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Original Article

Formation of the extracellular matrix during the epimorphic anterior regeneration of *Owenia fusiformis:* autoradiographical and *in situ* hybridization studies

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ABSTRACT During post-traumatic regeneration of the polychaete annelid Owenia fusiformis, the extracellular matrix (ECM) formation was studied by light and electron microscopy and by histo-autoradiography after incorporation of tritiated proline as marker for collagenic proteins. Three days after amputation, a new basement membrane was reformed in the blastema between the ectoderm and the mesoderm. At the same time, the cytoskeleton and the anchoring structures (hemidesmosomes) were differentiated in the basal part of the ectodermal cells. Four days after amputation, collagen fibers appeared in the extracellular matrix newly reformed between the ectodermal and mesodermal layers. The existence of a proximo-distal gradient in the organization of the new extracellular matrix and the accumulation of molecules labeled by ^{3}H-proline was shown. This accumulation started at the level of the injured segment of the stump. Differences in labeling intensity were seen in the regenerate. Within specific organogenetic zones, *i.e.* the epidermal gland anlagen, the branchial buds and the stomodeal invagination, the labeling between the ectodermal and mesodermal layers was less intense than in other parts of the regenerate. In the mesodermal connective septa (dissepiments), located between consecutive segments, the labeling and the accumulation of extracellular material occurred later than the formation of the ectodermal basement membrane. *In situ* hybridization of a DNA molecular probe corresponding partially to the coding region of the collagen-like gene Ocg8, showed a spatio-temporal expression of this gene. Northern blot analysis showed a single transcript of 6.6 kb. Four days after amputation the accumulation of this transcript was exclusively localized at the level of the ectodermal layer during differentiation of the regenerate. The ectoderm was thus shown to play a dynamic role during the first stages of traumatic regeneration, although it did not seem to be directly involved in the early events of the metameric process.

KEY WORDS: *regeneration, invertebrates, annelids, extracellular matrix, collagen*

Introduction

The extracellular matrix (ECM) plays a crucial and dynamic role in developmental morphogenetic events in embryos and in adult animals by ensuring the stability of tissue specific structures and functions. Permanent instructions and permissive information seem to be exchanged between cells through the ECM. The quantitative and qualitative microheterogeneity and the spatio-temporal variations of this structure, as well as its pathological modifications, contribute to cellular behavior (migration, proliferation and differentiation) by means of differential accumulation of soluble factors (growth factors) and/or specific ECM molecules, according to the»dynamic reciprocity model» proposed by Bissell *et al.* (1982).

During development, interactions between cell surface and ECM molecules were involved in gastrulation of insect (Knibiehler *et al.*, 1990), sea urchin (Wessels *et al.*, 1984), amphibians (Boucaut and Darribère, 1983; Lee *et al.*, 1984; Nakatsuji, 1984; Boucaut *et al.*, 1985; Johnson *et al.*, 1990), chicken (Duban and Thiéry, 1982) and early mouse embryos (Vartiovaara and Vaheri, 1980). ECM molecules control the migration of neural crest cells (Thiéry *et al.*, 1985; Bronner-Fraser and Lallier, 1988; Löfberg *et al.*, 1989; Newgreen, 1989; Bronner-Fraser, 1990; Perris *et al.*, 1990) and growth of nerve endings (Letourneau, 1975; Collins and Garrett, 1980; Sanes, 1989, for review). The secretion of orientated ECM molecules was involved in morphogenetic pattern formation in a lower invertebrate

Abbreviations used in this paper: ECM, extracellular matrix

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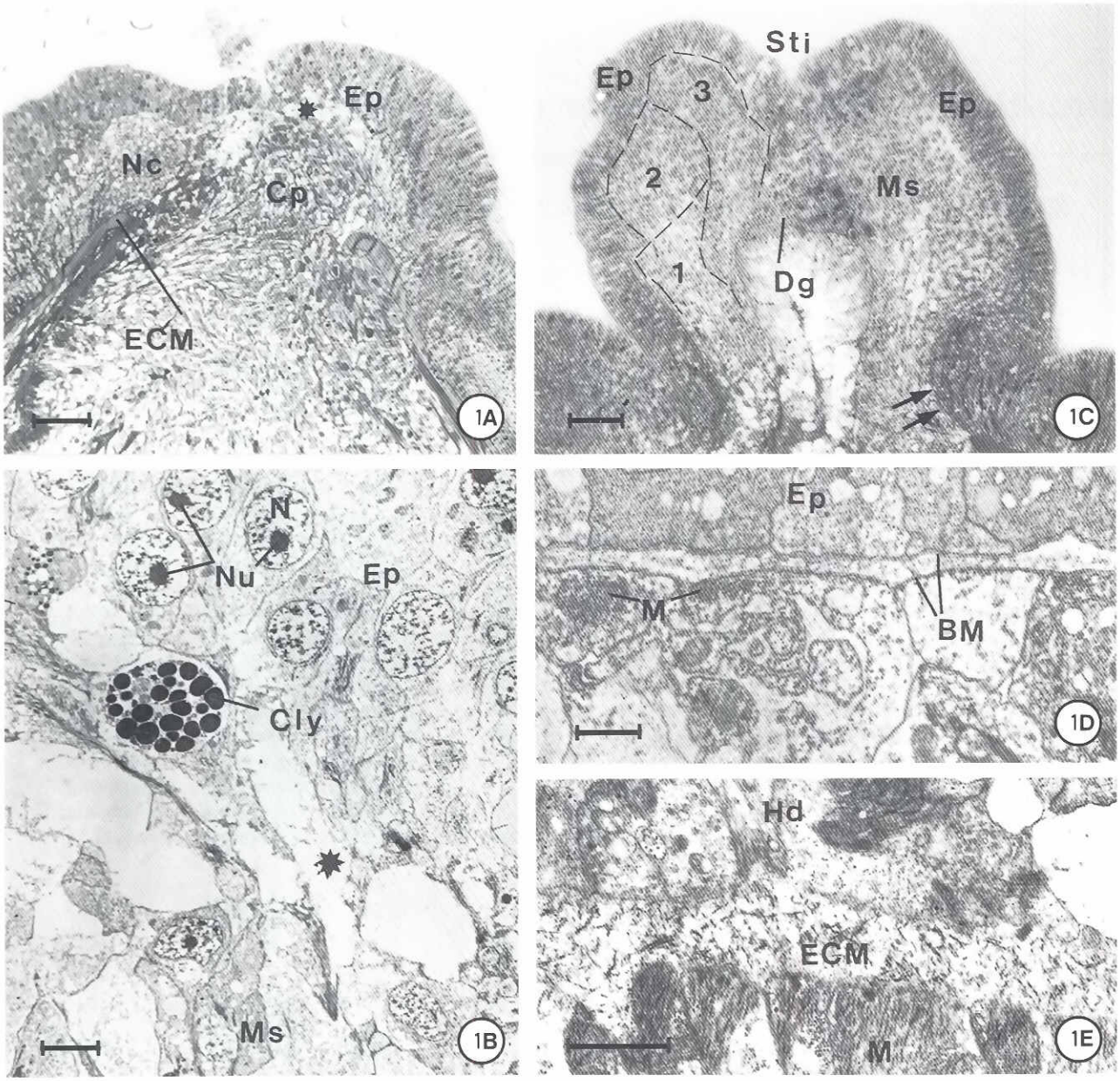


