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De novo expression of a type IV collagen gene in *Drosophila* embryos is restricted to mesodermal derivatives and occurs at germ band shortening

CHRISTIAN MIRRE, JEAN-PIERRE CECCHINI, YANNICK LE PARCO and BERNARD KNIBIEHLER

Laboratoire de Biologie de la Différenciation Cellulaire, UA CNRS 179 Case 901, Faculté des Sciences de Luminy, 13288 Marseille Cedex 9, France

Summary

We have examined directly the expression of one collagen gene (*DCg1*) during *Drosophila melanogaster* embryogenesis by means of *in situ* hybridization. Transcripts of this gene, which were demonstrated to encode a basement membrane type IV collagen chain, began to accumulate specifically in mesodermal derivatives at stages 12–13 of embryogenesis, and not before. Cells expressing this gene overlap, or are closely intermingled with, somatic and visceral mesoderm in stages 12–14. In stages 15–17, in addition to the strongly positive fat bodies, highly labelled cell spots are found scattered around all the parts of the gut and symmetrically on each side of the ventral nerve cord. They correspond to circulating mesodermal cells which we consider to be haemocytes or mesoblasts.

Key words: type IV collagen, *Drosophila*, gene expression, mesoderm, haemocytes, *in situ* hybridization, transcription.

Introduction

The role of collagenous matrices during developmental processes is now better documented and it is evident that they are involved in cytodifferentiation, cell adhesion, cell migration and cell microenvironment (Hay, 1984; Bissell *et al.* 1982). It is now a current concept that basement membrane components, including type IV collagen, are important guiding factors in morphogenetic movements of cells and tissues (Reddi, 1976; Hay, 1984), but little is known about their specific role. The importance of this function was thought to be crucial during embryogenesis since, in early embryos, basement membranes segregate into layers of cells that have separate lineages and different biochemical properties (ectoderm, endoderm, mesoderm).

Type IV collagen, a major structural component of basement membranes, appears first in the mouse embryo at the 2-cell stage (Sherman *et al.* 1980). According to Leivo *et al.* (1980), its appearance in the mouse blastocyst coincides with differentiation of the primitive endoderm and assembly of the first embryonic basement membrane. It forms a specific lattice network by end-to-end interactions, the C-terminal noncollagenous domain (NC1) having been shown to be one of the major cross-linking sites (Timpl *et al.* 1981; Kühn *et al.* 1981; Yurchenko *et al.* 1986; Weber *et al.* 1984).

By means of sequence analysis of cDNA and genomic DNA clones for the 3′ end of the collagen gene *DCg1*, characterized solely in *Drosophila melanogaster* (Monson *et al.* 1982; Le Parco *et al.* 1986*a*,*b*), we have given the first direct evidence that type IV collagen exists in this organism (Cecchini *et al.* 1987). The data we have reported revealed a high degree of similarity between the noncollagenous domains (NC1) of *Drosophila* and of human and mouse α1 (IV) chains, including an unusual internal homology. The extent of both interspecies and intramolecular homologies strongly suggests the maintenance, both

in vertebrates and in invertebrates, of an ancestral specific function.

Taking into account the relative similarity of *Drosophila* and vertebrate type IV collagens, and the extensive knowledge of the morphogenetic events occurring during *Drosophila* embryogenesis, we were prompted to study type IV collagen gene expression during the onset of *Drosophila* life cycle.

The results presented in this paper are based on *in situ* hybridization experiments on tissue sections probed with labelled coding fragments of the *Drosophila DCg1* collagen gene. Thus, we examined directly the expression of this gene during embryogenesis by detecting the sites, and the onset, of specific mRNA accumulation. The results suggest a fundamental role of circulating mesodermal cells during morphogenetic processes.

Materials and methods

DCg1 *collagen gene*

We previously reported the identification and characterization of members of the *Drosophila* collagen gene family (Le Parco *et al.* 1986*a*,*b*). Specifically, we studied the *DCg1* gene, previously characterized by Monson *et al.* (1982), and we showed that it is a single-copy gene located at the 2L25C locus. It mainly encodes a 6·4 kb transcript, differentially accumulated during the course of *Drosophila* development (Le Parco *et al.* 1986*a*,*b*; Knibiehler *et al.* 1987). By sequence analysis of cDNA and genomic DNA clones for the 3′ end, we were the first to demonstrate that *DCg1* gene encodes a type IV basement membrane collagen chain (Cecchini *et al.* 1987).

