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Gene expression pattern

Developmentally controlled expression patterns of intermediate filament proteins in the cephalochordate *Branchiostoma*

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Abstract

Expression of cytoplasmic intermediate filament (IF) proteins starts in the gastrula with three keratins (k1, Y1, D1) and protein X1. The number of IF proteins expressed increases at the neurula and early larval stages to seven and 11, respectively, and reaches 13 in the adult. Using antibodies specific for a single IF protein the expression patterns of nine of the 13 IF proteins were analyzed at different developmental stages. Keratin k1 of the larval epidermis is replaced in the juvenile by keratin E1. Protein C1 of the larval epidermis persists only weakly and only in the most ventral part of the adult. While down-regulated in the adult epidermis k1 and C1 are major proteins in the atrial epithelium which forms in the later larva. B1 is currently the only IF protein expressed in mesodermally derived tissues such as the muscle tails and some coelomic epithelia. Two-dimensional gels confirm that keratins are the major IF proteins in the nerve cord. Immunogold electronmicroscopy shows that proteins X1 and C2 are present in epidermis and nerve cord in keratin IF.

Keywords: *Amphioxus*; Epidermis; Intermediate filament; Keratin; Nerve cord

1. Results

Developmental expression of the different IF transcripts was assessed by cDNA hybridization followed by sequence analysis (Table 1).

From the heterogeneous population of free-swimming and feeding *Branchiostoma lanceolatum* in the plankton, specimens of 3–6 mm length were selected. Immunofluorescence on longitudinal frozen sections identified at least two groups. Fig. 1A and Table 2 summarize the results on the first group. Both epidermis and nerve cord stained strongly for C2, D1, E2 and X1. Additional strong staining for k1 was observed in the epidermis while E1 was weakly expressed. Y1 staining occurred exclusively in the nerve cord. No epithelial structures or immunoreactivity were observed in the position that subsequently yields the atrial epithelium. This stage of development most likely corresponds to the pre-metamorphic larva which still lacks the atrium (Ruppert, 1997; Stokes and Holland, 1995).

Fig. 1B gives the results on the second group of specimens. The presence of the atrium (see below) and the visible preoral cirri (toluidine blue stain) classifies these specimens either as the late metamorphic larva or the early juvenile (Stokes and Holland, 1995; Ruppert, 1997). Immunostaining results of this developmental stage differ from those observed in the pre-metamorphic larva. Now strong expression of keratin E1 is present both in the epidermis and the nerve cord while epidermal keratin k1 expression is weak. Furthermore, keratins k1 (type I) and D1 (type II) are present in the atrial epithelium surrounding the gill slits and the intestine (right panel in Fig. 1B).

Expression of B1 was examined on longitudinal sections of late larva and the juvenile (Fig. 2). The B1-specific antibody strongly decorated the muscle tail bundles, which make ventrolateral contacts with the nerve cord (Ruppert, 1997), and weakly the coelomic epithelia surrounding the body musculature. This staining pattern was also present in adults (Fig. 2F,H). In the adult lancelet B1 was found in some mesodermally derived coelomic epithelia and weakly in the centre of the nerve cord (Fig. 2F,H; Riemer et al., 1998).

In adults C1 seemed exclusively expressed in the atrial epithelia (Karabinos et al., 2000). Interestingly, in the early