Fig. 1. Morphological events of ECM formation. (A) *Semi-thin sagittal section 24 h after amputation at the first abdominal segment level. The wound is closed by the cell plug (Cp) originating from the mesoderm. The healing process involves migration of epidermal cells (Ep) which cover the cell plug. Nc= nervous cord, ECM= extracellular matrix. (Bar= 50 μm).* **(B)** *Electron micrograph of the same region (*) showing the absence of basement membrane (BM) and the discontinuous contacts between epidermal and mesodermal cells. N= nucleus; Nu= nucleolus; Cly= cytolysomes; Ms= mesoderm. (Bar= 0.25 μm).* **(C)** *Semi-thin parasagittal section of 3-day-old blastema showing three clusters of mesodermal cells corresponding to the regenerated parts of the body: 1= anterior part of the first abdominal segment; 2= thoracic segment; 3= cephalic part. A thin reorganizing BM separating the ectoderm from the mesoderm is present in continuity with the ECM of the stump (arrows). Sti= stomodeal invagination; Dg= digestive gut; Ep= epidermis; Ms= mesoderm. (Bar= 50 μm).* ***(D)*** *4-day-old regenerate. The reforming basement membrane (BM) is seen on this electron micrograph showing at this stage the presence of a sparse fibrillar material. M= muscle; Ep= epidermis. (Bar= 0.2 μm).* **(E)** *7-day-old regenerate. The new extracellular matrix (ECM) exhibits normal structural features, with abundant characteristic collagen fibers. Muscles (M) and basal hemidesmosomes (Hd) are well reorganized. (Bar= 2 μm).*

(Garrone, 1984) and in vertebrate chondrogenesis (Rooney *et al.*, 1984). During organogenesis, the role of the ECM has also been demonstrated in many biological systems (Bernfield and Banerjee, 1978; Hay, 1984). Following injury, the basement membrane, a specific epithelial ECM, was found to be involved in wound healing, scar formation and regeneration as a substate for cell migration, proliferation and differentiation in the different species studied (Timpl and Dziadek, 1986; Clark, 1989, for review).

In annelids, a thick ECM is present between the epiderm and the mesoderm, and between the mesoderm and the endoderm. Between coelomic cavities of consecutive segments, ECM forms a connective septum in the dissepiment. ECM structures provide the animals with a scaffold that determines their anatomical shape.

In *Owenia fusiformis*, epimorphic regeneration involves first the formation of a blastema from which the exact number of amputated segments is reproduced (Thouveny, 1967). We have previously shown that ECM was lacking in the early blastema (Coulon *et al.*, 1989) but was progressively restored during differentiation of the regenerate. During this process a basement membrane and then an ECM were formed between the three different layers, the ectoderm, the mesoderm and the endoderm. Later, when the coelomic cavities develop in the regenerate, ECM was formed in the connective rows (dissepiments) according to a well defined metameric pattern (Thouveny *et al.*, 1988). Ultrastructural and immunohistochemical data (Coulon, 1979; Coulon *et al.*, 1989), have shown that collagen is a major component of the ECM of *Owenia*.

We have studied the expression of a partially characterized collagen-like gene isolated from a genomic library of *Owenia fusiformis*. *In situ* hybridization of the related probe corresponding to the helicoidal domain of the molecule has shown a specific accumulation of the transcripts in the epidermal cells. This accumulation was correlated with the accumulation, in the basement membrane, of tritiated proline, which was used as a marker for collagenic proteins.

The involvement of the related Ocg8 gene in morphogenetical events of regeneration during the main stages of ECM remodeling was discussed.

Results

ECM remodeling during anterior regeneration

During anterior epimorphic regeneration of *Owenia fusiformis*, we chronologically observed the reorganization of the ECM after amputation at the level of the first abdominal segment. The ectodermal cells localized on the edge of the wound lose their anchoring structures and migrate on a mesodermal plug to form the wound epithelium (Fig. 1A). The wound healing process brings into apposition the ectodermal and mesodermal layers without intercalation of any detectable extracellular component. One day after amputation, the healing epiderm covered the entire surface of the wound, but its inner surface showed irregular cytoplasmic expansions, containing cytoskeletal bundles, thus making discontinuous contacts with the mesodermal cells (Fig. 1B).

Three days after amputation, a thin reorganizing basement membrane (about 0.1 µm) was observed between the ectoderm and the mesoderm linked to the ECM of the stump (Fig. 1C). No collagen fibrils were present (Fig. 1D). At this stage, the clusters of mesodermal cells do not have coelomic cavities and, between them, no connective boundaries can be observed.

At 6-7 days after amputation, a more complex extracellular matrix was observed. The thickness of the extracellular matrix was increased (about 1µm) by associating with organized bundles of collagen fibers. Correlatively, the anchoring structures (desmosomes and hemidesmosomes) were formed in epidermal and mesodermal (muscle) cells (Fig. 1E). Later, (7-day-old regenerate), metameric structures, such as epidermal glands and parapodial structures (uncini), differentiated. Typically, in the axial part of the regenerate, the stomodeal invagination and the endodermal digestive tube were joined together at the limit between the thorax and the first abdominal segment level. In the mesoderm, dissepiments provided by ECM components isolate the coelomic cavities of the segments (see Fig. 2E).

