BIOCHEMICAL AND BIOPHYSICAL RESEARCH COMMUNICATIONS **238,** 334–337 (1997)
ARTICLE NO. RC977288

Spatial Distribution of Collagen Type I mRNA in *Paracentrotus lividus* Eggs and Embryos

Roberto Gambino,[1] Daniele P. Romancino, Melchiorre Cervello, Aiti Vizzini, Maria Gabriella Isola, Lucrezia Virruso, and Marta Di Carlo
Istituto di Biologia dello Sviluppo, C.N.R., Via Ugo La Malfa 153, 90144 Palermo, Italy

Received August 4, 1997

We have identified the presence of type I collagen (COLL1α) mRNA in *Paracentrotus lividus* unfertilized egg, indicating a maternal origin of this mRNA. By in situ whole mount hybridization the spatial distribution of COLL1α mRNA in egg and embryo at different developmental stages was established. Moreover, the presence of COLL1α gene in *Paracentrotus lividus* genome was analyzed by Southern blot experiments. The localization pattern indicates that the maternal mRNA is placed in the fertilized egg in a fixed position, relative to the embryonic axes. Furthermore, the embryonic expression is spatially restricted during development, suggesting involvement in sea urchin embryo cell specification events. The presence of two bands in Southern blot hybridization may indicate that two genes specific for COLL1α are present in the sea urchin genome.

The animal-vegetal axis is already established in sea urchin unfertilized egg (1). Maternal cytoplasmic molecules, such as mRNAs and proteins, are localized in a particular region of the egg, and are asymmetrically distributed during embryonic cleavage (2, 3). This may be considered to be the mechanism by which initial assignment of different fates is established in the early embryo. The identification of molecules that may play a role in early determinative events is important for understanding the mechanism that determines egg polarity. In other systems, such as Drosophila and Xenopus, several mRNAs relevant to axis determination have been identified (4, 5). Although the sea urchin was one of the first organisms regarding which the problem of axis determination was raised, it is only recently that a few examples of maternal localized mRNAs have been described in the literature. In particular, three maternal mRNAs, called *bep1*, *bep3* and *bep4*, coding for cell surface proteins, have been found localized in the animal part of *Paracentrotus lividus* (*P. lividus*) eggs and embryos (6, 7). Furthermore, another maternal mRNA, referred to as SpCOUP-TF, transcribing for a transcription factor, is placed in the egg in a fixed position, at 45° to the oral-aboral axis (8).

Signals transduced by cell-cell and cell-extracellular matrix interactions are certainly involved in cell fate determination. Among molecules present in the extracellular matrix collagens are of fundamental importance. They have a major role in determination of temporal-spatial differences of the extracellular matrix. In sea urchin there have been isolated and sequenced four cDNAs coding for collagens: two for fibrillar collagens, called COLL1α and COLL2α (9), and two for non-fibrillar collagens, called COLP3α and COLP4α (10, 11).

In this report we demonstrate that the COLL1α mRNA, coding for a fibrillar-forming collagen, of the sea urchin *P. lividus*, is already present in the unfertilized egg and is spatially restricted in the fertilized egg and during embriogenesis.

MATERIALS AND METHODS

RNA extraction and Northern blot hybridization. RNA was extracted from eggs and embryos following the protocol published by Spoerel et al. (1986) (12). Poly(A)* RNA was isolated from total RNA by binding to oligo d(T)-cellulose (13). 10 μg of egg poly(A)$^+$ RNA and 10 μg of total prism RNA were separated on denaturing agarose gel, and after transfer to a nylon membrane (Hybond N, Amersham), hybridized with the *labeled Bgl*II-*Eco*RI fragment of COLL1α (9), utilizing standard procedures (13).

In situ identification of collagen mRNA. The fragment BglII-*Eco*RI of COLL1α (9) was subcloned in the same restriction sites of the bluescript vector. This construction was linearized at the *Bgl*II site and transcribed by utilizing T7 polymerase, according to the instructions of the manufacturer of the digoxygenein (DIG)-RNA labeling kit (Boehringer, Germany). After transcription the ribo-probe was nicked by digestion at 70°C in 40 mM sodium bicarbonate/60 mM sodium carbonate to obtain fragments in the range 150-250 bp. Whole mount in situ hybridization of the probe utilized (4 ng/ml) was carried out according to Montana et al. 1996 (7).

[1] To whom correspondence should be addressed at Istituto di Biologia dello Sviluppo, Consiglio Nazionale delle Ricerche, via Ugo La Malfa 153, 90144 Palermo, Italy. Fax: ++ 39-91-6809-548.

0006-291X/97 $25.00

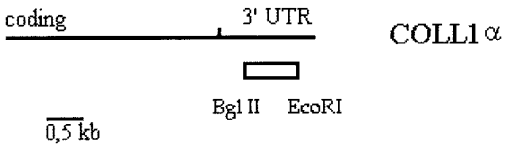

FIG. 1. *Schematic representation of COLL1α cDNA clone.* The coding region and the 3′ untranslated region are indicated. The box corresponds to the fragment obtained after digestion with *Bgl*II and *Eco*RI utilized as probes for Northern and Southern blot and for in vitro transcription.

