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Comparative Analysis of Fibrillar and Basement Membrane Collagen Expression in Embryos of the Sea Urchin, *Strongylocentrotus purpuratus*

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ABSTRACT—The time of appearance and location of three distinct collagen gene transcripts termed 1α, 2α, and 3α, were monitored in the developing *S. purpuratus* embryo by *in situ* hybridization. The 1α and 2α transcripts of fibrillar collagens were detected simultaneously in the primary (PMC) and secondary (SMC) mesenchyme cells of the late gastrula stage and subsequently expressed in the spicules and gut associated cells of the pluteus stage. The 3α transcripts of the basement membrane collagen appeared earlier than 1α and 2α, and were first detected in the presumptive PMC at the vegetal plate of the late blastula stage. The PMC exhibited high expression of 3α at the mesenchyme blastula stage, but during gastrulation the level of expression was reduced differentially among the PMC. In the late gastrula and pluteus stages, both PMC and SMC expressed 3α mRNA, and thus at these stages all three collagen genes displayed an identical expression pattern by coincidence. This study thus provides the first survey of onset and localization of multiple collagen transcripts in a single sea urchin species.

INTRODUCTION

Collagen molecules are a diverse group of glycoproteins, which have in common characteristic triple helical domains and the ability to form a wide range of morphologically distinct supramolecular aggregates (van der Rest and Garrone, 1991). Collagens are an essential constituent of the extracellular matrix and are known to perform a variety of important functions in cell differentiation and morphogenesis (Hay, 1991). More than 19 different collagen types have been described in the vertebrate and a comparable heterogeneity is expected to exist in invertebrates (Weckmann and Cabral, 1996; van der Rest and Garrone, 1991).

Echinoid embryos have been a particularly useful model for understanding aspects of early development. During early sea urchin embryogenesis, collagens within the blastocoel play crucial roles. Metabolic inhibitors for these molecules result in the block of gastrulation and spiculogenesis (Mizoguchi and Yasumasu, 1983; Blankenship and Benson, 1984; Wessel and McClay, 1987; Wessel *et al.*, 1991). However, the number, identity and function of sea urchin collagens remain largely unknown.

The existence of multiple collagenous proteins has been implicated by immunoblotting and radiolabeling studies (Wessel *et al.*, 1984; Benson *et al.*, 1990; Nemer and Harlow, 1988; Saitta *et al.*, 1989; Tomita *et al.*, 1994). More recently, four different collagen chains (1α to 4α) have been identified in three sea urchin species, *Paracentrotus lividus*, *Strongylocentrotus purpuratus* and *Hemicentrotus pulcherrimus* (D'Alessio *et al.*, 1989, 1990; Exposito *et al.*, 1992a, 1992b, 1993, 1994; Tomita *et al.*, 1994; Venkatesan *et al.*, 1986; Wessel *et al.*, 1991). Two of the polypeptides (1α and 2α) belong to the fibrillar group of collagens, whereas the others (3α and 4α) display features of basement membrane collagens. Based on *in situ* hybridization, all of the transcripts so far analyzed have been expressed in the mesenchymal lineage (Angerer *et al.*, 1988; D'Alessio *et al.*, 1989, 1990; Wessel *et al.*, 1991). However, our descriptive knowledge has been limited because these data were gathered using different sea urchin species and only a few typical developmental stages were examined.

In the present study, we examined the expression pattern

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This study is dedicated to the memory of Dr. Michael Solursh. The authors also wish to appreciate late Dr. Katsuma Dan and his work.

of three of these collagen genes at various stages of developing *S. purpuratus* embryo. The two fibrillar collagen genes (1α and 2α) were first detected simultaneously during the late gastrula stage. We confirmed the earlier onset of transcription of the 3α collagen gene (Angerer *et al.*, 1988; Wessel *et al.*, 1991) and provide a more detailed analysis of its tissue distribution during gastrulation. Our results showed that both primary and secondary mesenchyme derivatives express all three collagen genes with a remarkably similar expression pattern.

