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ORIGINAL ARTICLE

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The *caudal* gene of the barnacle *Sacculina carcini* is not expressed in its vestigial abdomen

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Abstract We report the characterization of a *caudal* gene from the rhizocephalan cirripede *Sacculina carcini* and its embryonic and larval expression patterns. Cirripedes are maxillopodan crustaceans that are devoid of any complete abdominal segment at the adult stage. We currently explore the genetic basis of this peculiar body plan. In a previous study we have shown that they probably lack the *abdominalA* gene, while possessing the other Hox genes shared by arthropods. However, at least a part of the genetic program might be conserved, since the *engrailed.a* and *engrailed.b* genes are expressed in a posterior region that we interpret as a relic of an ancestral abdomen. Here we show first that the *Sacculina caudal* gene is expressed early in embryogenesis, which makes it the earliest genetic marker evidenced in the development of *Sacculina* and of any other crustacean species. It is expressed later in the embryo in the caudal papilla, a posterior proliferating zone of cells. During the larval stages, the *caudal* gene is first expressed in the whole thoracic region; then its expression regresses to the posterior end of the larva. Surprisingly, it is never expressed in the vestigial abdomen. This lack of expression of the *Sacculina caudal* gene in a posterior region, at odds with what is known in all other studied metazoan species, might be correlated with the defective development of the abdomen.

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Introduction

Starting with the discovery of the homeobox (McGinnis et al. 1984; Scott and Wiener 1984), the past 15 years or so have been marked by the astonishing discovery that many, if not most, developmental genes first isolated in *Drosophila* are conserved across the whole metazoan kingdom. Several of these show related patterns of expression within a wide variety of animals belonging to different phyla. Since the nineteenth century, animals have been classified into phyla according to the shared characteristics of their body plans: animals presenting similar body architectures belong to the same phylum, whereas animals belonging to different phyla are quite divergent in morphology. Comparative developmental genetics is now faced with a new challenge: how to explain the generation of such a diversity of body plans using the same set of homologous genes (Gellon and McGinnis 1998)?

We chose to address this question at the level of intraphylum, rather than inter-phyla, diversity. Besides insects, crustaceans are probably those animals that share with *Drosophila* the more-numerous and the more-similar developmental genes. Crustaceans show a wide variety of body plans. Among crustaceans, cirripedes show a striking feature: they are devoid of complete abdominal segments at any stage of their development. The exact status of putative abdominal segments is a matter of controversy: some authors state that there is no abdominal segment at all (Borradaile and Potts 1958; Turquier 1972), while others include the penis as a vestige of an abdominal segment (Schram 1986; Anderson 1994). As early as 1905, Gruvel reported the presence of a very reduced abdomen in the well-known thoracican cirripede *Lepas* (the so-called goose barnacle), at a particular larval stage, the cypris, which is the settlement stage of barnacles (Fig. 1A; Calman 1909). This observation was confirmed only very recently by a scanning electron mi-

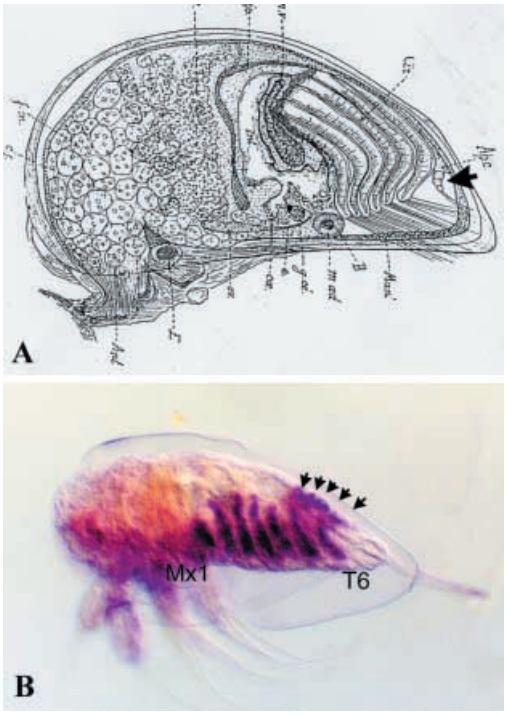

Fig. 1A,B The vestigial abdomen of cirripedes**. A** Drawing by Gruvel (1905) showing a vestigial segmented abdomen (*arrow*) in the cypris of the pedunculate barnacle *Lepas australis*. **B** Expression of *engrailed.b* in a *Sacculina* nauplius IV larva revealing eight large stripes corresponding to the last two cephalic segments, Mx1 and Mx2, and to the six thoracic segments. Five small stripes are also visible in the dorsal posterior region (*arrows*). We interpret them as the progenitors of the vestigial abdominal segments

crograph of the cyprid larva of an acrothoracican cirripede species (Kolbasov et al. 1999).