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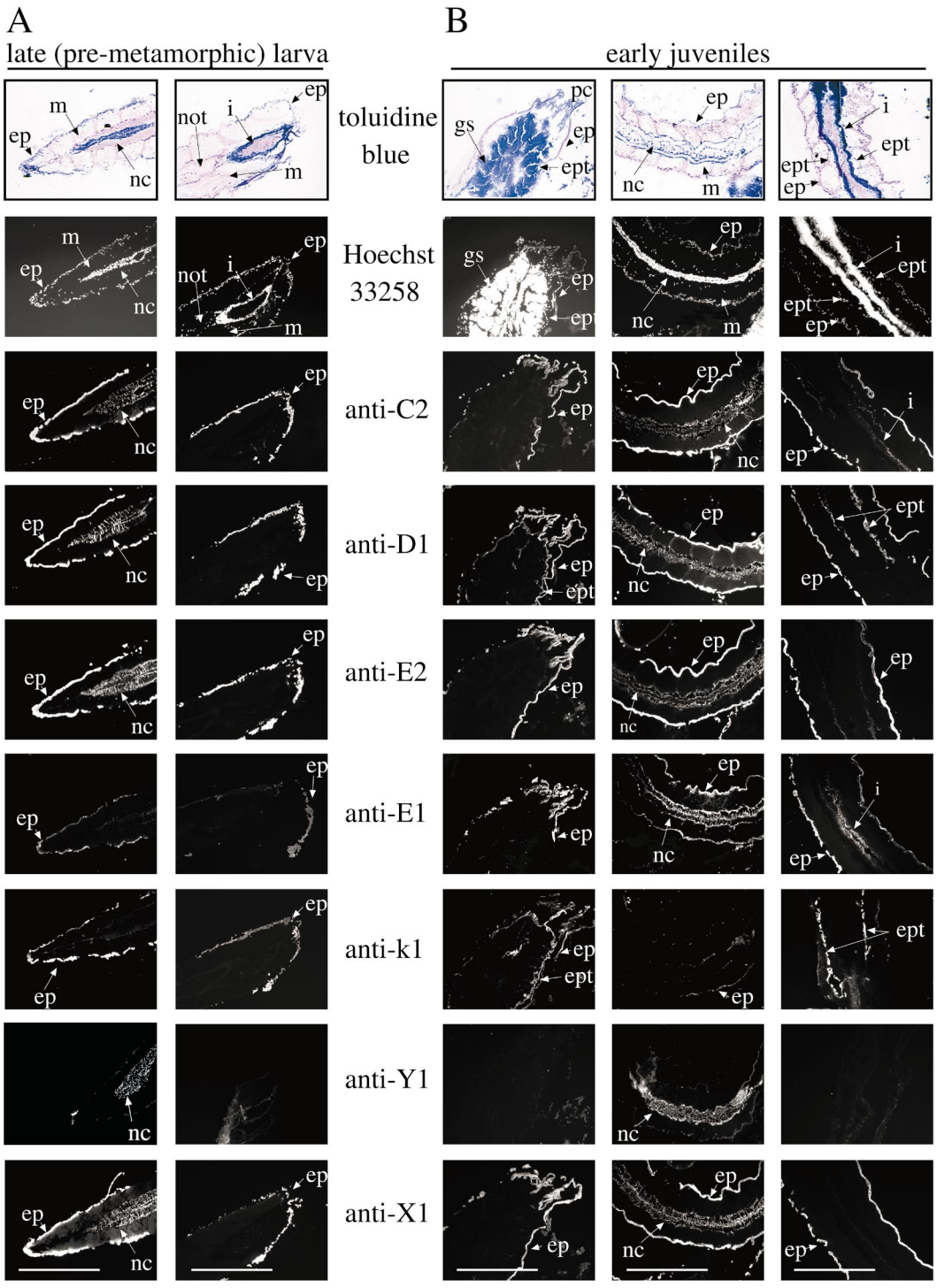

Fig. 1. Tissue-specific expression of IF proteins in pre-metamorphic larvae (A) and the early juvenile (B). Frozen longitudinal sections were stained with toluidine blue or with Hoechst 33258 (DNA staining) to facilitate the identification of cells and tissues. Immunofluorescence microscopy of parallel sections labelled with anti-C2, -D1, -E2, -E1, -k1, -Y1 and -X1 antibodies as indicated. (A) The epidermis and the nerve cord are strongly stained with antibodies against C2, D1, E2 and X1 proteins. The epidermis shows weak expression of k1 and strong expression of E1. The Y1 keratin is detected exclusively in the nerve cord. (B) The immunostaining differs from that in panel A in strong expression of E1 in the epidermis and nerve cord tissue as well as in the weak epidermal expression of keratin k1. ep, epidermis; ept, epithelium; gs, gill slit; i, intestine; m, body musculature; nc, neural cord; not, notochord; pc, preoral cirri. Note the weak spotty staining of C2 and E1 antibodies in the intestinal cells (right panel of B). Scale bars: 300 μm.

