and Buss 1994; Irvine et al. 1997). However, it is not known whether these protostomian class 3 Hox genes are expressed in the typical Hox register as in vertebrates, or whether they play a different role as in insects. In the study described here, we have analysed a class 3 Hox gene orthologue from the spider *Cupiennius salei* (*Cs-Hox3*). The spiders are representatives of the chelicerates, which arose very early during arthropod radiation. In a previous study we have found that the

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anterior expression boundaries of orthologues of other Hox genes are expressed in the same register and at the same segmental position as in *Drosophila* (Damen et al. 1998). This suggested that the Hox gene expression register found in the spider and in insects reflects the ancestral mode of segmental specification in arthropods. Thus, the comparison of the *Cs-Hox3* and *zen* genes, both with respect to their sequence and their expression patterns, are of particular interest for the understanding of the evolution of the Hox3 genes.

Materials and methods

Embryos

We used embryos of the Central American wandering spider *Cupiennius salei* Keys. (Ctenidae, Araneida) from a colony bred by Ernst-August Seyfarth in Frankfurt am Main (Germany). Ctenid spiders carry their cocoons attached to their spinnerets. The cocoons of *C. salei* contain several hundred embryos. Embryos were removed by making a small incision into the cocoon. After removing the number of embryos needed, the cocoon was sealed with a small strip of adhesive tape and returned to the spider.

Cloning of *Cs-Hox3*

A fragment for the spider Hox3 orthologue was obtained by reverse transcriptase (RT)-PCR with RNA from germ-band stage embryos. The oligonucleotide primers used were: *Hox3-forward* AARMGNGCNMGNACNGCNTWYAC and *Hox3-backward* TCYTTYTTRTAYTTCATNCKNCKRTT. The PCR fragment was cloned and sequenced and then used to recover a cDNA from an embryonic *C. salei* cDNA library in λ ZAPII (Stratagene). Two clones were recovered and the sequence was determined from both strands on an ABI-377XL automated sequencer (Applied Biosystems). The full nucleotide sequence data will appear under the accession number AJ005643.

In situ hybridization

Whole-mount in situ hybridizations were essentially performed as described previously for *Drosophila* (Tautz and Pfeifle 1989) using RNA probes (Klingler and Gergen 1993). The wash steps and incubation steps were extended for the large spider embryos. In short, the embryos were dechorionated in a 50% commercially available bleach solution (about 2% natriumhypochlorite) and then fixed overnight in a 1:1 mixture of heptane and 5.5% formaldehyde in PEMS buffer (100 mM Pipes, 1 mM EDTA, 2 mM $MgSO_4$, pH 6.9) under gentle agitation. After fixation, the embryos were placed into methanol and the vitelline membranes were removed manually using Dumont-5 forceps. The embryos were stored in methanol at –20°C for at least a few days before using them. For the in situ hybridizations, the embryos were rehydrated in PBS-T (phosphate-buffered saline plus 0.1% Tween 20) in several steps. After a 20 min post-fixation in 4% paraformaldehyde in PBS and three subsequent washes in PBS-T, the embryos were digested with 10 µg/ml proteinase K and then fixed again in 4% paraformaldehyde in PBS. After four additional washes in PBS-T the embryos were transferred into hybridization solution (Klingler and Gergen 1993) and prehybridized at 65°C for 1 h. The embryos were hybridized overnight with the anti-sense DIG (digoxigenin) labelled RNA probes at 65°C. The probe was removed by a 30 min wash in hybridization solution at 65°C, a 10 min wash in a 1:1 mixture of hybridization solution and PBS-T at room temperature (RT) and several washes in PBS-T for a total of 1 h. Subsequently, the embryos were blocked in PBS-T with 1% bovine serum albumin (BSA) and 2% normal sheep serum for 30 min, followed by a 4-h incubation with the 1:2000 diluted anti-DIG antibody (Boehringer) in the same buffer. After this incubation, the embryos were washed in PBS-T for at least 16 h, with several exchanges of the washing buffer. After three washes in the staining buffer the embryos were stained for from one to several hours, until the staining was visible.