Preparation of the probes

The DNA sequences used for our *in situ* hybridization experiments were the restriction fragments of *DCg1* gene shown in Fig. 1. Since all the results were similar, we have chosen to use routinely the 1·5 kb long *Eco*RI–*Bam*HI fragment (black boxes). The methods used for fragment DNA preparation were as described by Maniatis *et al.* (1982). The probe was labelled by the method of 'random-primed' DNA labelling, developed by Feinberg & Vogelstein (1984), which enables labelling to high activities and to an equal degree along the entire length of the input DNA. With all the three ^{3}H-labelled deoxynucleotides (^{3}H-dATP, ^{3}H-dCTP, ^{3}H-dTTP, approximately 50 Ci mM, NEN) we routinely obtained up to 3×10^{8} disints min^{-1} μg^{-1} DNA. This method thus permits shorter exposures (about 10 days), usually only obtained with ^{32}P-labelled probes, but without ^{32}P disadvantages (Knibiehler *et al.* 1987).

In situ *hybridization*

Frozen tissue sections were prepared from living embryos which were previously dechorionated and freed of their vitelline membrane before being embedded in OCT compound (Tissue teck). The 6 μm-thick sections were processed as described by Hafen *et al.* (1983) and finally acetylated with acetic anhydride as described by Hayashi *et al.* (1978) and dehydrated. All subsequent treatments of the slides were as previously described (Knibiehler *et al.* 1987).

Embryonic stages

The embryos were harvested every 3 h (0–3, 3–6, 6–9, 9–12 h) in early development stages and every 6 h in late development (12–18, 18–24 h) at 22 °C. Numbering of the embryonic stages was that proposed by Campos-Ortega & Hartenstein (1986) and their identification was based on the same morphological criteria.

Results

We have performed *in situ* hybridization experiments on serial frozen tissue sections of embryos selected at sequential stages of development. They were probed with different restriction fragments of *DCg1* genomic DNA clone, located upstream and downstream of the 1·5 kb *Eco*RI-*Bam*HI fragment (black boxes in Fig. 1). The results we obtained with each fragment were strictly comparable, emphasizing the great specificity of DCg1 mRNA detection by *in situ* hybridization.

No signal has been detected during early embryogenesis, even after the longest exposures. That is, the transcripts specified by type IV collagen *DCg1* gene do not appear to be accumulated anywhere (or at a level below the detection capacity of the technique) (Fig. 2). The first signals appeared abruptly at the end of stage 12 (or at the beginning of stage 13), i.e. approximately in 9·5 h-old embryos.

Stage 12–14 embryos (9·5–12 h)

The germ band retracts during stage 12. During this process, the fusion of anterior and posterior midgut

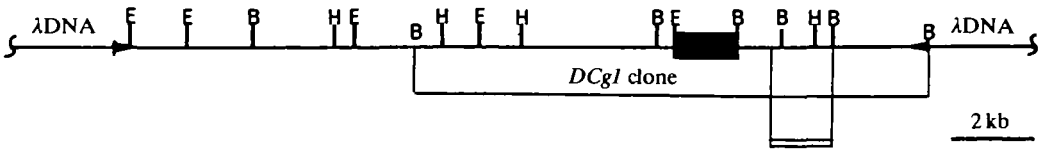

Fig. 1. Restriction endonuclease map of about 20 kb of genomic DNA overlapping genomic DNA *DCg1* clone. B, *Bam*HI; E, *Eco*RI; *H*, *Hin*dIII. Black box represents the 1·5 kb fragment, the sequence of which had not yet been determined by Monson *et al.* (1982). It was used for *in situ* hybridization as well as the other BB or BE fragments covering the coding region of *DCg1* clone. White box represents the region of *DCg1*, the cDNA sequence of which we have determined (Cecchini *et al.* 1987).