Table 1
Expression of keratin type I and II and other IF proteins in *Branchiostoma* development[a]

Stage		Gastrula (5–6 h)	Neurula (26 h)	Early larva (2–4 days)	Adult
Type I	E1	−	−	(+)[c]	+[c]
	k1[f]	+[e]	+	+[d]	+
	Y1	+[e]	+	+	+
Type II	D1	+[e]	+	+[b]	+
	E2	−	−	−	+
A/B	A1	n.d.	n.d.	+[e]	+N[b]
	A2	−	−	+[b]	n.d.
	A3	n.d.	n.d.	+[b]	n.d.
	B1	−	+	+[b]	+
	B2	−	+	+[b]	+
C/X	C1	−	+	+[b]	+
	C2	−	−	−	+[e]
	X1	+[e]	+	+[e]	+[e]

[a] Dr Linda Holland kindly provided a 2–4-day larval λZap II cDNA library of *B. floridae* and the RNA for the gridded 5–6 h and 26 h cDNA libraries. To exclude false positives due to cross-hybridization, eight hybridization-positive clones from each experiment were randomly picked and characterized by DNA sequencing. B1 from the A/B group seems related to vertebrate type III proteins; C1, C2 and X1 have no obvious homologs among the four subfamilies of vertebrate cytoplasmic IF proteins (see text); n.d., not determined; N, expression determined by Northern blot analysis; (+), weak expression. References to previous cDNA cloning results are made by the following:
[b] Riemer et al., 1998.
[c] Karabinos et al., 1998.
[d] Luke and Holland, 1999.
[e] Karabinos et al., 2000.
[f] Here we noted the presence of two distinct k1 transcripts which show 98% identity on the amino acid sequence level.

(1–2 day) larva, which still lacks the atrium (see above), the entire epidermis expresses C1 (Fig. 3). In the adult, weak C1 expression is restricted to the most ventral side of the epidermis (Fig. 3). Thus C1 expression is down-regulated in the adult epidermis and up-regulated in the epithelia of the newly formed atrium (Table 2).

Fig. 4A shows the purity of dissected nerve cords by microscopical criteria. Nerve cords were extracted with Triton X-100 and the insoluble residue was subjected to gel electrophoresis (Fig. 4B). IF protein X1 and the four keratins D1, E1, E2 and Y1, identified by immunoblotting, were recognized as major IF proteins. The lack of a C2 signal is most likely due to proteolysis during the long preparation and extraction.

The electronmicrograph of the epidermal section (Fig. 5A) shows the lack of membranes due to the Triton extraction. The double decoration for keratins E1 (large gold) and D1 (small gold) along the IF is somewhat difficult to see at the magnification used for this overview. Double Immunogold labelling showed that the minor IF proteins C2 and X1 are present in filament bundles consisting of bona fide keratins labelled by keratin D1 antibodies (Fig. 5B) and keratin E2 antibodies (Fig. 5C). Similar results were obtained on sections of extracted nerve cord (Fig. 5D,E). In the nerve cord keratin Y1 is also present along the IF (data not shown).