We addressed the question of the genetic mechanism underlying this peculiar feature. We analyzed the complement of Hox genes in a sample of cirripede species representative of each super order (Thoracica, Rhizocephala, and Acrothoracica). We gained circumstantial evidence for the lack of the *abdominalA* gene in all cirripede species tested (Mouchel-Vielh et al. 1998), whereas it is present in *Ulophysema oeresundense*, a maxillopodan crustacean belonging to the Ascothoracica, supposed to be the sister-group of the Cirripedia clade (Schram and Hoeg 1995). We also studied the *engrailed* genes of cirripedes (Gibert et al. 1997; 2000; Queinnec et al. 1999). Small stripes of *engrailed* expression were revealed in nauplius larvae in a region located behind the last thoracic segment and in front of the telson (Fig. 1B; Gibert et al. 2000). We took this as evidence for the determination of a vestigial abdomen.

It was worth looking for other developmental genes putatively involved in abdomen formation. We report here the cloning, sequencing, and expression pattern of a partial cDNA of the *caudal* gene of *Sacculina carcini*. It is the earliest genetic marker ever reported in crustacean development. More specifically, although showing posterior expression in agreement with the homologous *caudal* genes in all animal species studied to date, it is never expressed in the vestigial abdomen at any stage of development. This may be related to the lack of a fully developed abdomen in cirripedes.

Materials and methods

Biological material

Sacculina carcini specimens were collected at Roscoff (France) and raised as previously described (Queinnec et al. 1999). For in situ hybridization, larvae were fixed as described by Queinnec et al. (1999). Embryos were prepared as follows: *Sacculina* externae were detached from their host; the sacks were opened and fixed in freshly prepared 4% paraformaldehyde for 2 days at room temperature. They were carefully rinsed several times with phosphate-buffered saline (PBS). The embryos were then dissected from the ovisacs (Anderson 1994) and directly submitted to the hybridization procedure. For RNA extraction, larval or embryonic samples were rapidly frozen and kept in liquid nitrogen until use.

RT-PCR reaction, cloning, and sequencing

Total larval RNA was extracted with RNAzol reagent (Bioprobe). PolyA+ RNAs were isolated from 500 µg of total RNA by chromatography on poly(A) Quick columns (Stratagene). Synthesis of cDNA by reverse transcription was performed as described previously (Mouchel-Vielh et al. 1998) using polyA+ RNAs from nauplius II larvae. To initiate the synthesis of the first strand of the cDNA we used an oligo-dT primer (5'GAGAGAACTAGTCTCGAG$(T)_{18}$-3').

PCR was performed on cDNA synthesized from larval nauplius II mRNA. The protocol used was as described by Mouchel-Vielh et al. (1998). We performed two successive reactions of PCR with nested 5'-primers and the same 3'-primer. Sequences of both 5'-primers were deduced from the partial sequence of the *S. carcini caudal* homologue previously isolated (Mouchel-Vielh et al. 1998): primer Cd1 (5'CAACTACATCACGATCAAGCG-3') which encodes the NYITIK peptide and primer Cd2 (5'ATCAAGCGCAAGCTGGAGCTG-3') which encodes the IKRKLEL peptide. The 3'-primer for both PCRs was the oligo-dT primer used in cDNA synthesis. A single PCR product of approximately 900 base pairs (bp) in length was obtained. This PCR product was purified and cloned in a T-overhang vector prepared as described elsewhere (Mouchel-Vielh et al. 1998). Ten clones were sequenced using the Thermosequenase sequencing kit (Amersham) and migrated on an automatic ALF express sequencer (Pharmacia).