Results and discussion

The spider class 3 Hox gene

A candidate for the spider Hox3 orthologue was initially identified by PCR using degenerate oligonucleotides directed against the class 3 homeodomain. The PCR fragment was then used to screen a cDNA library. The longest clone recovered contained a 2.6-kb insert. Sequencing showed that it contains two open reading frames of 616 and 1112 bp respectively, separated by a 49-bp intron sequence. Another shorter cDNA did not contain this intron sequence, indicating that the intron in the longest cDNA clone is the result of incomplete mRNA processing. Intronic sequences were also identified in cDNAs for other genes isolated from this embryonic spider cDNA library (unpublished observation). The deduced protein sequence is 576 amino acids long. The homeodomain is located at position 210-269. This position is similar to that found for chordate Hox3 proteins, but dissimilar to the *zen* genes, where the homeodomain is closer to the N-terminus (Falciani et al. 1996). Alignment of the full homeodomain shows three to five mismatches when compared to the Hox3 homeodomains from chordates (Fig. 1A). In addition, it shows high sequence similarity to the partial homeodomain sequences of three other protostome species, two annelids (*Ctenodrilus* and *Chaetopterus*) and another chelicerate (*Limulus*). On the other hand, it deviates at diagnostic positions from the Hox class 2 homeodomains, which are thought to be most closely related to the class 3 genes (Schubert et al. 1993; Zhang and Nei 1996; Fig. 1A).

We also find a hexapeptide motif separated from the homeobox by an intron. The hexapeptide is a conserved sequence stretch present upstream of the homeodomain in Hox proteins (Duboule 1994). Figure 1B shows an alignment of the hexapeptides and the neighbouring amino acids. Again, the spider sequence shows a high similarity to the respective region of other class 3 Hox genes. The insect ZEN proteins, on the other hand, do not contain a hexapeptide motif (Falciani et al. 1996). The boxes of sequence similarity outside the homeodomain that were identified for the *zen* genes (Falciani et al. 1996) could not be found in the spider Hox3 gene.

In a phylogenetic analysis using the homeobox sequences, the spider sequence groups closely with the chordate class 3 genes and is clearly different from the class 2 genes and the *zen*-like sequences (Fig. 2). We conclude from this that our clone represents a clear Hox class 3 orthologue from the spider, which is much more similar to the class 3 Hox genes in chordates than to the

A

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Antp        Dm    RKRGRQTYTRYQTLELEKEFHFNRYLTRRRRIEIAHALCLTERQIKIWFQNRRMKWKKEN

Cs-Hox3     Cs    A--A-TA--SA-LV-----------C-P----M-NL-N-S-------------Y---Q
Hox3        Amph  G--A-TA--SA-LV-----------C-P--V-M-AM-N---------------Y---Q
HoxA3       Mm    S--A-TA--SA-LV-----------C-P--V-M-NL-N---------------Y--DQ
HoxB3       Mm    S--A-TA--SA-LV-----------C-P--V-M-NL-N-S-------------Y--DQ
HoxD3       Mm    S--V-TA--SA-LV-----------C-P--V-M-NL-N---------------Y--DQ
Chaetopterus                        ------C-P----M-AL-N--------
Ctenodrilus                         ------C-P----M-AL-S-S------
Limulus                             ------C-P----M-NL-N--------

zen         Sg    S--A-TA--SQ-LI----D-SM----C-P----L-AQ-G---------------Y---K
zen         Tc    G--A-TA--SA-LV---R---HGK--S-P---Q--EN-N-S-------------H---Q
zen         Dm    V-LK-TAF-SV-LV---N--KS-M--Y-T------QR-S-C---V----------F--DI
z2          Dm    S--S-TAFSSL-LI---R---L-K--A-T-----SQR-A-----V----------L--ST

pb          Dm    PR-L-TA--NT-L----------K--C-P------AS-D-----V-V--------H-RQT
HoxA2       Mm    SR-L-TA--NT-L----------K--C-P--V---AL-D-----V-V--------H-RQT
Cs-pb       Cs              NT-L----------K--C-P------AS-D-----V-V---
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B

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consensus          IYPWMK
Cs-Hox3     Cs     LQKPIYPWMVDSRHNTKSRQQQ
Hox3        Amph   ANTK-----KE--Q-S-Q---P
HoxA3       Mm     VG-Q-F---KE--Q---QKTSG
HoxB3       Mm     -T-Q-F---KE--QTS-LKNSS
HoxD3       Mm     IS-Q-F---KE--Q-S-QKNSC

pb          Dm     DSV-E----KEKKTSR--SNNN
HoxA2       Mm     --P-E----KEKKAAK-TALPP
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Fig. 1 **A** Alignment of the homeodomains of different class 3 Hox genes (*Dm Drosophila melanogaster*, *Cs Cupiennius salei*, *Amph* Amphioxus, *Mm* mouse, *Sg Schistocerca gregaria*, *Tc Tribolium castaneum*). The accession numbers for the sequences are available on request. **B** Alignment of the hexapeptide sequence and neighbouring amino acids from the different class 3 genes

zen genes in insects. The gene is therefore designated as *Cs-Hox3* in the following.