DNA extraction and Southern blot. High molecular weight genomic DNA was prepared from sperm of individual *P. lividus* adult as described by Kedes et al. (1975) (14). For DNA blotting 10 μg of DNA were completely digested with *Eco*RI or *Hin*dIII restriction enzymes, fractionated on 1% agarose gel, and transferred to nylon membrane (Hybond N, Amersham). The Southern blot was hybridized with the ^{32}P-labeled *Bgl*II-*Eco*RI fragment of collagen utilizing standard procedures (13).

RESULTS

Expression of the Collagen Type I in P. lividus Eggs

In previous paper, by Northern blot analysis we detected a faint signal for different collagen mRNAs in sea urchin eggs utilizing as a probe a fragment containing the conserved region of the collagen genes (9). Here, to obtain a specific signal only for the type I collagen mRNA present in P. lividus egg, we used the 750 bp fragment, containing part of the 3′ untranslated region (UTR), obtained after digestion of the COLL1α cDNA clone with *Bgl*II-*Eco*RI (Fig. 1) (9). After labeling, this fragment was utilized in Northern blot experiments, using a filter containing an overloaded amount of poly(A)$^+$ RNA from eggs and total RNA from prism. The autoradiogram shown in Fig. 2 reveals the presence of a unique transcript of about 6 kb, demonstrating that this type of collagen is already present in the egg.

Spatial Expression of Type I Collagen mRNA

In order to investigate the possibility of a different territorial distribution of type I collagen mRNA within the egg and during development, we prepared an antisense RNA labeled by incorporation of DIG, utilizing as a probe the *Bgl*II-*Eco*RI fragment and employing it for whole mount in situ hybridization of *P. lividus* eggs and embryos. A gradient of staining, in about three fourths of the unfertilized egg was observed (data not shown), whereas following fertilization a region of the egg, close to the cortex, was intensively stained (Fig. 3A). After the second cleavage the highest collagen mRNA concentration was found in two of the four blastomeres (Fig. 3B). Three cell divisions later, at the 16-cell stage, the collagen mRNA was detected in two of the eight mesomeres, in two of the four macromeres and in two of the four micromeres (Fig. 3C). At the blastula stage (Fig. 3D) the collagen mRNA was present in some primary mesenchyme cells, staining was absent in the cells that were to give origin to pigmented cells, and in the cells derived from the two unstained micromeres. At the gastrula stage (Fig. 3E), the staining was present on one side of the ectodermal cells, and in some primary mesenchyme cells especially on one side of the archenteron. However, at this stage it is possible to assume that the detected transcript includes newly synthesized embryonic mRNA and preexisting maternal mRNA. At the pluteus stage (Fig. 3F) the most intense signal was in the oral arms and in one of the anal arms, whereas the other cells showed little or no staining. No staining was detected when sense mRNA was utilized as a negative control (data not shown).

Southern Blot Analysis of COLL1α Gene

In order to determine the number of COLL1α genes present in *P. lividus* genome, we digested *P. lividus* DNA with *Eco*RI and *Hin*dIII restriction enzymes. After electrophoresis the Southern blot was hybridized with the labeled *Bgl*II-*Eco*RI fragment, containing the 3′ UTR. Figure 4 shows two hybridization bands, with small differences in the molecular weight in both lanes, suggesting the presence of two genes. However, we cannot role out the possibility that there is only a single copy of the coding gene, and the two bands may be due to the presence of a pseudogene with a different restriction site.

DISCUSSION

The results reported in this paper clearly prove that the COLL1α mRNA is already present in the egg and is localized at a fixed position relative to the two embryonic axes. Collagen molecules are composed by a highly conserved Gly-Xaa-Yaa repeated sequences (15) and it is difficult, using as a probe a cDNA coding for this conserved region, to discriminate which type of collagen is expressed at a particular moment of development and in which part of the embryo. Moreover, this

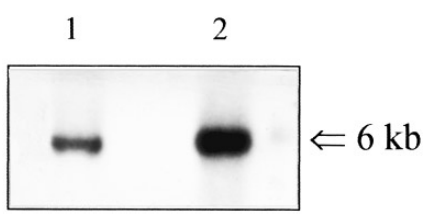

FIG. 2. *Northern blot analysis of COLL1α m RNA expression.* Poly(A)$^+$ RNA extracted from eggs (1) and total RNA extracted from prism stage (2) hybridized with labeled *Bgl*II-*Eco*RI fragment of COLL1α cDNA clone.