In situ hybridization

The digoxygenin-labeled RNA probe was synthesized according to the manufacturer's instructions (Boehringer Mannheim) from a 664-bp fragment isolated by restriction hydrolysis of the 963-bp *caudal* partial cDNA. This 664-bp fragment has the last amino acid and the 3'-untranslated region deleted. In situ hybridization of larvae and embryos was performed as described by Gibert et al. (2000).

Results

Cloning and sequence analysis of a partial *Sacculina carcini caudal* cDNA

Using a partial *caudal* sequence isolated previously (Mouchel-Vielh et al. 1998), we isolated and cloned a

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Dme     CTSRYITIRRKSELAQTLSLSERQVKIWFQNRRAKERTSN

Sca     RFNN----K--L--SRI-G-TD---------------KQK
Pcl     HY-------------SM-G--------
Aga     HYT--------A----N-Q----------------D-KQK
TcaA    YY---------A---NS-G------------------KQK
TcaB    FV-K----K------EN-G-----I------------KQ-
Bmo     HY-R-------A---VS-G------------------KQV
Aka     HY--------------A-N--------
Cva     HY---------A----S-Q--------
Lsa     HY-----MN--A---KS-D-T---I------------KI-
Cel     HT-PF--SD---Q-STM-S-T---I----------D-RDK
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Fig. 2 Partial *caudal* homeodomains from *Sacculina carcini* and other protostome species**.** Amino acids 21–60 of the homeodomain are aligned. *Dme*: *Drosophila melanogaster* (fly) (Mlodzik et al. 1985); *Sca*: *Sacculina carcini* (Mouchel-Vielh et al. 1998; and this study); *Pcl*: *Procambarus clarkii* (crayfish) (Abzhanov and Kaufman 2000b); *Aga*: *Anopheles gambiae* (mosquito) (Genbank accession number AF119382); *Tca*: *Tribolium castaneum* (flour beetle) (Schulz et al. 1998); *Bmo*: *Bombyx mori* (silk moth) (Xu et al. 1994); *Aka*: *Akanthokara kaputensis* (onychophoran) (Grenier et al. 1997); *Cva*: *Chaetopterus variopedatus* (annelid) (Genbank accession number U68274); *Lsa*: *Lineus sanguineus* (nemertine) (Kmita-Cunisse et al. 1998); *Cel*: *Caenorhabditis elegans* (nematode) (*ceh-3/pal-1*; Burglin et al. 1989; Genbank accession number Z46241). (– mino acid identical at this position to that of Dme)

single fragment of 963 bp (Genbank accession number AF213985). The cloned fragment contains half of the homeodomain (from amino acid 35 to 60), followed by a 190-amino acid carboxy-terminal domain and a 246-bp untranslated region ending with a polyA sequence. When this sequence is compared with the sequences of other *caudal* genes, similarity is only found within the homeodomain. Figure 2 shows alignment of the *S. carcini caudal* partial homeodomain (from amino acids 21 to 60) with the *caudal* sequence of other protostomes (arthropods, annelids, nematodes, and nemertines). In spite of a great variability, amino acids that characterize *caudal* sequences can be identified.

Embryonic expression

The *caudal* gene of *Sacculina* is expressed in the embryo (Fig. 3). The earliest expression of *caudal* was detected in massive cells located at the periphery of the embryo (Fig. 3A). Later, at pre-hatching stages, *caudal* is clearly expressed in the posterior part of the embryo (Fig. 3B, C). At that time, the *caudal* expression domain coincides with a region morphologically defined as the caudal papilla (Turquier 1967; Anderson 1994).