MATERIALS AND METHODS

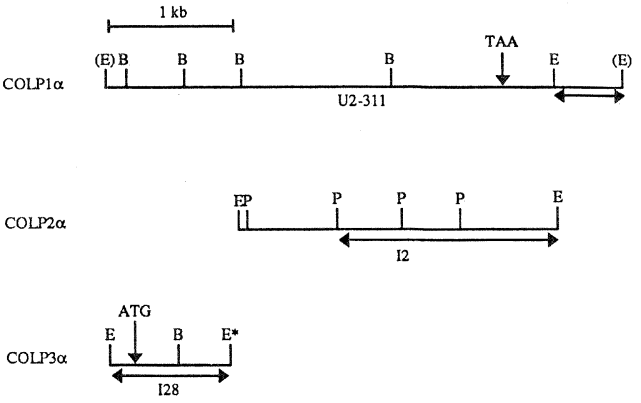
Northern blot and *in situ* hybridization

Fertilization and embryo culture of *Strongylocentrotus purpuratus* in artificial sea water were carried out as previously described (Lane and Solursh, 1991). Briefly, embryos at less than 2 ml greatly packed in 50 ml artificial sea water were cultured at 11°C in a plastic dish (Falcon 3025, 150 mm) with rotation (65 rpm). The culture medium was changed daily. For RNA blot hybridization, 1 μg of poly(A)$^+$ RNA from pluteus stage embryos was fractionated through a 1% agarose gel containing 2.2 M formaldehyde, transferred onto a nitrocellulose filter (Millipore), and hybridized sequentially with collagen probes as described (Saitta *et al.*, 1989). For *in situ* hybridization experiments, synchronously developing embryos were collected at different times, fixed in 1% glutaraldehyde (Angerer *et al.*, 1987), and processed for paraffin sections. The *in situ* hybridization protocol using ^{35}S-labeled sense and antisense RNA probes has been described (Suzuki *et al.*, 1991). Collagen probes used for all hybridization experiments (Fig. 1) were cDNA fragments corresponding to the 3' untranslated sequence (1α, 358 bp), coding region for the carboxy-propeptide (2α, 700 bp) or amino-terminal propeptide (3α, 940 bp) (Exposito *et al.*, 1992a, 1992b, 1993). These cDNA fragments were subcloned into the polylinker sequence of the transcription vector pT7/T3-19 (Life Technologies Inc., Gaithersburg, MD). Sense and antisense riboprobes were synthesized on 1 μg of the linearized template using either T7 or T3 RNA polymerase. Hybridization was carried out in 0.3 M NaCl, 50% formamide, 20 mM sodium acetate pH 5-6, 1 mM EDTA, 1 × Denhardt's, 250 μg/ml yeast tRNA, 10% dextran sulfate, 0.1 M dithiothreitol and 0.3 μg RNA probe/kb/ml at 50°C for 16 hr. Stringent washes were performed in 2 × SSC (1 × SSC; 0.15 M NaCl, 0.015 M sodium citrate), 50% formamide at 60°C for 1 hr, followed by 0.1 × SSC at 50°C for 15 min. For autoradiography, sections were dipped in Ilford K.5D emulsion and lightly stained with hematoxylin after development. The exposure time was 6 days for all three probes which resulted in signal levels at comparable range at late gastrula/pluteus stage, with longer probe (3α, 940 bp) producing more signal than shorter probes (2α, 700 bp or 1α, 358 bp). The mounted sections were photographed using phase-contrast and dark field microscopy.

RESULTS AND DISCUSSION

Three collagen genes of *S. purpuratus*, 1α, 2α and 3α, were analyzed in the present study. In order to assure specificity in the hybridization, divergent portion of each gene was used as a probe as shown without cross-hybridizing species in the northern blot hybridization (Fig 1). It should be noted that less-defined pattern of the 2α probe is due to alternative splicing of the primary transcript (Exposito *et al.*, 1992b).