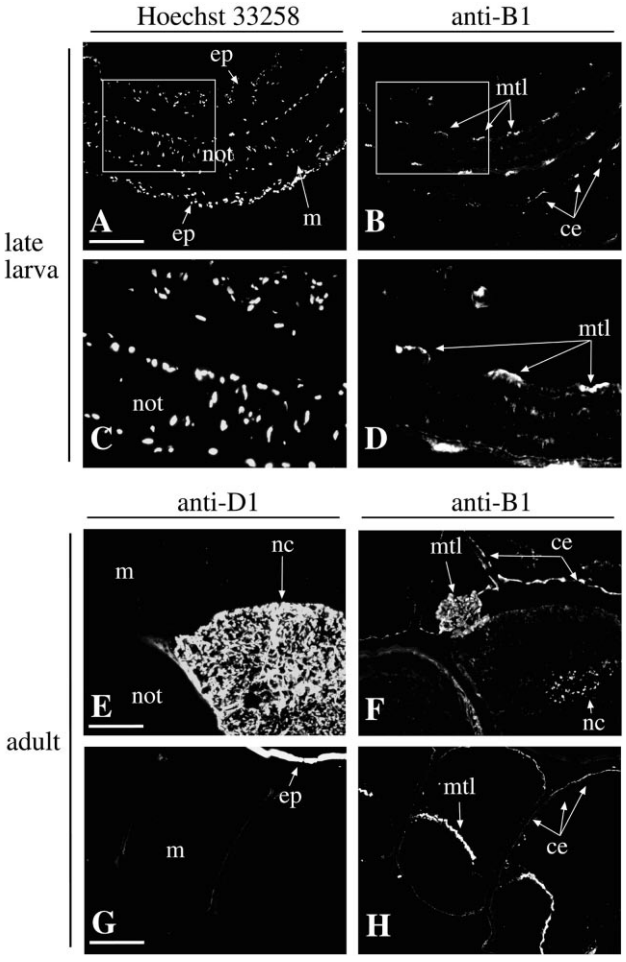

Fig. 2. Tissue-specific expression of the B1 IF protein in late larva and adult. Longitudinal (A–D) sections through the larva notochord (not) and cross-sections through the adult lancelet body (E–H) were labelled with Hoechst (left A,C) and guinea-pig D1 antibody (left E,G) to facilitate the identification of cells and tissues. Sections B,D,F,H were labelled with rabbit anti-B1 antibody. C and D are a higher magnification of the boxed regions of A and B, respectively. B1 decoration occurs in muscle tails, coelomic epithelia surrounding body muscle and in the centre of the nerve cord. The intense staining of D1 antibody in nerve cord (E) is clearly distinguished from the B1 reactivity in this tissue (F). The repetitive staining pattern of the muscle tail bundles is not bilaterally symmetric in agreement with the asymmetric arrangement of the individual muscle tail producing myomeres in the body of the lancelet. Both B1 expressing tissues, muscle tails and the decorated coelomic epithelia, are specialized parts of the myomeres responsible for their innervation (Ruppert, 1997). Thus B1 is so far the only lancelet IF protein expressed in the mesoderm. Moreover, recombinant B1 forms, like vertebrate type III proteins, homopolymeric IF in vitro (Karabinos et al., 1998) and is currently the best candidate for a type III protein. Type III proteins are typical for muscles of vertebrates (Fuchs and Weber, 1994; Parry and Steinert, 1995) and urochordates (Riemer and Weber, 1998; Wang et al., 2000). ce, coelom epithelium; m, body musculature; mtl, muscle tail bundles; nc, neural cord; not, notochord. Scale bars: 100 μm (A,B,G,H) and 50 μm (E,F).

Table 2
Tissue-specific expression of keratin type I and II and other IF proteins in the late pre-metamorphic larva, early juveniles and adult *Branchiostoma*[a]

Tissue		Epidermis	Atrial epithelia	Nerve cord	Muscle tails	Coelomic epithelia	Cutaneous canals
Pre-metamorphic larva							
Type I	E1	(+)	−	−	−	−	n.f.
	k1	+	−	−	−	−	n.f.
	Y1	−	−	+	−	−	n.f.
Type II	D1	+	−	+	−	−	n.f.
	E2	+	−	+	−	−	n.f.
	B1	−	(−)	−	+	+	n.f.
C/X	C1[b]	+	−	−	−	−	n.f.
	C2	+	−	+	−	−	n.f.
	X1	+	−	+	−	−	n.f.
Early juveniles							
Type I	E1	+	−	+	−	−	n.f.
	k1	(+)	+	−	−	−	n.f.
	Y1	−	−	+	−	−	n.f.
Type II	D1	+	+	+	−	−	n.f.
	E2	+	−	+	−	−	n.f.
	B1	−	(−)	−	+	+	n.f.
C/X	C1	n.d.	n.d.	n.d.	n.d.	n.d.	n.d.
	C2	+	−	+	−	−	n.f.
	X1	+	−	+	−	−	n.f.
Adult[c]							
Type I	E1	+	−	+	−	−	+
	k1	−	+	−	−	−	−
	Y1	−	−	+	−	−	+
Type II	D1	+	+	+	−	−	+
	E2	+	−	+	−	−	+
	B1	−	(−)	(+)	+	+	−
C/X	C1[b]	(+)	+	−	−	−	−
	C2	+	+	+	−	−	+
	X1	+	-	+	-	-	+

[a] n.d., not determined; n.f., not found; (+), weak expression.
[b] C1 staining was performed on *B. floridae*; all other experiments used *B. lanceolatum;*; (−) some B1 expression was observed in the area of the atrial epithelium.
[c] References to previous expression analyses of individual IF proteins on the adult lancelet: Riemer et al., 1998; Karabinos et al., 1998, 2000; Note that antibodies specific for a single IF protein, which are also suitable for immunofluorescence microscopy, are currently available for nine of the 13 lancelet IF proteins (Table 1). The four IF proteins for which we lack such antibodies are the three closely related proteins A1 to A3 and B2, which is a close relative of B1 (Karabinos et al., 1998; Luke and Holland, 1999). These four IF proteins form together with B1 the A/B branch of the lancelet IF protein tree (Karabinos et al., 2000). It remains to be seen whether some of the tissues, which are not decorated by the nine IF antibodies currently available (body musculature, digestive and respiratory systems and the gonads) contain one or more of the IF proteins A1, A2, A3 and B2, or even express additional IF proteins.