Tritiated proline incorporation

Incorporation of tritiated proline was used to study the distribution of proline-rich components during the formation of the ECM in regenerates. Longitudinal sections of labeled animals fixed after a 6h period of chase in cold proline showed a diffuse signal on the whole epidermis (data not shown). After a 24h period of chase, silver grains were concentrated in the newly differentiating basement membrane of the blastema and of the regenerate (Fig. 2A, B and E).

In the 3-day-old blastema, labeling was detected in the thin basement membrane formed in the continuation of the ECM of the stump. No labeling was seen in the undifferentiated apical part of the blastema (Fig. 2A).

Five days after amputation, the signal was intense over the ECM, between the epiderm and the mesoderm throughout the regenerate. However, the intensity of the signal was less pronounced in the anterior region. The labeling was rarely observed in the dissepiment present between the first abdominal and the thoracic segments (Fig. 2B). No signal was noticed in the epidermal gland anlagen (Fig. 2C and D). The variability of the signal presumably reflects regional differences in the level of proline-containing extracellular molecules within the basement membrane.

Seven days after amputation, all the structures corresponding to the extracellular matrix of the regenerate exhibited significant labeling (Fig. 2E). In none of the early stages did the cuticular structures show any labeling (Fig. 2A, B and C).

Characterization and expression of a collagen-like sequence

Characterization of Ocg8 «collagen-like» clone

The restriction map of Ocg8 selected recombinant phage and the orientations of the DNA fragments used for sequencing are presented in Fig. 3. The 5 kb Hind III fragment cross-hybridized to Dcg1 and the adjacent 1.1 kb Hind III-Eco R1 fragment cross-hybridized to Cg 45. Further, the size of the subfragments shown does not represent the actual limits of the collagen gene. The 2.5 kb Hind III fragment of Ocg8 was extensively cut by Sau 96 I, a restriction enzyme often used to identify coding sequences for collagen (Gordon et *al.*, 1987; Vasios *et al.*, 1987), because it recognizes specifically and cuts the sequence -GGNCC- predicted to be abundant in the triple-helical region. The digestion pattern of this fragment (data not shown) corresponded to what might be expected for a collagen-like sequence.

The 1.3 kb Hind III-EcoR1 and the 1.1 kb Eco R1-Hind III subfragments generated by the 2.5 kb Hind III fragment were subcloned in both orientations into M 13 vectors mp10 and mp11. Fig. 4 represents the sequence of 315 nucleotides of the insert in M 13 mp11. The first G nucleotide would be required in the exon splicing. The translation configuration results in a sequence encoding 98 amino-acids. An A+T-rich intervening sequence intron flanks the 5' end of the exon. This intron is bounded by the consensus splicing signal TYYAG/G (Sharp, 1981). Other sequence matrices (Fig. 4, arrows) have multiple termination codons in the three reading frames.

The most striking findings were first, the encoded amino-acid sequence composed of the (Gly-Xaa-Yaa) repeats (x29), and second, the two interruptions in the collagenic sequence in positions