The spatial and temporal expression patterns of collagen transcripts were analyzed by the *in situ* hybridization. Unlike



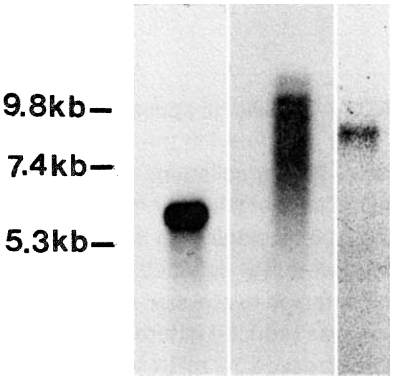


Fig. 1. (**Top**) Partial restriction maps of the collagen cDNA probes used in the hybridization experiments. The arrowed lines indicate the regions used as probes for the *in situ* hybridization. E = *Eco*RI, B = *Bam*HI, P = *Pvu*II, (E) = adapter, E* = linker. (**Bottom**) Northern blots of poly(A)$^+$ RNA from pluteus stage embryos probed with the 1α (left), 2α (middle), and 3α (right) probes, with molecular weight markers indicated on the left.

the *P. lividus* 1α gene (D'Alessio *et al.*, 1989), the transcripts of 1α collagen was not detected at the mesenchyme blastula stage (not shown) nor the early and mid gastrula stages (Fig. 2a, b). It was first detected at late gastrula/early prism stage (56 hr 30 min, Fig. 2c, d). Signal was seen in both primary mesenchyme cells (PMC) and secondary mesenchyme cells (SMC). In addition, signal was not uniform and some mesenchyme cells of either PMC or SMC did not express 1α message. The expression continued in the pluteus stage embryo (98 hr and 116 hr, Fig. 2e-h) where signal was localized in the skeletal mesenchymal cells and cells associated with the esophagus.

The expression pattern of the 2α collagen was identical to the 1α collagen. Though a weak signal was first detected at mid-gastrula stage (not shown), it was at late gastrula/early prism stage when PMC and SMC were labeled clearly (Fig. 3a-d). The 2α signal in the positive cells was also variable. The expression persisted in the pluteus stage where signal was seen in the skeletal mesenchymal cells and cells