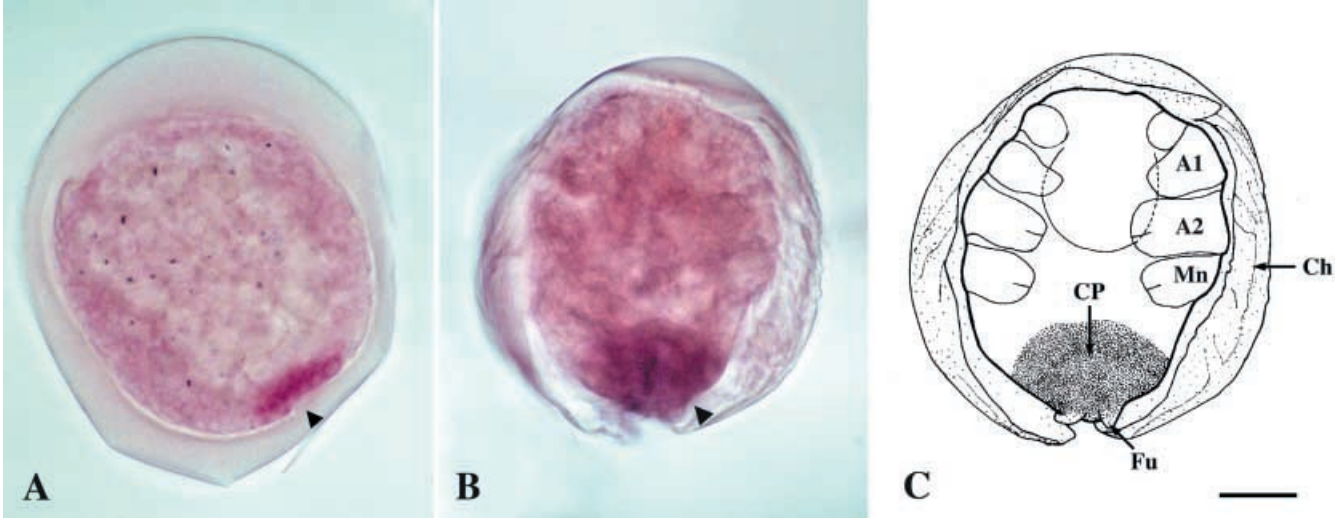
In crustaceans, the anterior head and the first three cephalic appendages are formed early in embryonic development (Anderson 1973; Schram 1986), giving rise to the nauplius larva. This is observed in barnacles as well (Anderson 1994). In most anamorphically developing crustaceans, the more posterior cephalic, thoracic, and abdominal segments are progressively formed during the larval stages from a posterior growth zone located just in front of the telson. Cell proliferation, *engrailed* expression, and segment formation all occur during the larval stages (Dohle and Scholtz 1997).

In contrast, in barnacles, cell proliferation occurs in the embryo in the caudal papilla formed before hatching (Turquier 1967); *engrailed* expression as well as segment determination and formation occur later, during the naupliar stages, within cells that are already in place (Queinnec et al. 1999; Gibert et al. 2000).

Larval expression pattern

During the first two larval stages (nauplius I, Fig. 4A; nauplius II, Fig. 4B), *caudal* is expressed in all the large

Fig. 3A–C *caudal* expression in *Sacculina carcini* embryos. **A** Embryo, about 8th–9th cell cycle stage; **B** pre-nauplius embryonic stage; **C** interpretative drawing of Fig. 3B. *Arrowheads caudal* expression; *A1* antennular bud; *A2* antennal bud; *Ch* chorion; *CP* caudal papilla; *Fu* furca; *Mn* mandible bud. The vitelline membrane is closely applied to the chorion (Anderson 1994). Scale bar 20 µm