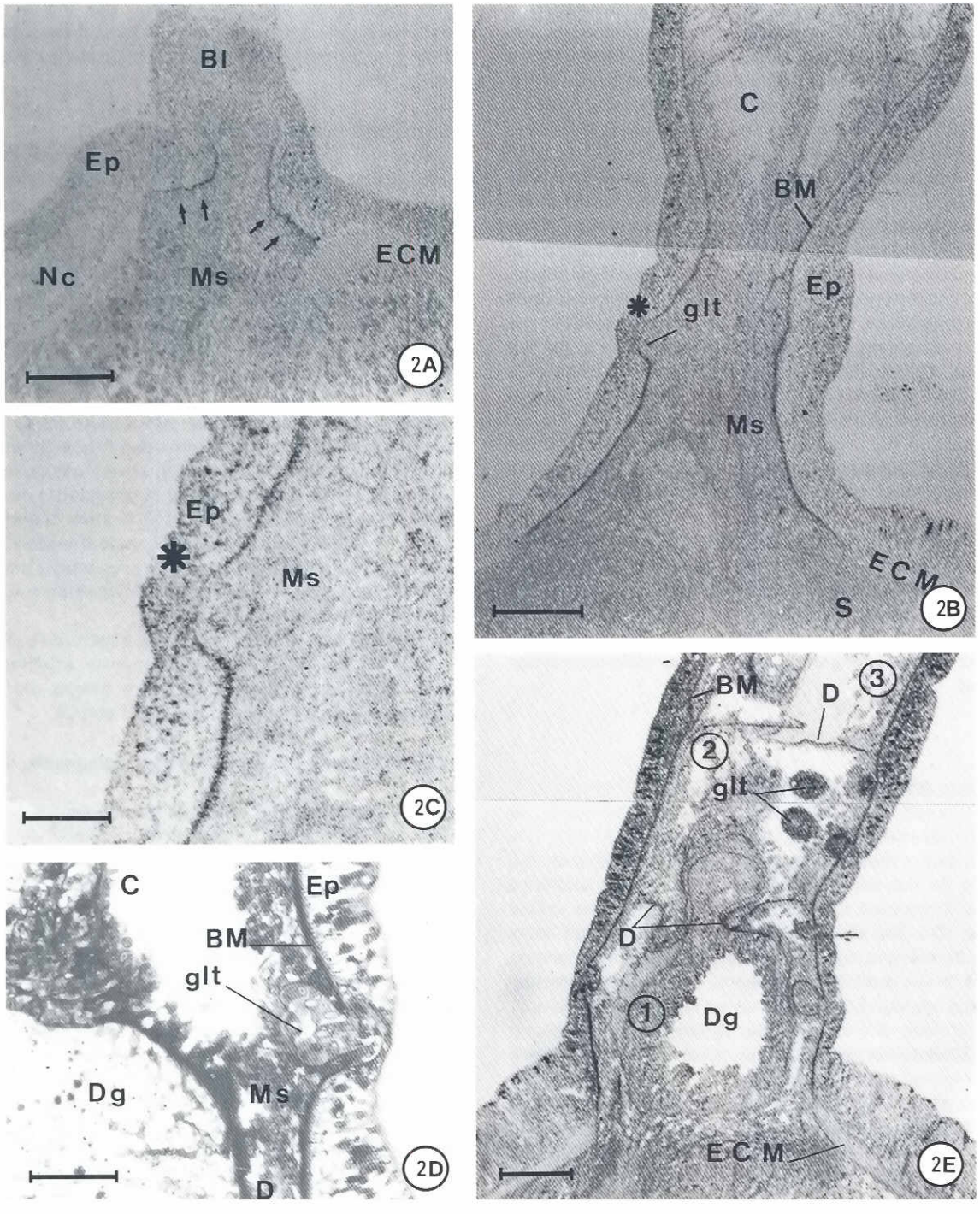


Fig. 2. Autoradiographs after tritiated proline incorporation. (A) *Semi-thin section of 3-day-old blastema showing the selective accumulation of silver grains on the new reforming basement membrane (arrows) linked to the extracellular matrix (ECM) of the stump. Bl= blastema; Ep= epidermis; Nc= nervous cord; Ms= mesoderm. (Bar= 100 μm).* **(B)** *Semi-thin section of a 5-day-old regenerate. The labeling is seen at the interface between the epidermis (Ep) and the mesoderm (Ms) of the whole regenerate. Note the decrease in intensity of the labeling in the anterior part of the regenerate. C= coelom, BM= basement membrane; S= stump; glt= secretory epidermal gland of the tube. (Bar= 100 μm).* **(C)** *Higher magnification of Fig. 2b, showing the discontinuity of the labeling at the level of the secretory gland of the tube (glt). Note the absence of silver grain accumulation on the cuticle. (Bar= 50 μm).* **(D)** *Semi-thin section stained with Unna blue, at the level of the formation of the secretory gland of the tube (glt), showing the invagination of ectodermal cells into the mesoderm (Ms). C= coelom; Ep= epidermis; BM= basement membrane; Dg= digestive gut. (Bar= 50 μm).* **(E)** *Semi-thin section of a 7-day-old regenerate showing the three anatomical regions. The labeling is seen on the basement membrane (BM) of the regenerate, around the secretory gland of the tube (glt) and along the dissepiments (D). Dg= digestive gut. (Bar= 100 μm).*

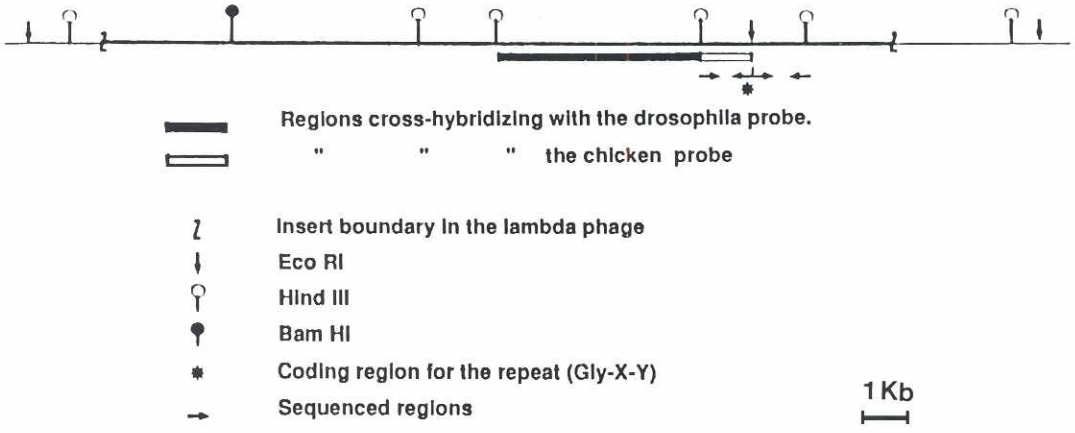


Fig. 3. Restriction enzyme map of the Ocg8 recombinant phage containing *Owenia fusiformis* collagen sequences. *The white box represents the fragment of* Owenia *DNA that cross-hybridizes to pCg45; the dark box, the fragment that cross-hybridizes to Dcg1. The arrows indicate the sequenced portions.*

244 and 307, which were, respectively, 8 and at least 2 aminoacids long. In addition, proline constitutes 19% and alanine 16% of aminoacids. Histidine, cysteine and tyrosine are absent.

Genomic Southern

Ocg8 restriction fragments were labeled with α^{32}P and hybridized under stringent conditions to a whole genomic southern blot of *Owenia fusiformis* DNA digested with Pst I. Ocg8 exhibited self-hybridization only to a single band of about 6.5 kb (Fig. 5A), and thus constitutes a very specific DNA probe to be used in specific mRNA detection experiments, by *in situ* hybridization.

Northern blot analysis

Northern blots of 5-day-old regenerate poly(A)+ RNAs showed that the Ocg8 probe hybridized to a single mRNA species, about 6600 bases long (Fig. 5B, lane 1). Northern blots with poly(A)+ RNAs extracted from non-regenerating tissues (normal animals) gave very few, if any, positive results (data not shown). Poly(A)+ RNAs from *Drosophila* were used as negative controls (Fig. 5B, lane 2).

In situ *hybridization analysis*

We analyzed the expression of Ocg8 gene by *in situ* hybridization in sectioned regenerates at various stages of regeneration, using DNA α^{32}P-labeled probes that correspond to restriction fragments of Ocg8.

Ocg8 transcripts were not detectable during the first stages of regeneration into the 2- to 3-day-old regenerates when dedifferentiating processes and cell multiplication are more efficient (Thouveny *et al.*, 1988). A selective signal appeared in 4-day-old regenerates, and was only present in ectodermal cells (Fig. 6A and B). This labeling persisted during the later stages (5-7 days; Fig. 6C and D). The accumulation of grains was localized in all the ectodermal cells including the stomodeal invagination and epidermal glands (Fig. 6D, E and F). The mesodermal region and digestive gut displayed no labeling (Fig. 6D).

Control data demonstrated that non-specific binding of heterologous λ DNA was randomly distributed throughout the sections. On the other hand, sections treated with RNase I prior to hybridization with Ocg8 DNA showed an autoradiographic labeling reduced to background level (data not shown).

Discussion

In this paper we described the main stages of ectodermal basement membrane formation during post-traumatic anterior regeneration in the polychaete annelid *Owenia fusiformis*. The main findings can be summarized as follows:

Histological and ultrastructural data showed that the basement membrane appears during blastema formation and differentiates along with the reorganization of the epidermis and the underlying muscle tissue originating from the mesodermal cells of the blastema. New anchoring structures (hemidesmosomes and cytoskeleton) were observed in the epidermal and muscle cells 4-5 days after amputation just before collagen fibers begin to accumulate within the new basement membrane.