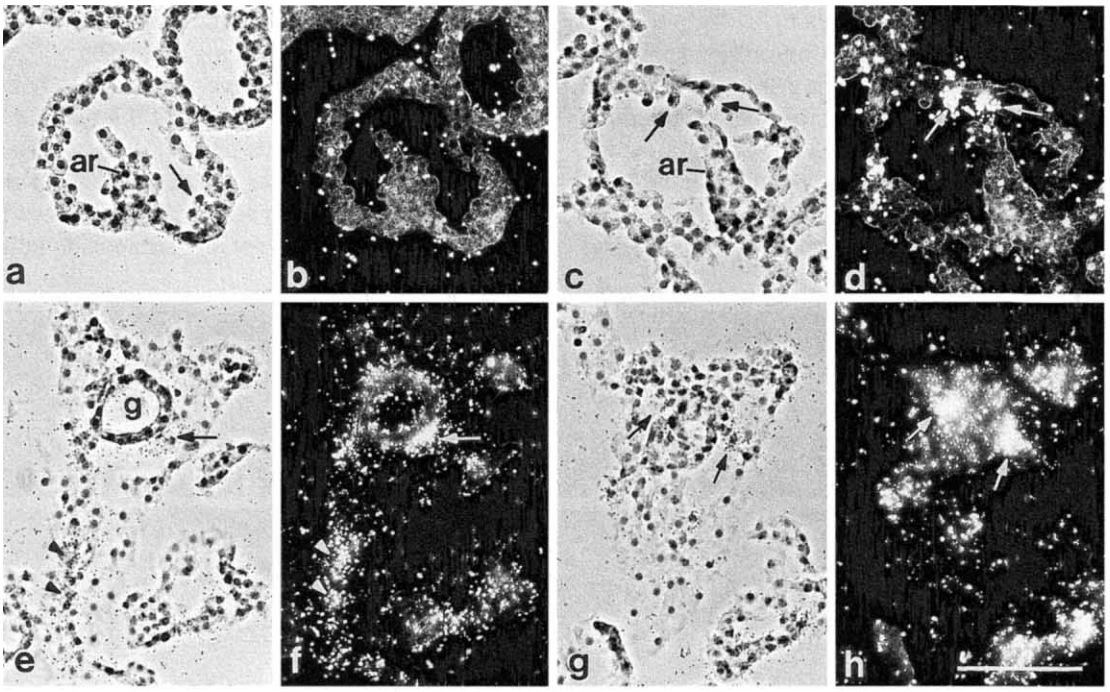


Fig. 2. *In situ* hybridization pattern of the 1α transcripts in various stages of *S. purpuratus* embryos using the 358 bp probe. (**a**, **b**) Phase-contrast and dark field view of the early gastrula stage embryo. The PMC (arrow) are negative. The conical archenteron (ar) and blastocoel wall are also negative. (**c**, **d**) Late gastrula/early prism stage embryo. Two of the secondary mesenchyme cells (arrows) near the animal pole region are clearly labeled. (**e**, **f**) Pluteus stage embryo. Signal can be seen in the skeletal mesenchymal cells (arrowhead) along the edge of the body rod. Note that the calcareous spicules had washed out during the procedure. Signal can also be seen in the cells (arrow) associated with the gut (g). (**g**, **h**) Pluteus stage embryo at a different angle. Two clumps of cells (arrows) associated with the esophagus are labeled. Bar = 50 μm. No distinctive signal was detected with the sense probe (data not shown).