Expression of *Cs-Hox3* in a Hox-gene-like fashion

The expression pattern of *Cs-Hox3* was studied by whole-mount in situ hybridization on developing embryos. As it is currently not possible to prepare embryos at early stages, we started our analysis at the germband stage. At the early germ band stage, *Cs-Hox3* expression is found in the tips of the appendages of the pedipalp and leg-bearing segments and at the base of these appendages (Fig. 3A). No expression is seen in the cheliceres or any other segment, neither at this stage nor at later stages of development (Fig. 3). The expression is strongest in the pedipalps, and somewhat weaker in the legs. Within the appendages, the expression is not uniformly distributed, which becomes most obvious in embryos at mid germ band stage (Fig. 3B, C). In these, one finds a narrow staining at the tip and a broad domain at the base of the appendage. Furthermore, at these stages it also becomes clear that the corresponding segments show an expression of *Cs-Hox3* not only in the appendages, but also in their ectodermal parts (Fig. 3B, C). At a later stage, the so-called inversion stage (Foelix 1996), *Cs-Hox3* is expressed in a thin longitudinal stripe in the pedipalps and the four pairs of legs (Fig. 3D, E). In addition, there is again a clear expression in the corresponding segments themselves. No expression is found in the labrum, in the cheliceres or any other segment. Thus, *Cs-Hox3* is expressed in a defined domain along the anterior-posterior axis within the developing embryo, which is a typical hallmark of a Hox gene expression pattern.

In a previous study we have shown that other Hox genes are also expressed in both the segments and the corresponding appendages of the spider embryos (Damen et al. 1998). The orthologue for *labial* (*Cs-lab*) is expressed in the pedipalp segment and the four leg segments, whilst the *Deformed* orthologue (*Cs-Dfd*) is expressed in the four leg segments only (summarized in Fig. 3F). Interestingly, both of these are also expressed strongly in the legs, as has been observed for *Cs-Hox3*. The other Hox gene orthologues analysed are expressed more posteriorly, generally obeying the colinearity and the segmental spacing known from *Drosophila* (Damen et al. 1998). In comparison to this, *Cs-Hox3* is expressed somewhat differently, as it is expressed in the same segments as *Cs-lab*, which belongs to the class 1 Hox genes. Thus, *Cs-Hox3* expression violates the staggered colinearity rule which is found for most other Hox genes. However, there is also a violation of this rule for other genes in the anterior Hox gene classes. In mouse, the most anteriorly expressed Hox genes are the class 2 genes with the anterior boundary between rhombomere 2 and 3, followed by class 1 at the rhombomere 3/4 border and class 3 at the rhombomere 4/5 border (Krumlauf 1993). In *Drosophila*, the most anterior Hox gene is class 1 (*labial*) expressed in the intercalary segment,

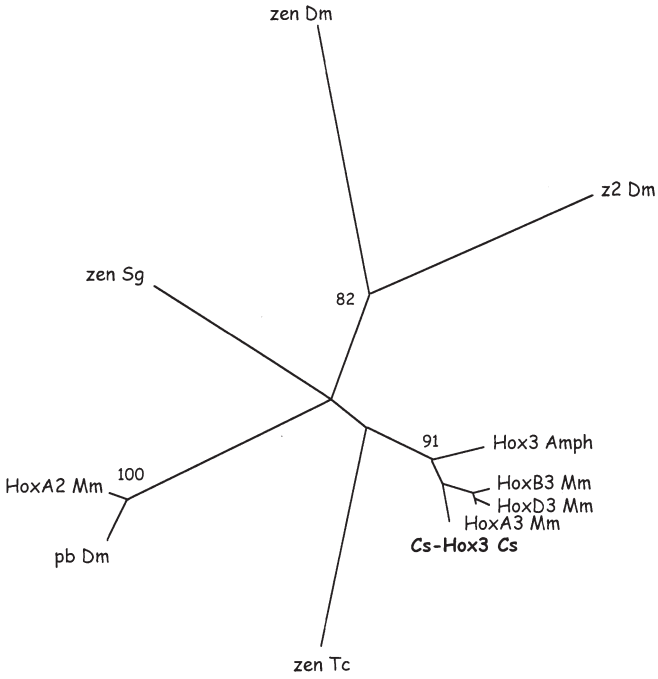

Fig. 2 Neighbour joining tree with the homeobox sequences generated using 500 bootstrap replicates

followed by class 4 (*Deformed*) with an expression in the mandibular and maxillary segments and by class 2 (*proboscipedia*) which is expressed in the maxillary and labial segments (Kaufman et al. 1990). Thus, *Cs-Hox3* gene expression may represent just another modification of this non-colinearity situation.