Autoradiography after ^{3}H-proline incorporation (pulse and chase experiments) showed that the epidermis synthesized labeled proteins from the 3rd day after amputation. Afterwards, these proteins accumulated in the newly formed basement membrane of 4-5-day-old regenerates. Proline is one of the major amino-acid components of collagen (12%) and its incorporation can be used as a marker for the synthesis of this protein. Our chronological data on this synthesis were in good agreement with those of Burke and Ross (1975), during regeneration of the oligochaete annelids. Our autoradiographic experiments suggested that the main product synthesized by the epidermis, labeled by ^{3}H proline, was targeted to the new basement membrane. The targeting of this(ese) protein(s) into the differentiating basement membrane started at the level of the stump and progressed into the regenerated segments following a proximo-distal gradient. It was noteworthy that the extracellular matrix was first deposited at the interface between the ectodermal and mesodermal layers and, later, at the boundaries between the coelomic cavities of two consecutive segments.

Ocg8 probe provided a good tool for *in situ* hybridization. This clone contains a 13 kb *Owenia* DNA insert which was characterized by Southern and Northern analyses and has been partially sequenced.

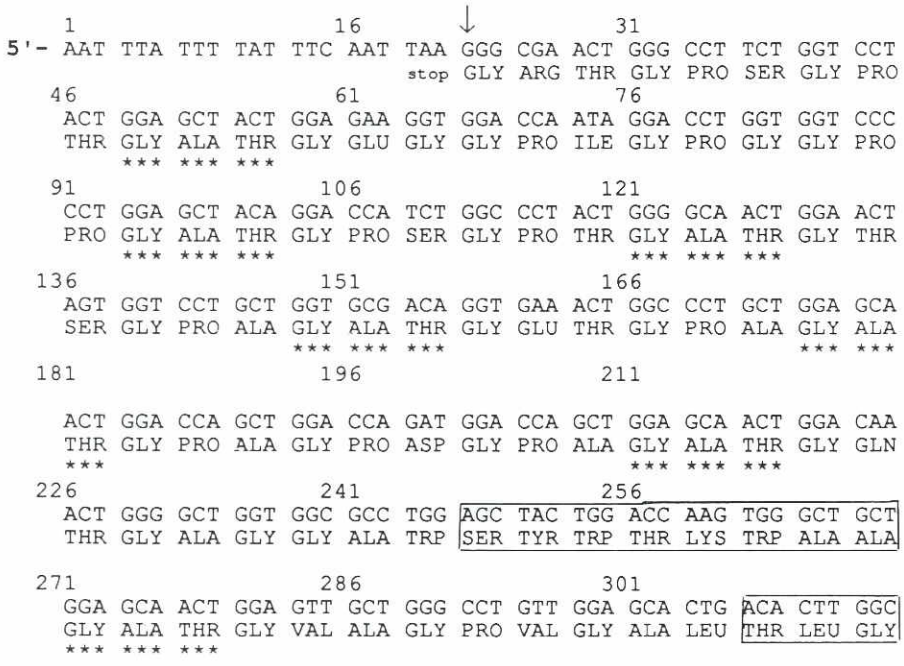


Fig. 4. Nucleotide and corresponding amino-acid sequences of a 315 nucleotide fragment. *Ocg8 contains an exon coding the potential triple-helical domain. The derived amino-acid sequence is shown below the appropriate codons. The numbering of nucleotides is for reference purposes only. The arrow indicates the location of the probable splice site in the consensus junction sequence of the intron-exon (underlined). The (Gly-Ala-Thr) repeat is frequently present (stars).*

The related nucleotide sequence clearly specified a collagenous peptide. It contains a stretch of 315 base pairs (bp) that codes for 29 Gly-Xaa-Yaa repeats and is flanked by the appropriate splice junction. The boundary of the intron was analogous to those reported for other genes and had a consensus sequence AG/G-exon. The donor splice site appeared to be located within the glycine codon which is found in type IV collagen genes of both vertebrates and invertebrates while in type I genes the starting glycine codon remains intact. This feature applies to other invertebrate collagen genes; for instance, cuticular genes of nematodes (Kramer *et al.*, 1982). On the other hand, there were two interruptions in the sequenced triple-helical coding related region, as in type IV or cuticular collagen genes. The exon of Ocg8 was at least 304 bp long and it probably extended beyond the 3' end of the 0.9 kb Eco R1-Hind III fragment. It differs from the vertebrate fibrillar collagen genes whose exons consist of 9 bp-multiple motifs which rarely exceed 108 bp. Northern blot analysis showed a single transcript of 6.6 kb which was comparable to those detected in other organisms which code for collagenic molecules. For example, a mRNA of 6.4 kb corresponding to type IV collagen was found in the early stages of development of Drosophila (Le Parco *et al.*, 1986). In sea urchins, several specific high molecular weight mRNAs (9 kb, 7 kb, 6 kb) were differentially expressed at blastula, gastrula and pluteus stages (Nemer and Harlow, 1988; Saitta *et al.*, 1989). In sponges however, non-fibrillar collagen mRNA has a lower molecular weight (1.6 kb) (Expósito and Garrone, 1990) and is similar to that of cuticular collagen of *Caenorhabditis elegans* (Cox *et al.*, 1981).

Finally, the genomic hybridization pattern generated by the Ocg8 clone suggests the existence of a single-copy gene. Therefore this sequence has the main characteristics required for a molecular probe used in *in situ* hybridization experiments.