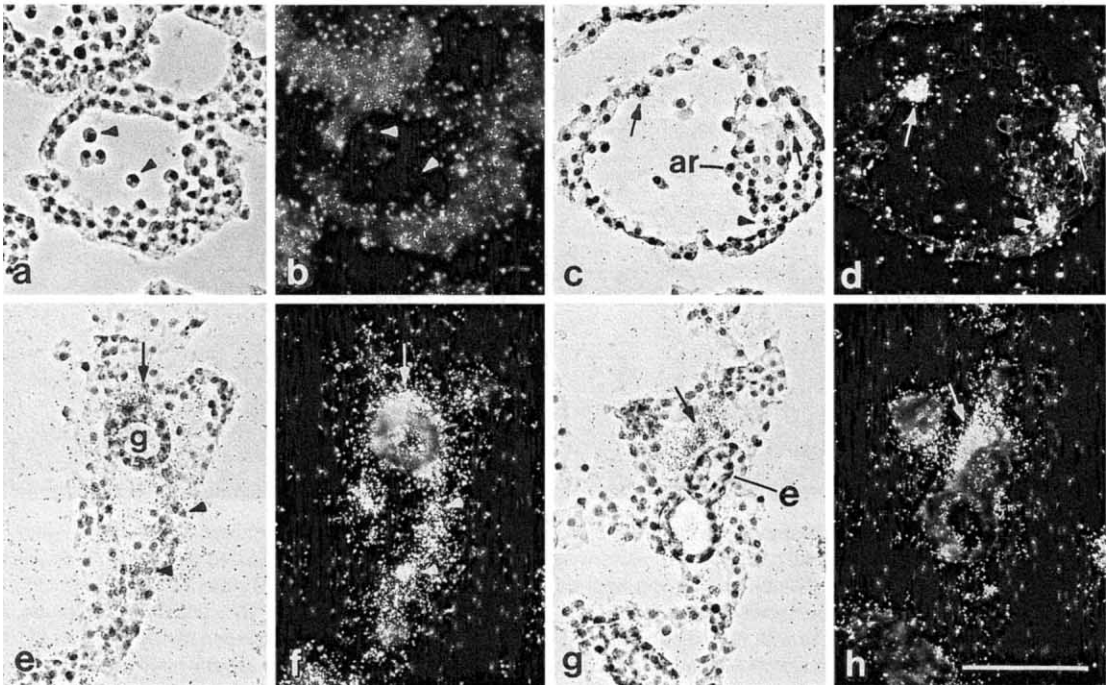


Fig. 3. Expression pattern of the 2α transcripts using the 700 bp probe. (**a**, **b**) Mesenchyme blastula stage embryo. PMC (arrowheads) and the blastocoel wall are negative. (**c**, **d**) Late gastrula/early prism stage embryo. Two SMC (arrows) and one PMC (arrow head) near the base of the archenteron (ar) are labeled. (**e**, **f**) Pluteus stage embryo. Signal can be seen in the skeletal mesenchymal cells (arrowheads) along the body rod as well as in the cells (arrow) associated with the gut (g). (**g**, **h**) Another section of the pluteus stage embryo showing signal (arrow) in the cells associated with the esophagus (e). Bar = 50 μm.

associated with the esophagus (Fig. 3e-h).

These two fibrillar collagens become expressed simultaneously at the late gastrula stage in the PMC and SMC lineages. This pattern of expression resembles our earlier reports on the Mediterranean species, *P. lividus*, where the *P. lividus* 1α and 2α transcripts were detected in the PMC and SMC at the late gastrula stage (D'Alessio *et al.*, 1989, 1990). Though we have suggested previously that the *P. lividus* 1α message appears first in the mesenchyme blastula stage (D'Alessio *et al.*, 1989), our reexamination of *P. lividus* slides confirmed that the actual level of signal was not significantly different from the background at the stage and thus the statement incorrect. In the present study, we established the onset of 1α and 2α genes and their continued expression in the pluteus stage, which have not been achieved previously. In *P. lividus*, similar onset and expression pattern can be