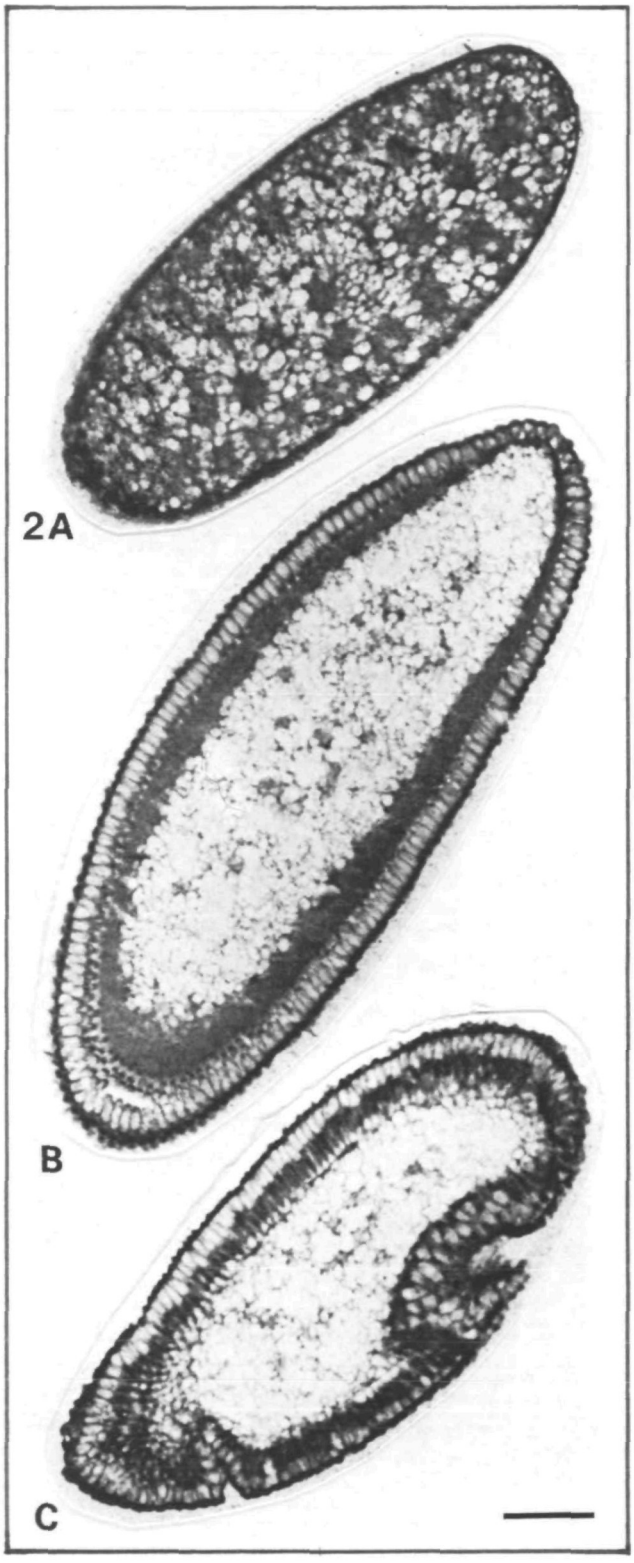

Fig. 2. Autoradiographs of *Drosophila* early embryos after *in situ* hybridization with the 1·5 kb *DCg1*-labelled fragment. (A) Stage 3; (B) stage 5, cellularized blastoderm; (C) stage 7, gastrulation. No signal was observed even after the longest (1 month) period of exposure. This negative response was observed up to stage 13. Bar, 50 μm.

takes place, and the definitive segmental furrows become apparent. At the end of stage 13, muscle cells become visible, inserting at incipient apodemes of the lateral epidermis. At completion of germ band shortening (stage 14), pharynx and oesophagus are clearly distinguishable and cytodifferentiation of axonal processes begins in sensory organs.

The autoradiographs corresponding to these stages are shown in Figs 3, 4. Globally, the repartition of hybridization can be divided into three different sets, which overlap or are closely intermingled with mesodermal derivatives.