According to Edwards *et al.* (1958), many if not all cell types might be involved in the secretion of collagenic structures. The data presented here identify the epidermal cells as the unique site responsible for the expression of Ocg8 gene. During traumatic regeneration in *Owenia*, extensive remodeling processes occurred in the cells participating in the formation of the blastema and the regenerate, including the reorganizing cuticle and the reforming basement lamina and extracellular matrix (Coulon *et al.*, 1989). Consequently, the translational products of Ocg8 might be an extracellular matrix or a cuticular collagen. The localization of collagen messenger RNAs in *C. elegans* by *in situ* hybridization showed that the cuticular collagen was synthesized by the underlying hypodermal cells and developmentally regulated before each moult (Edwards and Woods, 1983). In *Owenia*, ultrastructural and biochemical data (Dupin, 1990) showed that the cuticle does not contain collagen. Thus, on the basis of the present data, it can be assumed that Ocg8 expression in the epidermal cells is related to a fibrillar or a non-fibrillar collagen component of the epidermal basement membrane. *In situ* hybridization showed that the accumulation of the related transcripts is tissue-specific and spatio-temporally regulated. There was a good correlation between our *in situ* hybridizations and biochemical data previously obtained, particularly the increase of transcriptional activity at 4-5 days after trauma (Fontés *et al.*, 1979). Northern blot data using poly $(A)^+$ RNAs extracted from 4-day-old regenerates corroborated this finding. The results obtained by *in situ* hybridization were in favour of a renewed gene activation, expressed in the whole epidermis of the proximal stump and the regenerate, and were in good agreement with the autoradiographic and ultrastructural studies on the reorganization of the extracellular matrix during regeneration.

Materials and Methods

Animals

Polychaete annelids, *Owenia fusiformis*, were collected at the Marine Biological Laboratory of Roscoff (France). They were reared in a circulating

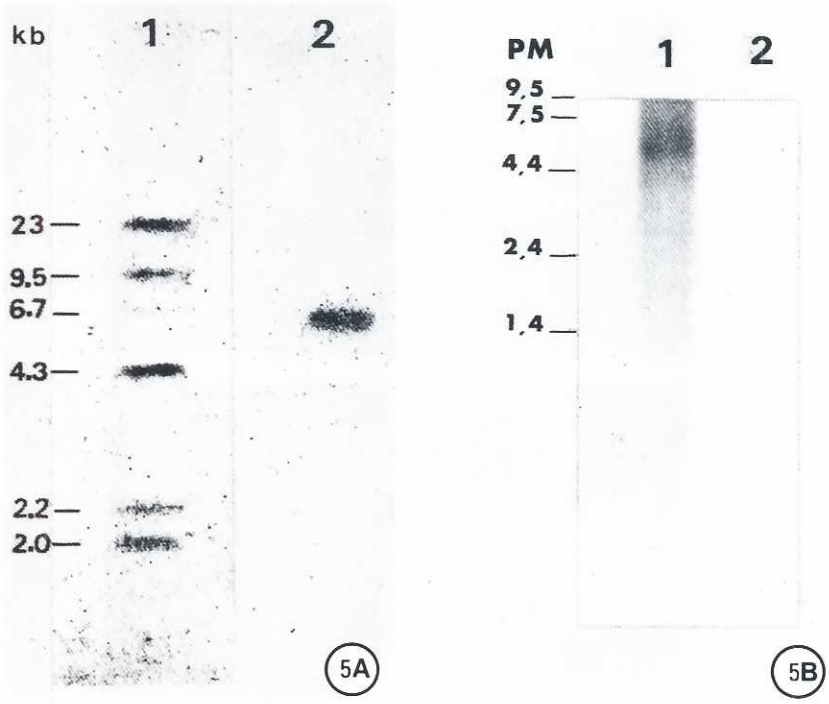


Fig. 5. Genomic Southern and Northern blots. (A) Owenia fusiformis *genomic hybridization pattern. The genomic DNA of a single animal was digested to completion with Pst I, electrophoresed through a 0.8% agarose gel, and transferred to nylon filter (Hybond-N from Amersham). (Lane 1)* ^{32}P *labeled size markers in kilobases (kb) derived from a Hind III digest of lambda phage. (Lane 2) the Ocg8 insert was* ^{32}P *labeled by «random priming» up to 1X 108 cpm/μg DNA, and hybridized to the Southern blot of digested DNA in a 30% formamide, 1xSSC hybridization solution at 50°C. The filter was washed in 0.1xSSC at 65°C.* **(B)** *Northern blot. poly (A)+ RNAs extracted from 4-day-old regenerates were electrophoresed and transferred onto a nylon filter and hybridized with* ^{32}P*-dCTP labeled Ocg8 probe (Lane 1). «Rapid hybridization» buffer (Amersham) was used. The filter was washed by 0.1xSSPE at 65°C. Lane 2: hybridization with the same probe was performed on poly (A)+ RNAs from* Drosophila. *PM= RNA molecular weight marker.*

sea water aquarium, under standard laboratory conditions. The amputation was done at the level of the anterior part of the first abdominal segment. Regeneration was always complete (Thouveny, 1967), following a morphological and biochemical pattern previously described (Fontés *et al.*, 1983).

Electron microscopy

Samples excised from normal animals and from 2 to 7 day-old-regenerates were prefixed with 2% glutaraldehyde in 0.05M cacodylate pH 7.5 and artificial sea water for 1h at 4°C. Specimens were subsequently washed and post-fixed for 1h in 1% OsO_4 in the same buffer. Pieces were dehydrated using a graded ethanol series and embedded in Araldite M.

Semi-thin sections were stained with Unna blue. Ultra-thin sections were stained with uranyl acetate and lead citrate, and examined in an HU600 electron microscope operated at 75kV.

Autoradiography

Regenerating animals (3,5,7 days after amputation) were incubated for 2h in sea water containing 2,3-^{3}H L-proline (Amersham, specific activity: 26 Ci/mM, concentration: 25μCi/ml). After incubation, samples were washed for 6 to 36h in 1% cold proline, and fixed according to the procedure described above.

Semi-thin sections were coated with Kodak NTB2 emulsion, exposed for 10 days and developed in Kodak D19b.

Screening and selection of collagen-like* Owenia fusiformis *genomic clones

We have constructed a library of random *Owenia* genomic fragments (about 15 Kb) in the laboratory of Dr. V. Pirrota in EMBL, using a partial Sau 3A digest of adult *Owenia* DNA. The fragments were ligated into Bam H1-digested L 47 λ vector (Bakalara *et al.*, 1990).

Screening of the library was first performed according to the method of Benton and Davis (1977). The 2.6 Kb Hind III insert of the recombinant plasmid pCg 45 of the chicken λ (2)-procollagen cDNA was used as heterologous probe for hybridizations (Lehrach *et al.*, 1978); it contains the carboxy-terminal propeptide region and the last half of the triple helical domain cloned into pBR 322. We also used the Bam H1 restriction fragments of the Dcg1 clone, previously sequenced by Monson *et al.* (1982), corresponding to the *Drosophila* type IV collagen gene (Cecchini *et al.*, 1987). These fragments were purified from agarose slab gels (see below) and ^{32}P labeled by nick-translation (Rigby *et al.*, 1977). Filters were prehybridized and hybridized as described by Monson *et al.* (1982).