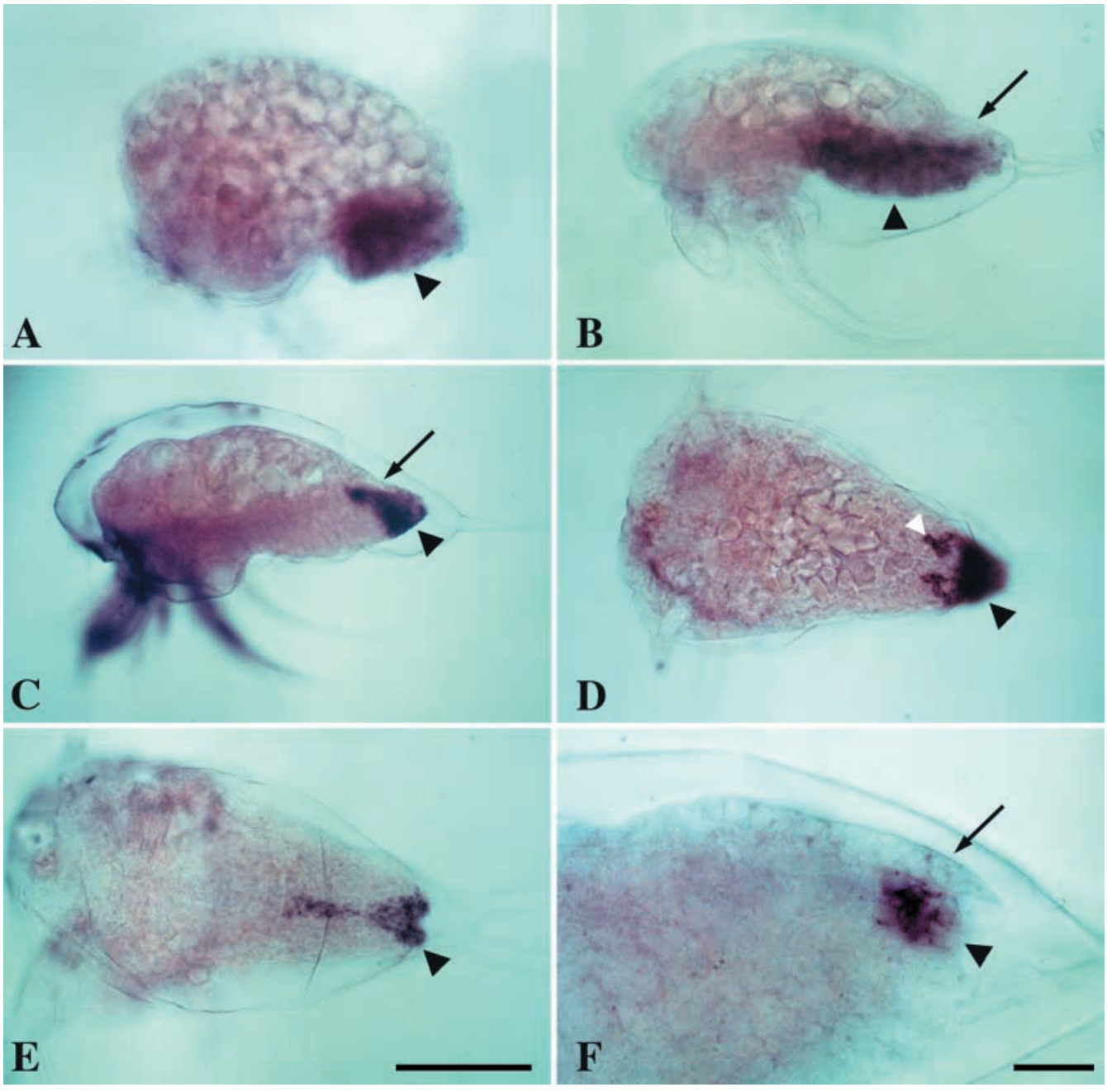


Fig. 4A–F *caudal* expression in *Sacculina carcini* nauplius larvae. **A**: Nauplius I; **B** late nauplius II; **C** and **D** nauplius III; **E** and **F** nauplius IV. **A**, **B**, **C**, **F** Lateral views, anterior to the left; dorsal up. **D**, **E** ventral views, anterior to the left. *Black arrowheads* thoracic staining; *white arrowhead* neural cells; *fine arrows* vestigial abdomen. The vestigial abdomen appears dorsally. Scale bars: **A**–**E** 50 µm; **F** 10 µm

cells that will eventually form the whole thorax. Then, staining is suddenly restricted to the most posterior region (Fig. 4C, D). This posterior staining is accompanied by more anterior dorsal cells. Restriction to the posterior part of the larva continues, and during the fourth nauplia r stage (metanauplius), *caudal* expression persists in the telson (Fig. 4E). At the end of the metanauplius stage (pre-cypris), it is restricted to a few posterior cells only. At the cyprid stage *caudal* expression is no longer detected. Surprisingly, even when it is expressed in the whole thorax, *caudal* is never expressed in the vestigial abdomen, at any larval stage (*arrows* in Fig. 4B, C, F).