Evolution of class 3 Hox genes

Our results clearly show that there is a Hox3 orthologue in chelicerates that is very similar to the vertebrate Hox3 genes. In the mouse, genetic experiments have shown that the Hox3 genes have a homeotic function. Disruption of the *HoxA3* or *HoxD3* gene resulted in regionally restricted defects along the anterior-posterior axis (Chisaka and Capecchi 1991). In *HoxD3* mutant mice the first cervical vertebra, the atlas, is homeotically transformed into the adjacent anterior structure (Condie and Capecchi 1993, 1994). Regarding the high degree of conservation of *Cs-Hox3* and its Hox-like expression pattern, we assume that it has a function as a homeotic gene in the spider as well. As the chelicerates represent a basal arthropod group, this would imply that the common ancestor of the arthropods would also have had a Hox3 gene with a homeotic function. Somewhere in the lineage leading to the insects, the Hox3 gene must have lost its function in axis formation and must have become restricted to the expression in the extra-embryonic tissues. We cannot yet say whether extra-embryonic expression of *Cs-Hox3* also occurs in the spider in addition to embryonic expression. The earliest stages of embryogenesis in *Cupiennius* are not accessible to whole-mount in situ hybridization experiments as they are extremly fragile. Thus, it remains open at this point, whether the extra-embryonic expression of the *zen* genes was newly acquired concomittant with the loss of the embryonic expression, or whether this already existed. It will be useful to analyse different crustacean taxa in this respect, as the insects are thought to have evolved from a crustacean ancestor (Averof and Akam 1995; Friedrich and Tautz 1995).

The strong sequence divergence of the *zen*-like genes is noteworthy. In evolutionary terms, spiders should be closer to the insects than to the chordates. Still, the *Cs-*

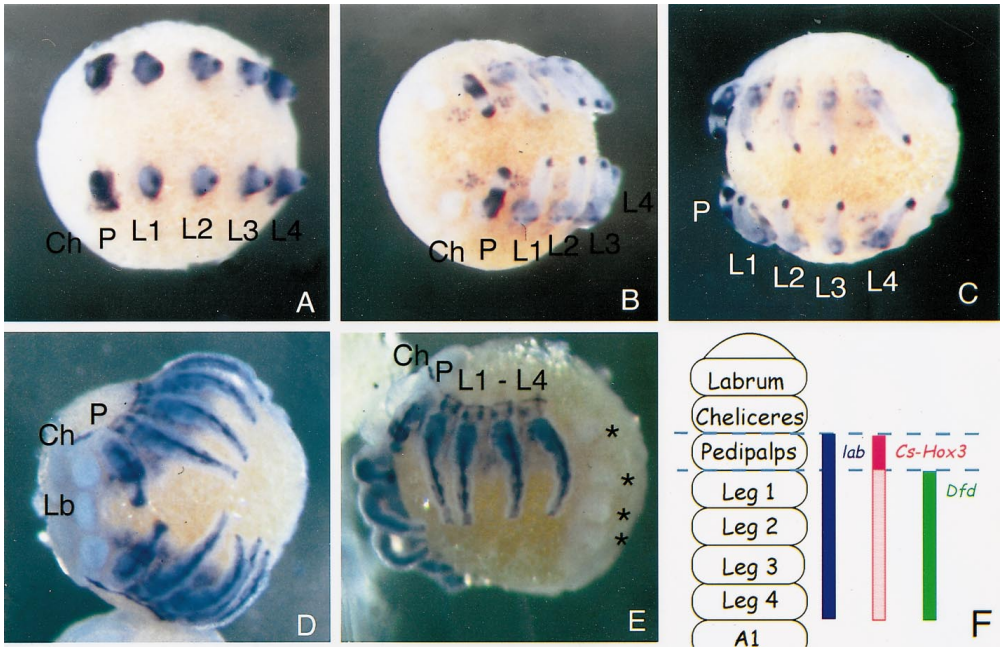

Fig. 3A–F *Cs-Hox3* expression in embryos of the spider *Cupiennius salei*. Whole-mount in situ hybridizations for *Cs-Hox3* at early germ band stage (**A**), at mid germ band stage (**B, C**) and at the inversion stage (**D, E**; *Lb* labrum, *Ch* cheliceres, *P* Pedipalps, *L1–L4* leg 1–4). The asterisks in **E** mark the abdominal appendages on the abdominal segments 2 to 5. **F** Schematic representation of the expression patterns for *Cs-labial*, *Cs-Hox3* and *Cs-Deformed*

Hox3 homeobox is much more similar to the chordate genes, implying that it might be acting within a homologous gene network with conserved binding sites. By inference, one would then assume that the *zen* homeoboxes would have become strongly modified because they had to be integrated into a different gene network with different binding sites. It would thus be interesting to test whether the spider Hox3 gene can replace the function of a mouse Hox3 gene and whether this capacity has been lost for the *zen* genes.

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