The first set of results corresponded to strongly labelled, discrete spots each one overlapping one or a few cells. In 10 h-old embryos they are either arranged in tightly packed slender layers or are present as single entities, scattered all around the different parts of the gut (Fig. 3A,B,D). These accumulation sites of *DCg1* transcripts strictly coincide with visceral mesoderm, a derivative of the inner splanchnic layer which, at these stages, detaches as single cells from the mesodermal primordium (Campos-Ortega & Hartenstein, 1985; Beer *et al.* 1987; Poulson, 1965).

The second set also had the appearance of intensely labelled spots and was well visible, as shown in Fig. 3C. Its segmented nature is sometimes difficult to observe owing to the plane of the sections. When sagittal sections were obtained, the spots appeared to be regularly arranged between the ectoderm and the ventral cord and, also, symmetrically between the ventral cord and endoderm. This second set of labelling corresponds to cells of the segmented somatic mesoderm. This derivative originates from single cells of the somatopleura, contacting both the developing nervous system and ectoderm.

The last set of hybridization signals was mostly visible in frontal and transverse sections (Figs 4A,B, 3D). It consists of a slightly labelled, thin, dorsolateral layer of cells extending on either side along the embryo between the gut and the somatic musculature. It overlaps strictly the mesodermal derivative that will give rise to the fat bodies extending from the gonad to the level of the brain hemispheres.

Stage 15, 16, 17 embryos (12–24 h)

During these stages, dorsal closure and epidermal segmentation are accomplished, the gut completely contains the yolk sac (stage 15), shortening of the ventral cord occurs and the fat body can be seen laterally extending from the gonads to the anterior thoracic levels. The larval pattern of somatic musculature becomes distinguishable (stage 16). During stage 17, no conspicuous differences can be distinguished compared to stage 16.