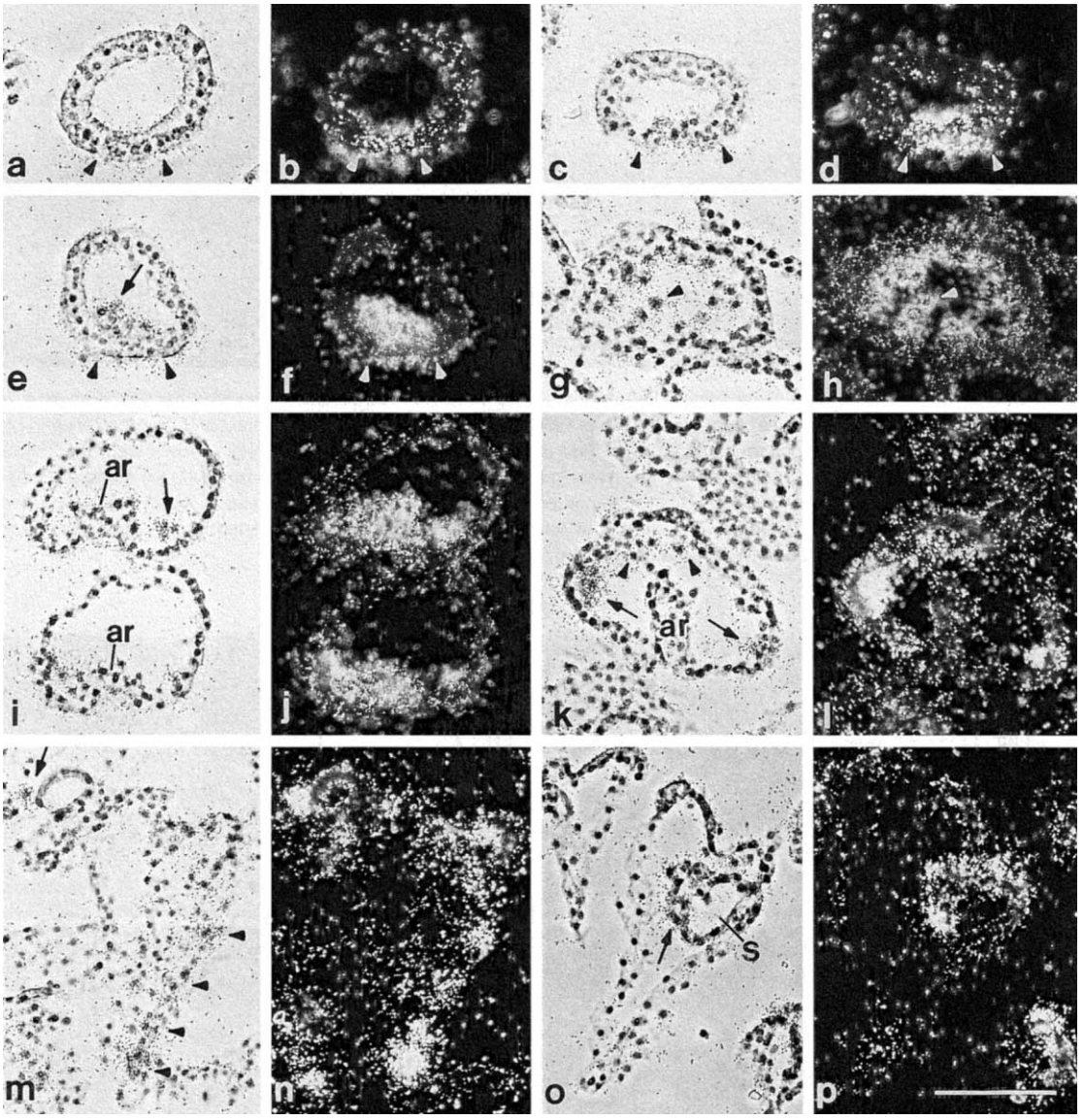


Fig. 4. Expression pattern of the 3α transcripts using the 940 bp probe. (**a**, **b**) Unhatched blastula embryo (20 hr 40 min after fertilization at 11°C). Weak signal can be seen in the vegetal plate (between arrowheads). At this time, the group consisted of mostly unhatched embryos and 5-10% hatched embryos, with fertilization membranes having washed off during the procedure. (**c**, **d**) Hatched blastula (24 hr). Signal in the vegetal plate increased significantly (between arrowheads). The group consisted of 90% hatched and 10% unhatched embryos, with the inner surface of the blastocoel wall being smooth with no indication of ingressing cells. (**e**, **f**) Very early mesenchyme blastula (27 hr). PMC are beginning to ingress into the blastocoel and clearly labeled (arrow). Residual signal can be seen in the vegetal plate (between arrowheads). (**g**, **h**) Mesenchyme blastula (33 hr). PMC were non-motile in the previous stages (27-30 hr), but have become migratory with strong signal (arrowhead, cf. with Fig. 3 a, b). (**i**, **j**) Early gastrula (43 hr). PMC (arrow) are clearly labeled, while the archenteron (ar) seems also positive in two embryos. (**k**, **l**) Late gastrula/early prism (56 hr 30 min). PMC (arrows) and SMC (between arrowheads) are labeled. (**m**, **n**) Pluteus stage embryo (98 hr). Signal can be seen along the body rod (arrowheads) and also in the cells associated with the gut of another embryo (arrow). (**o**, **p**) Oblique section of pluteus stage embryo showing signal (arrow) in the cells associated with esophagus and the upper part of the stomach (s). Bar = 50 μm.

expected for 1α and 2α genes, while the SMC of *P. lividus* gave more pronounced signal as compared to *S. purpuratus* at the late gastrula stage.