Several positive plaques (20) of varying signal intensity were obtained under low stringency conditions. Six of these recombinant clones (Ocg6, 7, 8, 9, 16 and 19) which presented higher hybridization signals with both the collagen Cg45 and Dcg1 probes were isolated and amplified using *E. coli* Q358 strain.

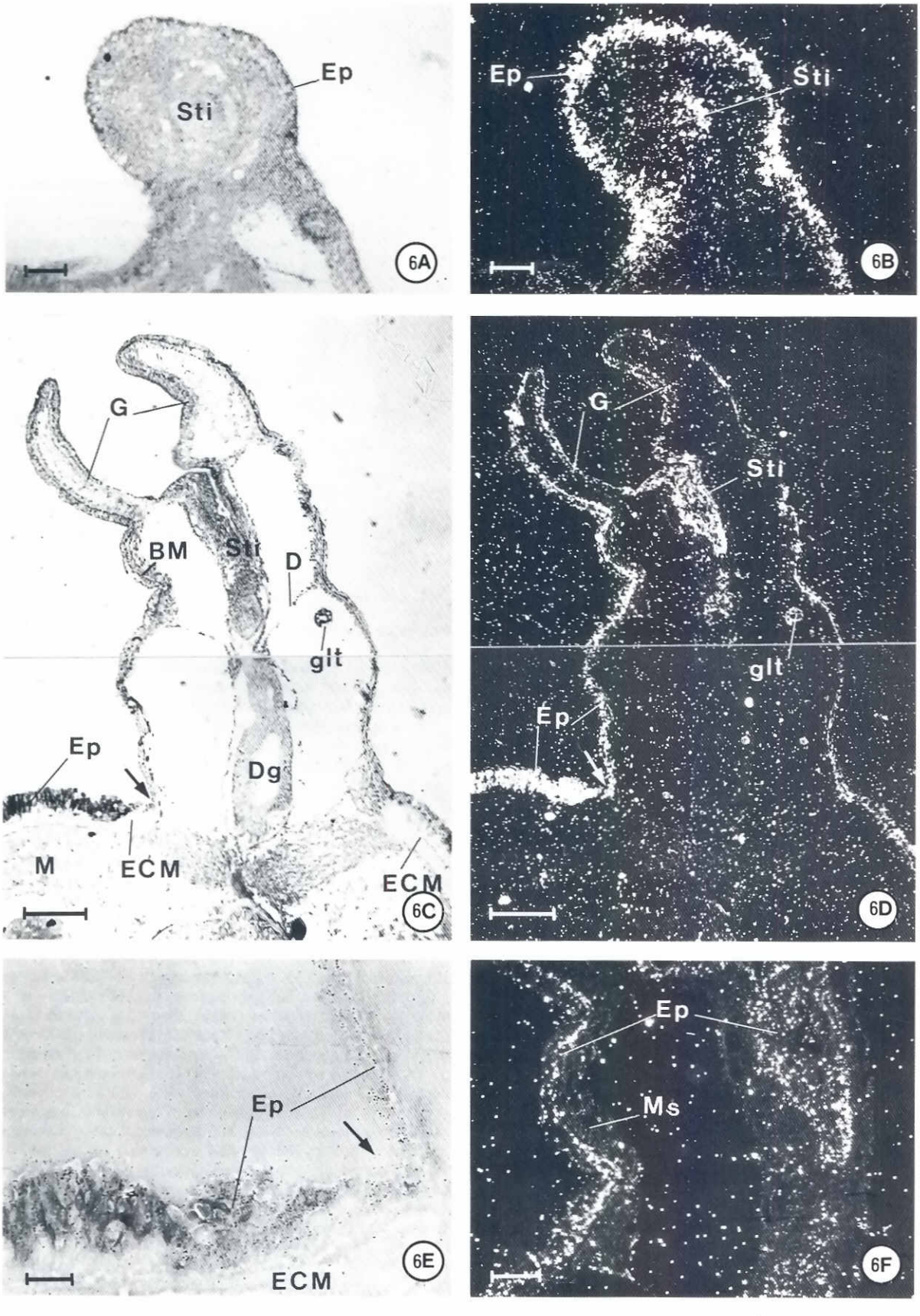
Southern blots of these genomic clones cleaved with Eco R1, Bam H1, Hind III restriction endonucleases were hybridized with ^{32}P labeled Cg45 in order to localize the subfragments which are homologous to the chicken collagen cDNA probe. We repeated the experiment using the BamHI restriction fragments of the genomic Dcg1 clone.

DNA procedures

The lambda phage DNA was purified essentially as described by Yamamoto *et al.* (1970) and Blattner *et al.* (1977), for further analysis. The preparation of Bluescript (BS) plasmid and M13 phage was carried out, using the method of Miller (1972). Bluescript plasmid DNA was finally purified by precipitation using 10% polyethylene glycol, 500 mM NaCl, for 30 min in ice, and banded on CsCl-ethidium bromide density gradients.

Genomic DNA was prepared from pure isolated nuclei using the method described by Schwartz and Cantor (1984), with minor modifications. The annelid pure nuclei were purified prior to embedding in agarose. DNA was transferred onto nitrocellulose filters (Hybond C, from Amersham) according to Southern (1975). Hybridization probes were labeled with ^{32}P-dCTP by «random primer» elongation (Feinberg and Vogelstein, 1983). Hybridizations and washes were essentially done as described in Maniatis *et al.* (1982).

For restriction enzyme analysis, samples of DNA were dissolved in 10mM Tris-HCl pH8.1 mM EDTA, and digested with enzymes (4 units/μg DNA), at 37°C for 1h in appropriate buffers. Isolation and purification of given restriction fragments were performed as follows: restriction digests of recombinant phage or plasmid DNA were size-fractionated by electrophoresis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on 1% agarose gel, the slices containing a given fragment were recovered and purified using a Geneclean kit (Ozyme).