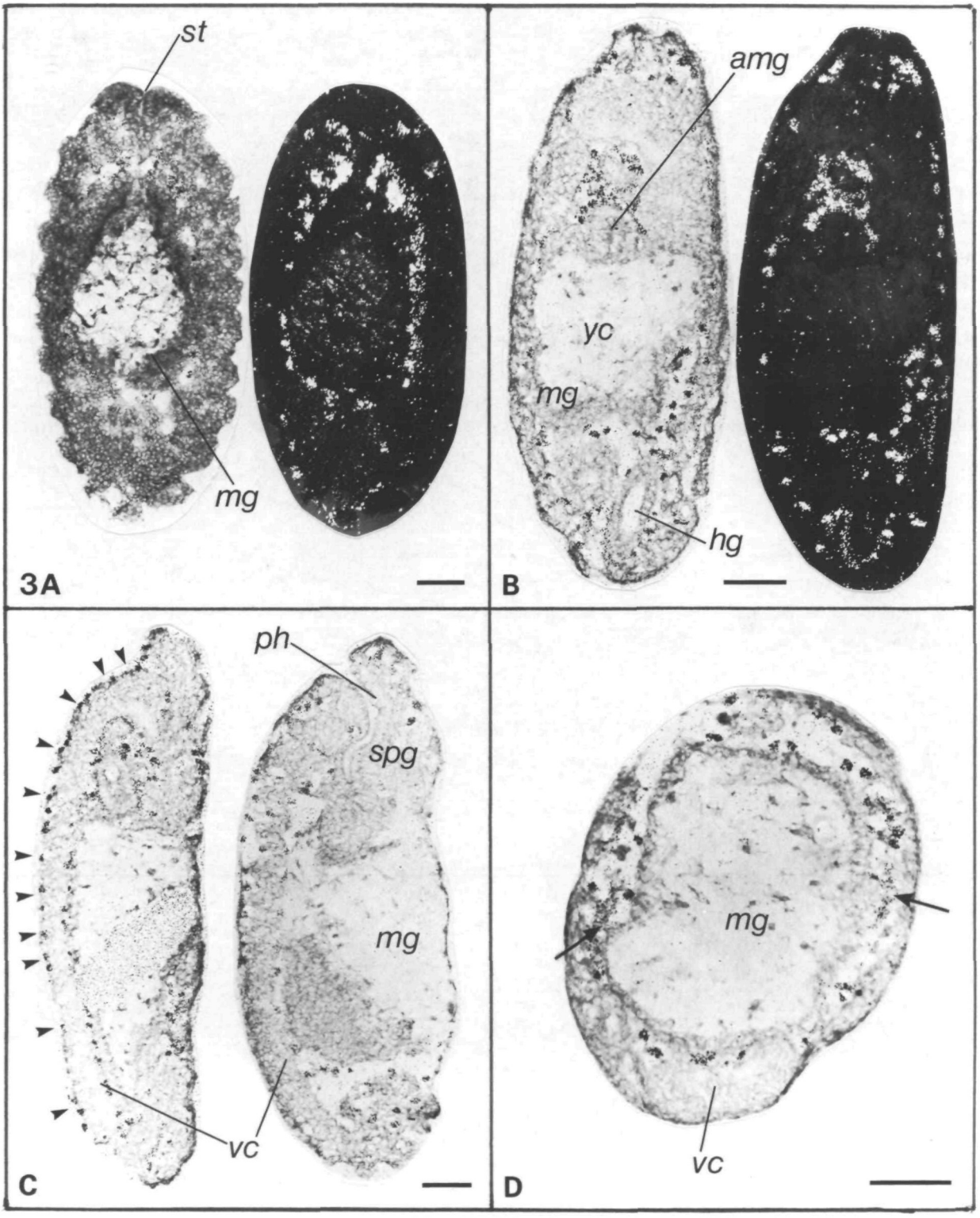

Fig. 3. Autoradiographs of 9–12 h-old embryos after *in situ* hybridization. The sections were probed with a *DCg1* DNA fragment labelled up to 3×10^8 disints min^{-1} μg^{-1} DNA. Exposure time was 15 days at 4°C. (A) Midhorizontal and (B) horizontal sections of stage-13 embryos with bright-field and dark-field illumination. The intense labelled spots were scattered around all the parts of the gut. (C) Parasaggital and saggital sections of stages 13 and 14 in which the segmented nature of the second set of labelled spots was well visible (arrowheads). (D) A transverse section of a stage-13 embryo. In addition to the intensely labelled spots, a slightly labelled lateral layer of cells was also observed (arrows). *Amg*, anterior midgut; *Hg*, hindgut; *Mg*, midgut; *ph*, pharynx; *spg*, supraoesophageal ganglion; *st*, stomodeum; *vc*, ventral nerve cord; *yc*, yolk cells. Bar, 50 μm.

On the autoradiographs corresponding to these stages, the signals previously detected in younger embryos are reproduced and amplified (Fig. 5); they confirm the mesodermal nature of the cells actively accumulating *DCg1* transcripts, i.e. (i) strongly labelled single cells of the visceral mesoderm, scattered over the entire embryo, surrounding all parts of the gut, the brain lobes and other organs (Fig. 5); (ii) intense spots on somatic mesoderm cells arranged regularly in each segment beneath the epidermis and also on each side of the ventral nerve cord (Fig. 5D), where they seem to be associated with each of the paired nerves innervating the corresponding segment of the body; (i) lastly, the very active fat bodies extending on each side of the embryo (Fig. 5B,C,F).

If we exclude the fat body cells, we can conclude that *DCg1* transcripts are specifically accumulated during *Drosophila* late embryogenesis by single circulating mesodermal cells. These cells do not overlap with the forming muscles and are still present even after muscle formation (stages 15, 16). This type of circulating mesodermal cell corresponds to the definition *lato sensu* of a haemocyte which, as mentioned by Jones (1962), 'is a mesodermal cell which sometimes during its life circulates in the haemolymph' and which originates from the midventral epineural sinus (Mori, 1979).

According to this definition the myocytes, too, might be considered as transformed haemocytes. However, the term 'haemocyte' is now mainly used to characterize circulating cells involved in phagocytosis (Crossley, 1975) so we will use the more general term of mesoblast to define all the cells belonging to the family of mesodermal circulating cells (J. A. Hoffmann, personal discussion).

Discussion

We have used a fragment of a DNA sequence encoding a type IV basement membrane collagen chain in *Drosophila* as probe in *in situ* hybridization experiments to examine directly the spatiotemporal expression of the corresponding *DCg1* gene during *Drosophila* embryogenesis. The main results of this investigation are (i) the *DCg1* gene only begins to be expressed at developmental stages 12, 13 (9·5 h-old embryos) and (ii) its transcription products are specifically accumulated in mesodermal derivatives, i.e. fat bodies and mesenchymal circulating cells which we call haemocytes or mesoblasts.