2. Experimental procedures

B. lanceolatum (larvae, juveniles and adult) were from Helgoland. Adult *B. floridae* were from Tampa, FL. Eggs from electrically stimulated animals were fertilized and various developmental stages were collected (Panopoulou et al., 1998). Hybridization probes for C2 and E2 were amplified from an adult *B. floridae* Marathon cDNA library using primers derived from the corresponding *B. lanceolatum* cDNAs. Other probes were as described before (Karabinos et al., 1998, 2000; Riemer et al., 1998).

Except for B1 all rabbit antibodies used were previously shown to be specific for a single IF polypeptide in immunoblots of total lancelet extracts (Karabinos et al., 2000; Riemer et al., 1998). The new B1 rabbit antiserum was obtained using the synthetic peptide corresponding to the carboxyterminal 15 residues of B1. Two additional antisera were obtained in guinea pigs using synthetic peptides corresponding to the carboxyterminal 15 or 14 residues of D1 and X1 from *B. lanceolatum*. Antigen-affinity purification and immunofluorescence microscopy on frozen sections were as described (Karabinos et al., 2000; Riemer et al., 1998).

The thawed frozen section technique (Tokuyasu, 1986) was used. Approximate IgG concentrations for the first (rabbit) antibodies were: 10 μg/ml (anti-E1 1:2, anti-E2) and 30 μg/ml (anti-C2); approximate IgG concentrations for both guinea pig antibodies were 50 μg/ml. The concentration of both second antibodies (10-nm gold particles conjugated anti-rabbit IgG and 5-nm gold particles conjugated anti-guinea pig; British BioCell International, UK)

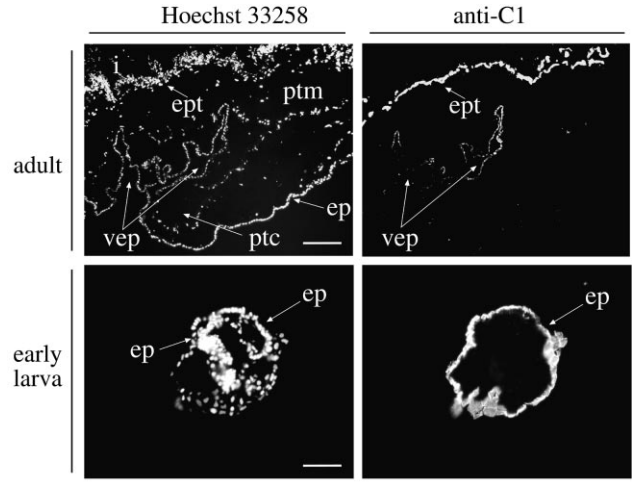

Fig. 3. Expression of C1 in tissues of the adult and the early larva. Frozen cross-sections were labelled with C1 antibody and simultaneously with Hoechst 33258 to visualize DNA (left panels). Weak expression in the epidermis of the most ventral part of the adult animal is indicated in the upper panel. The epidermis of other body parts was negative. In the early *Amphioxus* larva (lower panel) strong expression of C1 occurs exclusively in the epidermis. ep, epidermis; ept, epithelium; ptc, pterygocoel; ptm, pterygial muscle; vep, ventral epidermis. Scale bars for adult and early larva panels are 40 and 20 μm, respectively.

was 2 μg/ml. Fixed and washed grids were stained in 4% uranyl acetate, pH 7.0, for 5 min.

Epidermis and nerve cord were dissected with surgical forceps under a stereo microscope at 4°C. Tissues were extracted with 1% Triton buffer. The resulting cytoskeletal residue was subjected to two-dimensional gel electrophoresis. Gels were stained or processed for immunoblotting using the various monospecific antibodies to IF proteins.