3α expression was in the mesenchyme lineage as the fibrillar collagens but displayed an earlier pattern as previously reported (Angerer *et al.*, 1988; Wessel *et al.*, 1991). Initially, transcript was detected weakly in the vegetal plate prior to hatching (Fig. 4a, b). Subsequently, 3α signal was seen clearly in the forming PMC (Fig. 4c-f). As these cells ingress from the blastula wall and migrate into the blastocoel, the vegetal plate became negative and the PMC exhibited high and homogeneous expression of 3α (Fig. 4g, h). As invagination takes place, signal was detected in the PMC and also in the SMC by the late gastrula stage (Fig. 4i-l). In addition, the level of expression decreased as compared from the mesenchyme blastula stage, and the 3α signal in the PMC or SMC was variable as described for 1α and 2α chains. In the pluteus stage, signal was localized in the skeletal mesenchymal cells and cells associated with esophagus (Fig. 4m-p).

All of the three collagen transcripts were localized in the mesenchyme lineage with a remarkably similar expression pattern. Primary functions of these collagens may be for formation and maintenance of spicules by the PMC (Okazaki, 1975; Blankenship and Benson, 1984; Benson *et al.*, 1990; Wessel and McClay, 1987; Wessel *et al.*, 1991) and the blastocoel microenvironment by the PMC and SMC (Tamboline and Burke, 1992). Though these genes share an identical expression pattern during late gastrula and pluteus stages, each collagen chain may be regulated independently. Fibrillar collagens of 1α and 2α, and the basement membrane 3α chain are the homologues of the vertebrate type I, type II and type IV collagens, respectively, and these chains may not be assembled into the same collagen trimer (Exposito *et al.*, 1992a, 1992b, 1993). In addition, the expression pattern of these collagens, especially of 3α, resembles that of another PMC-specific gene product, the cell surface glycoprotein msp130 (Harkey *et al.*, 1992), wherein high and homogeneous expression of msp130 at the mesenchyme blastula stage is followed by heterogeneous down regulation at the late gastrula stage.

All collagen genes were expressed by the PMC and SMC which derive from large micromeres and macromeres, respectively (Endo, 1966; Cameron *et al.*, 1991; Tamboline and Burke, 1992). There are two additional cell types in the mesenchyme lineage, the pigment and muscle cells (Gibson and Burke, 1985; Ishimoda-Takagi *et al.*, 1984; Burke and Alvarez, 1988). It is likely that the muscle cells, i.e., those constitute coelomic sacs and surround the esophagus, produce 1α and 2α (this study) and 3α collagens (Angerer *et al.*, 1988; this study), though rigorous identification is necessary among muscle cells, coelomic sacs and the blastocoelar SMC nearby (Pehrson and Cohen, 1986; Tanaka and Dan, 1990; Tamboline and Burke, 1992). Similarly, the possibility of pigment cells among the collagen-producing cells (e.g. Fig. 4i, j) remains to be demonstrated.

This study provides the first survey comparing the expression of fibrillar and basement membrane collagen genes in the sea urchin embryo. The data indicates the basic patterns of collagen gene expression, the production of basement membranes before fibrillar networks, and the restriction of gene expression to mesenchyme cells. The primary mesenchyme cell lineage has attracted considerable interest because these cells differentiate autonomously into skeletogenic cells, and can be used as a model system for studying cell commitment (Okazaki, 1975). In addition to the collagens, a number of other mesenchyme specific gene products have been described (Wessel and McClay, 1985; Benson *et al.*, 1987; Livingston *et al.*, 1991; Leaf *et al.*, 1987; Harkey *et al.*, 1992). Future studies using these well-characterized reagents should provide knowledge about molecular mechanisms which lead to cell commitment and determination in this unique biological system.

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