The absence of *DCg1* gene expression during early embryogenesis does not mean that there is no type IV

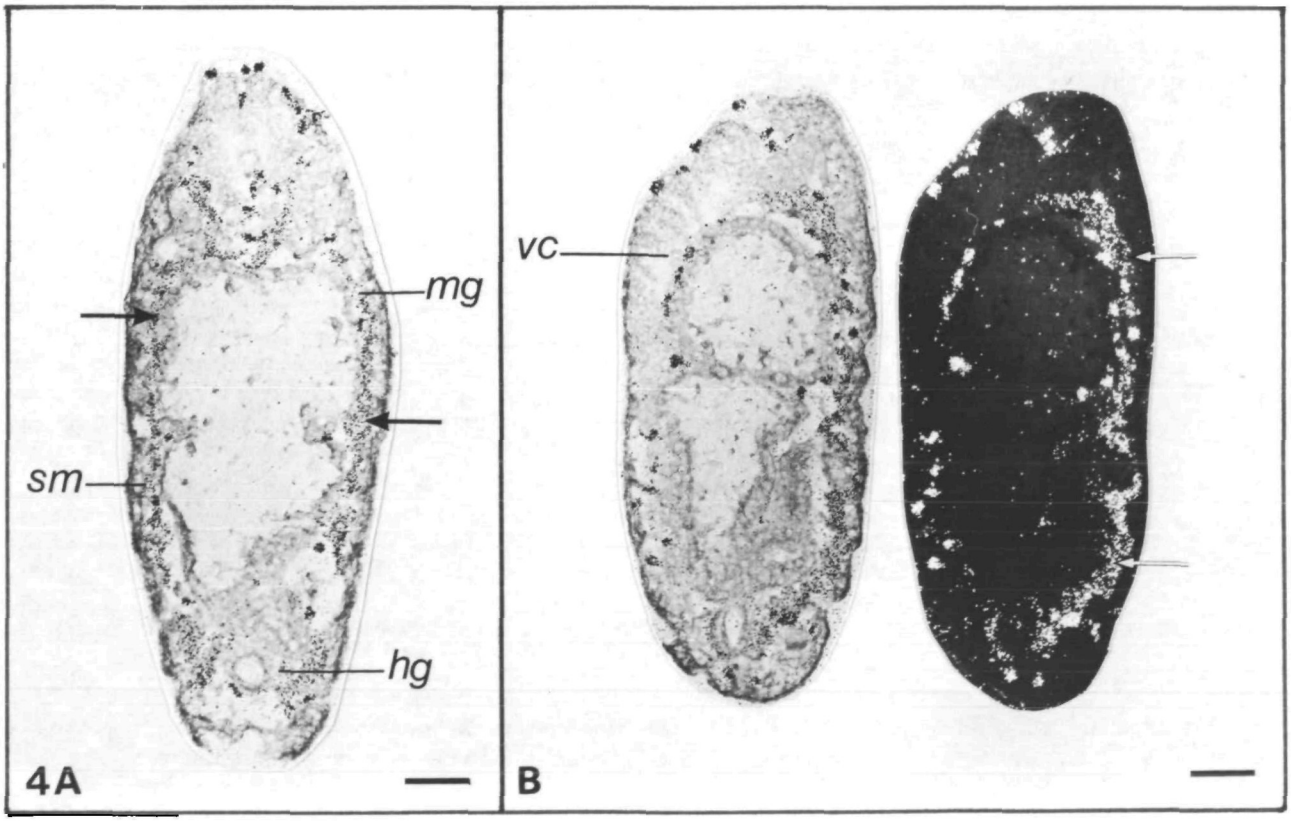

Fig. 4. Autoradiographs of 9–12 h-old embryo sections after *in situ* hybridization. In these frontal (A) and midtransverse (B) sections, a thin layer of labelled cells was observed (arrows) extending between the gut (*mg*) and the somatic musculature (*sm*). *hg*, hindgut; *vc*, ventral nerve cord. Bar, 50 μm.