Caudal expression in putative neural cells

During the nauplius III stage, two pairs of cells are clearly stained in the dorsal region of the thorax (Fig. 5A). These cells are symmetrically located with respect to the median line; *caudal* expression is maintained in these cells, while it fades in the neighboring cells due to the overall regression of the expression in the thorax. The

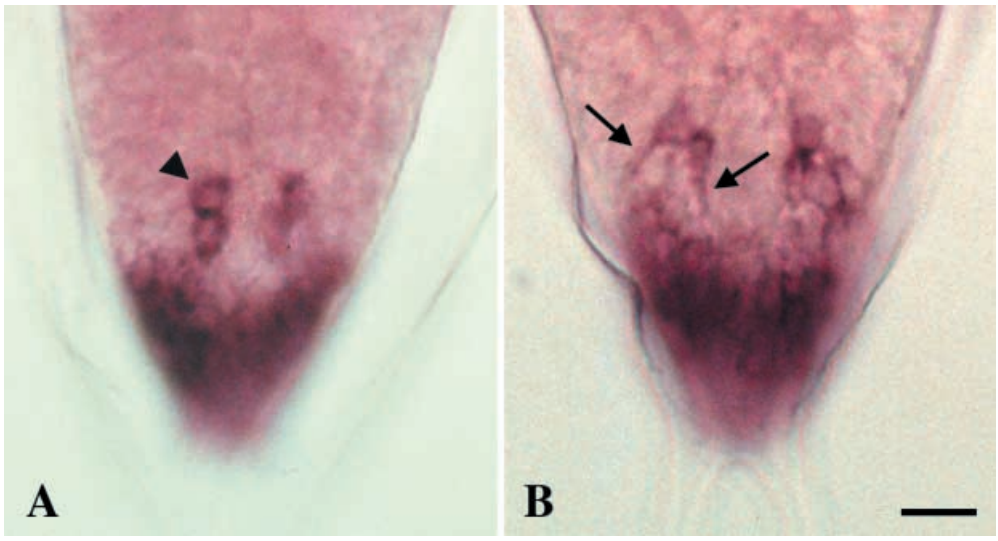


Fig. 5A,B *caudal* expression in putative neural cells**. A** Early nauplius III; **B** late nauplius III. Ventral views; anterior up. *Arrowhead* cellular body; *arrows* cellular extensions. Scale bar 10 µm

morphology of these labeled cells is rather dynamic, passing from a spherical to a drop-like form. Cytoplasmic expansions distinctly appear, mainly directed toward the posterior end of the body. The most posterior median cells send cytoplasmic expansions along the anterior to posterior axis of the body, whereas the most anterior external cells send out projections more laterally (Fig. 5B). Later, *caudal* expression disappears first from the most anterior cells, then progressively from the posterior ones. The morphological differentiation of these cells recalls neuroblast differentiation into neurons (the term "neuroblast" is here used in a broad sense as defined in Dohle and Scholtz 1997). In *Drosophila*, a similar observation has been reported (Macdonald and Struhl 1986). A few bilaterally symmetric pairs of neural cells are first labeled in the neuromeres of parasegments 1–14. Then, at germ-band shortening, *caudal* staining is retained in only 2–4 neuromeres of the thorax and anterior abdomen. However, in *Sacculina*, the *caudal*-expressing neural cells are dorsal. Hence, they may not be homologous to the *caudal*-expressing neuroblasts of the ventral nerve cord of *Drosophila*. The neuronal fate of these cells has to be confirmed by use of a neuronal marker.

Discussion

Early expression of the *Sacculina caudal* gene

Several developmental genes have been previously tested for expression in *Sacculina* by immunodetection (*engrailed.a*, Queinnec et al. 1999), in situ hybridization (both *engrailed* genes, Gibert et al. 2000; Hox genes, our unpublished results), and RT-PCR (some Hox genes, Mouchel-Vielh et al. 1999). Similarly, developmental genes have been studied in other crustacean species, such as *engrailed* (Manzaneres et al. 1993, 1996; Patel et al. 1989; Scholtz et al. 1993, 1994; Scholtz 1995), Hox genes (Averof and Akam 1995; Abzhanov and Kaufman 2000a, b), *Distalless* (Panganiban et al. 1995; Gonzales-Crespo and Morata 1996; Scholtz et al. 1998; Williams 1998), *pdm* and *apterous* (Averof and Cohen 1997), and *wingless* (Nulsen and Nagy 1999). To date, the expression of none of these genes has been reported in the early embryo. Recently, the expression of the *caudal* gene of the crayfish *Procambarus clarkii*, a malacostracan decapod crustacean, has been reported (Abzhanov and Kaufman 2000b). It is expressed in the posterior part of the telson. No early embryonic expression has been reported so far. The *Sacculina caudal* gene is thus the first gene that allows following of crustacean early embryonic development and determination.