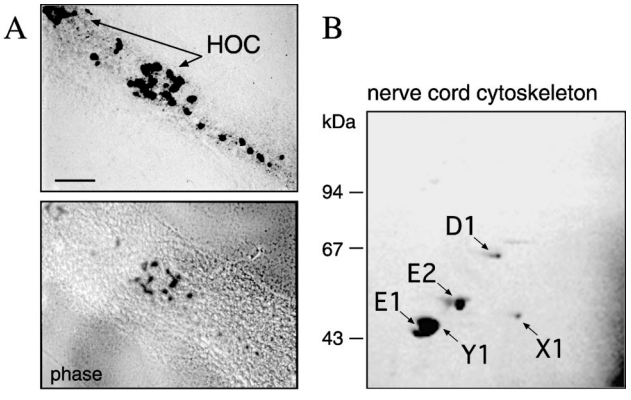

Fig. 4. Dissected nerve cord and the two-dimensional gel pattern of the cytoskeletal residue. (A) The dissected nerve cord was photographed without (upper panel) or with phase contrast (lower panel). The distribution of Hesse ocelli (HOC), pigment cells within the nerve tissue, is indicated by arrows. Scale bar: 100 μm. (B) Protein spots of the cytoskeletal residue (Triton X-100 insoluble material) separated in two-dimensional gels. Although we cannot exclude some proteolysis during the time-consuming dissection process, the gels clearly identify keratins D1, E2, E1 and Y1 as well as IF protein X1 by immunoblotting experiments and microsequencing of peptides released from the protein spots E1 and Y1 (data not shown).

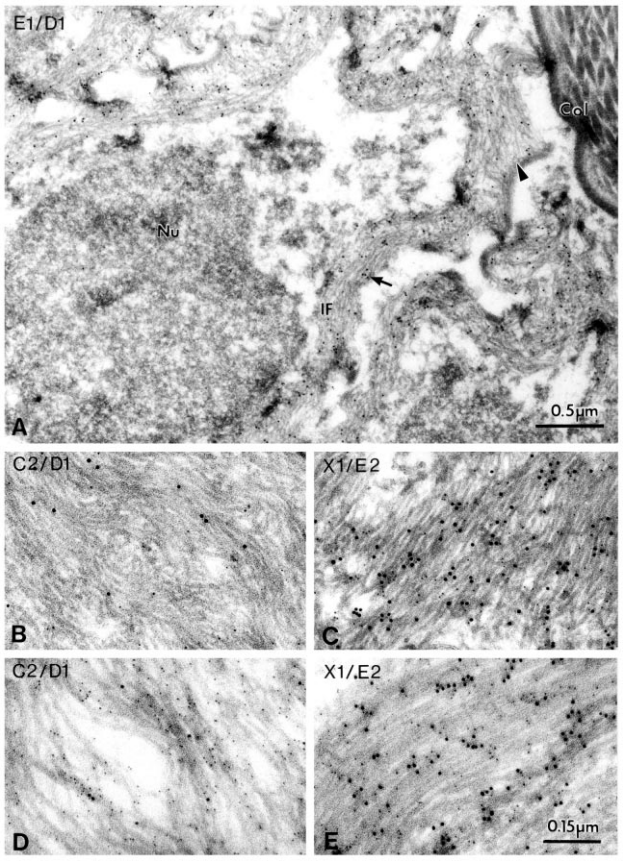

Fig. 5. Double Immunogold electronmicroscopy of IF proteins present in filament bundles of extracted epidermis (A–C) and nerve cord (D,E). Double labelling was performed using polypeptide specific rabbit (anti-C2, E1, E2) and guinea pig (anti-D1, X1) antibodies in the combinations indicated in the panels. Secondary anti-rabbit and anti-guinea-pig antibodies were conjugated with 10-nm (arrow) and 5-nm (arrowhead) gold particles, respectively. (A) Ultrathin cryosection of the extracted epidermal cells shows in a low-power overview a collagen mesh (Col), the nuclei (Nu) and surrounding IF bundles (IF). Membranes are lost due to Triton extraction. This sample had been treated with rabbit anti-E1 and guinea-pig anti-D1 antibodies. (B,C) Epidermal filament bundles labelled for C2/D1 (B) and X1/E2 (C), respectively. (D,E) Filament bundles from nerve cord labelled for C2/D1 (D) and X1/E2 (E), respectively. The micrographs show that both C2 and X1 are integrated into the filaments composed of true keratins. The relation of X1 and keratin filaments is also fulfilled in the gastrula since at this developmental stage the only other IF proteins detected by hybridization and cDNA cloning are keratins k1, Y1 and D1 (Table 1). Scale bars: 0.5 μm (A); 0.15 μm for other panels.

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