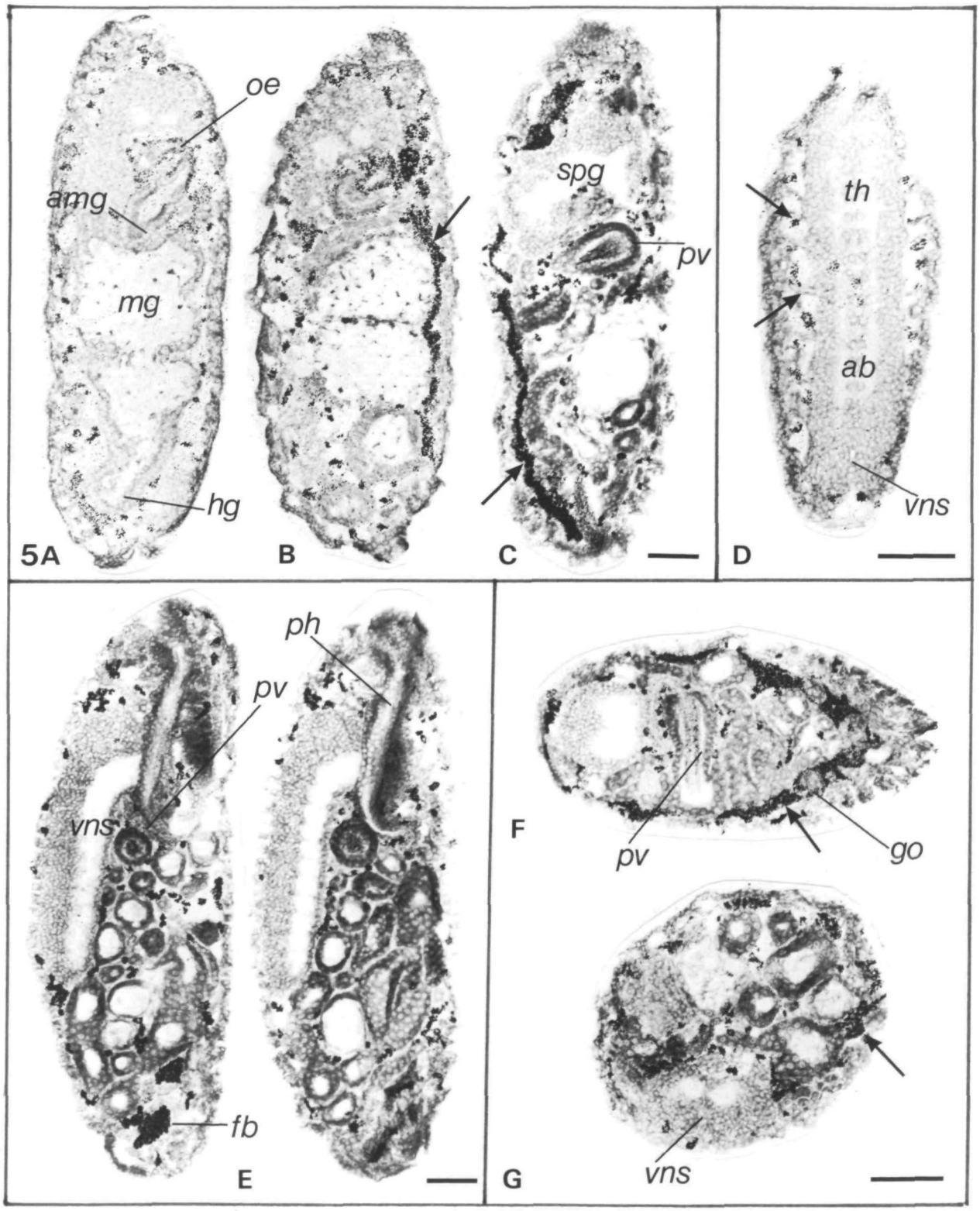

Fig. 5. Autoradiographs of 12–24 h-old embryo sections after *in situ* hybridization. (A–C) Midhorizontal sections of 12, 14 and 16 h-old embryos, respectively, in which the different sets of signal can be observed. The fat bodies (arrows) appear intensely labelled. (D) Horizontal section through the thoracic (th) and abdominal (ab) neuromeres. The arrows point to some segmental nerves with which intensely labelled cells are associated. (E) Two serial saggital sections of a 18 h-old embryo. (F,G) Transverse sections of older embryos showing the labelled spots and the fat bodies (fb) (arrows). *amg*, anterior midgut; *oe*, oesophagus; *hg*, hindgut; *mg*, midgut; *spg*, supraoesophageal ganglion; *pv*, proventriculus; *vns*, ventral nervous system; *ph*, pharynx; *go*, gonads. Bar, 50 μm.

collagen during these stages. Indeed, as in vertebrates, *Drosophila* type IV collagen molecules consist of triple-helical thread-like particles composed of homotrimers or heterotrimers of two different types of α chains (α1 (IV)) and α2 (IV)) (Bornstein & Sage, 1980; Timpl *et al.* 1981; Miller & Gay, 1982; Qian & Glanville, 1984; Fessler *et al.* 1984). Among the clones we have isolated (Le Parco *et al.* 1986*a*) one might correspond to the other type of α chain that could be involved in basement membrane composition during early embryonic stages. Work is in progress to check this hypothesis. Another possibility might be that type IV collagen has been stocked in the oocyte. The fact that follicular cells actively accumulate *DCg1* transcripts (Le Parco *et al.* 1986*b*) may favour this hypothesis. Work is in progress to obtain specific antibodies raised against a fusion protein containing a portion of the *DCg1* α IV chain.

Although it is currently admitted that basement membrane type IV collagen is deposited by the underlying epithelial cells (Hay, 1981) the mesodermal expression of *DCg1* gene during *Drosophila* late embryogenesis was not surprising since we have previously shown (Le Parco *et al.* 1986*b*; Knibiehler *et al.* 1987) that it is also differentially expressed during larval, pupal and adult stages in fat bodies, lymph glands and in circulating mesodermal cells. The mesodermal origin of these tissues and cells was recently confirmed by Lawrence & Johnston (1986) and Beer *et al.