Subcloning

The eluted restriction fragments were ligated with the vectors M13 mp10 or mp11 using double stranded (replicative form) DNA or with the BS plasmid. Transformation was carried out with *E. coli* strain JM107 or JM109 for M13 strains mp10 and mp11 and with *E. coli* strain JM101 for BS plasmid, using the calcium chloride procedure.

Sequence analysis

Single-stranded DNA from M13 mp10 and mp11 clones were used as templates and sequencing was performed by the dideoxy chain termination method (Sanger *et al.*, 1977) using the «universal» primer to initiate the sequencing reaction with the Klenow fragment of DNA polymerase I.

Isolation of RNAs and Northern blot analysis

Total RNAs were extracted from 5-day-old-regenerates using the guanidinium thiocyanate method (Chirgwin *et al.*, 1979).

Poly(A)+ RNAs were isolated by oligo(dT) cellulose chromatography; the sample (10µg) was then fractionated by electrophoresis on 1.5% formaldehyde agarose gel and transferred to a nylon filter (Hybond N Amersham) in 10x SSPE buffer (NaCl 1.5 M, NaH_2PO_4 0.1 M, EDTA 10mM).

The 2.25 Kb Hind III restriction fragment Ocg8 was labeled as a homologous probe to hybridize to the poly (A)+ blot at 42°C for 16h in a «rapid hybridization» buffer (Amersham). The filter was washed under high stringency conditions (0.1x SSPE, 0.5% SDS), dried and exposed to Kodak XAR film.

In situ *hybridization*

Tissue preparation

Samples excised from normal animals and 2-to-7day-old regenerates were either: i) fixed in Carnoy's solution (3 ethanol: 1 acetic acid), for 30 min at 4°C and then for 30 min at room temperature, dehydrated in 100% ethanol (2x15 min) and immersed in toluene (3x15 min), then infiltrated in paraplast (m.p. 55-57°C), (3x60 min), and embedded in fresh paraplast; or fixed ii) in 4% paraformaldehyde buffered with a solution containing 0.05 M cacodylate pH 7.5 and artificial sea-water, for 30 min at 4°C, and then for 30 min at room temperature. The pieces were washed for about 10 min in two changes of the same buffer, then dehydrated using graded ethanol series, and embedded in paraplast as described above. The two fixation procedures were compared in order to find conditions which yielded maximal retention of poly (A)+ RNAs and the best morphology. Of the two fixatives tested, Carnoy's fixative gave a better cell morphology than paraformaldehyde fixative (data not shown) and used further on.

Tissue treatment

The 6 µm-thick sections were placed on aminoalkylsilane-treated slides according to a method of Rentrop *et al.* (1986). In order to permeabilize the tissue sections and to render them accessible to the hybridization probe fragments, the following series of treatments were necessary. The deparaffined sections were first incubated in 0.2 N HCl at room temperature for 20 min and washed in DEPC treated water. They were then immersed in a solution containing 2xSSC (0.3 M NaCl, 0.03 M Na-Citrate, pH 7.0) prewarmed to 70°C and incubated for 30 min (Brahic and Haase, 1978) at the same temperature. Afterwards, they were rinsed in H_2O at room temperature and incubated in a fresh solution of proteinase K at a final concentration of 2 µg/ml in 20 mM Tris-HCl, pH 7.4, 2 mM $CaCl_2$ for 15 min at 37°C, then immediately rinsed in 1xPBS for 20 min at room temperature (Singer and Ward, 1982). Finally, they were acetylated with acetic anhydride following the method of Hayashi *et al.* (1978), and dehydrated in ethanol containing 300 mM ammonium acetate salt.

Serial sections were hybridized in a solution containing the selected probe. The probe was prepared from restriction fragments by nick translation or «random primer» elongation (Feinberg and Vogelstein, 1983) in the presence of (α^{32}P) dCTP, dATP, dGTP. The size of the fragments (50-100 nucleotides) was monitored by a subsequent DNAse I treatment which allowed better penetration of the probe within the tissues. About 100 µl of the final hybridization solution, containing roughly 10^5 cpm of denatured probe (approximately 10^6 cpm/µg) was spread over the sections. The hybridization was performed for 20 h in 50% formamide: 600 mM NaCl, 10 mM Tris-HCl, pH 7.5, 1 mM EDTA, 1xDenhard's mixed at 37°C. The slides were then washed for 24h in the same buffer at 37°C, dehydrated and dipped in Kodak NTB2 emulsion diluted 1:1; they were exposed 10-21 days at 4°C and developed in Dektol D 19b, then stained in Giemsa diluted 1:10 in PBS.

The specificity of the signal was checked by hybridization on sections pretreated with RNAse I (100 µl/ml RNAse I in 10 mM Tris-HCl, 1 mM EDTA pH 8 for 1h at 37°C), and by a parallel hybridization with Hind III I DNA fragments of similar specific activity and fragment length.

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Fig. 6. Autoradiographs after *in situ* hybridization using Ocg8 insert as 32P-labeled DNA probe. (A-B) *Bright-field and dark-field illumination of parasagittal sections of a 4-day-old regenerate cut at the first abdominal segment level. Significant accumulation of transcripts is detected in ectodermal cells including stomodeal invagination (St i). Ep= epidermis. (Bar= 100 µm).* **(C-D)** *Sagittal sections of a 5-day-old regenerate amputated at the same level.* **(C)** *Bright-field illumination showing the three regenerated anatomical regions (see Fig. 2E). In the stump, a thick extracellular matrix (ECM) separates the epidermis (Ep) from the underlying muscle tissue (M). In the regenerate, a thin reforming basement membrane (BM) is present between epidermal and mesodermal tissue (see Fig. 2B for comparison). Note the presence of a section of a tube secretory gland (glt). (Dg)= digestive gut; D= dissepiment; G= gills; Sti= stomodeal invagination. (Bar= 100 µm).* **(D)** *Dark-field illumination of the previous section. A hybridization signal is detected only in the epidermal cells of the stump near the section and in the ectodermal cells of the regenerate, including stomodeal invagination and secretory gland of the tube. Sti= stomodeal invagination. (Bar= 100 µm).* **(E)** *Higher magnification at the amputation level in the stump. The signal is only seen in the epidermal cells (Ep). (Bar= 20 µm).* **(F)** *Enlargement of figure 6D. A strong hybridization signal is restricted to the basal part of the ectodermal cells (Ep). Mesoderm (Ms) displays no labeling. (Bar= 20 µm).*

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