This early expression is consistent with *caudal* expression in insects (Mlodzik et al. 1985; Macdonald and Struhl 1986; Xu et al. 1994; Schulz et al. 1998), nematodes (Hunter and Kenyon 1996; Ahringer 1997), and chordates (Duprey et al. 1988; Joly et al. 1992; Epstein et al. 1997; Brooke et al. 1998).

The dynamic expression of *Sacculina caudal*

The temporal and spatial pattern of *Sacculina caudal* expression is partly similar to what is known in various other animals. Indeed in *Tribolium* (Schulz et al. 1998) and in various vertebrates (Duprey et al. 1988; Epstein et al. 1997), *caudal* expression is progressively restricted to the posterior end in pace with segment or somite formation. In *Sacculina*, a similar restriction is observed (Fig. 4).

However, although it is expressed in a posterior region in the early embryo, *caudal* is expressed in the whole thorax in young larval stages. At late nauplius stage II, where *caudal* is still expressed in the whole thorax (Fig. 4B), all six thoracic segments are determined, as shown by the striped expression of both *engrailed.a* and *engrailed.b* (Queinnec et al. 1999; Gibert et al. 2000). This was a quite unexpected feature, since such

thoracic expression is observed neither in insects (Mlodzik et al. 1985; Macdonald and Struhl 1986; Xu et al. 1994; Schulz et al. 1998), nor in the crayfish (Abzhanov and Kaufman 2000b). Since the caudal papilla, where *caudal* is expressed in the embryo, is thought to be a proliferating zone, it could mean that the larval thorax is derived from the caudal papilla of the embryo. At later larval stages, *caudal* expression is restricted to the most posterior region. This region corresponds to the telson. In all other crustaceans, the digestive tract ends up in the telson, and *caudal* expression in the telson has been reported in another crustacean, the crayfish *Procambarus* (Abzhanov and Kaufman 2000b). In fact, *Sacculina* larvae are devoid of any endodermal tissues and feed only on their yolk preserves (Anderson 1994). It would be useful to study *caudal* expression in other crustaceans, such as non-parasitic cirripedes (acorn barnacles, i.e., *Balanus*) or anamorphically developing malacostracans (peneid shrimps), who possess an endoderm.

The vestigial abdomen does not express *caudal*

We have previously described the expression of the *engrailed* genes during *Sacculina* development (Queinnec et al. 1999; Gibert et al. 2000). These markers permitted us to evidence the transient formation of a segmented structure located in a dorsal posterior position, between the thorax and the telson (Gibert et al. 2000). We interpret this structure as a relic of an ancestral abdomen, which is not present in any adult cirripede, but is present in most other members of the Maxillipoda. Similarly, recent scanning micrographs have shown the presence of a tiny and reduced abdomen in cyprid larvae of another cirripede, the acrothroracican *Lithoglyptes* (Kolbasov et al. 1999). Precise comparison between *caudal* and *engrailed.b* allowed us to show that, contrary to what happens in the thorax, the vestigial abdomen was never marked by *caudal*. Especially, the secondary anterior expansion of *caudal* expression during the metanauplius stage is ventral towards the vestigial abdomen (Fig. 4F). The lack of *caudal* expression in a far-posterior and possibly abdominal structure is an astonishing feature when compared to what is known in other metazoans, protostomes (Mlodzik et al. 1985; Macdonald and Struhl 1986; Xu et al. 1994; Hunter and Kenyon 1996; Ahringer 1997; Schulz et al. 1998), as well as deuterostomes (Duprey et al. 1988; Joly et al. 1992; Epstein et al. 1997; Brooke et al. 1998). Thus in barnacles, although at least a part of the genetic program needed in the abdomen for segment formation is clearly operative (i.e., *engrailed*, Gibert et al. 2000), another part, required for full development of the abdomen may be missing (i.e., *abdA*, Mouchel-Vielh et al. 1998) or inactive (i.e., *caudal*, this study).

These observations on cirripedes are in agreement with the speculation of Akam (2000) that, contrary to early enthusiastic expectations in the evo/devo field, gross modifications of the body plan as observed in evolution would not be correlated with a single key genetic change, but rather with the accumulation of multiple changes.

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