* (1987). The fat-body cells of all insects that have been examined are surrounded by a basement membrane (Ashhurst, 1979), which was assumed to be secreted by each cell. However, the insect fat bodies are responsible for a wide range of roles in the ontogeny of insects (Riski, 1978; Wyatt, 1980). These include the synthesis of the major haemolymph proteins. Thus, it may be possible, although we do not know the exact sites and the time of type IV collagen deposition, that fat bodies secrete into the haemolymph this component of basement membranes during the larval periods of growth.

The circulating mesoblasts are active in *DCg1* expression only during late embryogenesis and metamorphosis of *Drosophila*, both developmental stages in which specific morphogenetic processes occur. These 'fibroblastic-like' cells are intermingled with somatic and visceral mesoderm before muscle formation during stages 12–13 of embryogenesis, and, in addition, they surround the autolysing and reforming adult muscles during metamorphosis. These observations suggest that the type IV collagen that they synthesize may be incorporated into the forming basal lamina on the plasmalemma of the myotubes. This situation was recently described in vertebrate cell cultures in which fibroblasts were demonstrated to promote the formation of a continuous basal lamina by type IV collagen deposition during myogenesis (Sanderson *et al.* 1986; Kühl *et al.* 1984).

However, some mesoblasts, which are located between the nerve cord and the epidermis during late embryogenesis (Fig. 5D), may also be involved in the deposition of type IV collagen in an extracellular material necessary for later development of muscles and motoneurone growth cones. This was thought to be the case in grasshopper embryos in which it was suggested that the large mesodermal 'muscle-pioneers' erect such a scaffold (Ho *et al.* 1983). The absence of, or a delay in, the deposition of this extracellular material overlapping the period of the first muscular contractions was thought to explain the substantial disorders observed during embryonic development of the mutant lethal myospheroid l(1)mys described by Wright (1960).

We think that the results we report here agree well with this hypothesis and support the notion that the mesoblasts as 'fibroblastic cells' seem to be of crucial importance during developmentally critical stages in *Drosophila*.

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