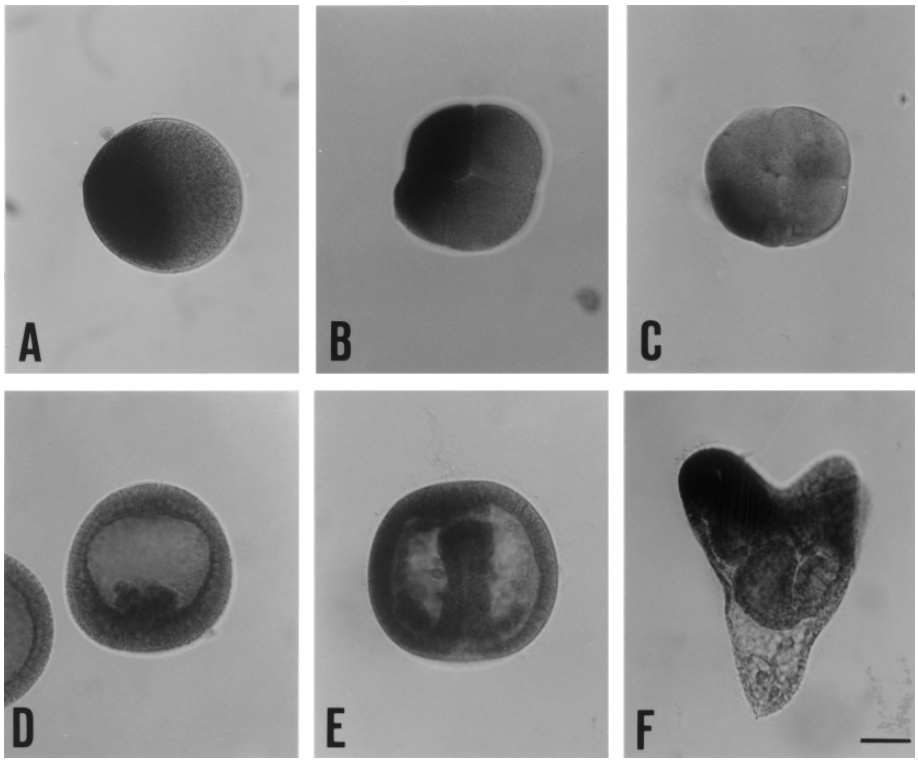

FIG. 3. *Localization of COLL1α mRNA.* Three-dimensional images of whole mount in situ hybridization with single-strand COLL1α probe of fertilized egg (A), 4-cell stage (B), 16-cell stage (C), mesenchyme blastula (D), gastrula (E) and pluteus (F). Bar, 30 μm.

problem is greater when after the blastula stage, for gastrulation and spiculogenesis processes, an increase in transcription of all collagen genes occurs. Here, we utilized the specific 3′ UTR region of COLL1α mRNA to discriminate a different expression of the collagen genes, and to understand why different type of collagens are present in *P. lividus* cells. In Northern blot experiments, utilizing a high amount of poly(A)$^+$, we detected the presence of a 6 kb band corresponding to COLL1α mRNA in *P. lividus* eggs.

Moreover, the presence of the COLL1α mRNA in the egg was confirmed by whole mount in situ hybridization: in *P. lividus* egg we detected the presence of collagen transcript localized in a particular region. During the cleavage stages this transcript is asymmetrically distributed, maintaining the staining at a fixed position relative to the two embryonic axes. At the blastula stage the mRNA is present only in the primary mesenchyme cells. At gastrula stage, the collagen mRNA is present in the ectodermal cells on one side of the blastocoel wall, the presumptive oral side as suggested by observing the prism stage, not shown here, and in some of the primary mesenchyme cells. Finally, at the pluteus stage, we detected staining only in the oral arms and in one of the anal arms. This is the first example of differential localization of RNA coding for a structural protein. This collagen localization on one side of the egg, which proves to be the oral side at the late stage, may suggest that this collagen is somehow involved in the determination of the oral-aboral axis. Moreover, this particular localization strongly suggests that this type of collagen may play a role in determining the diversity of the extracellular matrix, and consequently, in influencing the morphogenetic program and cellular activities. Thus we cannot role out the possibility that this type of collagen is relevant to initiating signal transduction events important for the differentiation of the cells with which it becomes in contact. Recently, it has been found that a cDNA for a β integrin is spatially distributed in *Strongylocentrotus purpuratus*

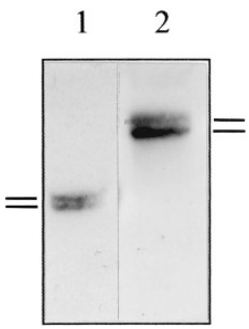

FIG. 4. *Southern blot analysis. P. lividus* DNA digested with *Eco*RI (1) and *Hin*dIII (2) restriction enzymes and hybridized with labeled *Bgl*II-*Eco*RI fragment.

eggs and embryos, and the authors suggested that probably this integrin is a receptor for a particular type of collagen (16). Moreover, it will be of interest to understand the mechanism that allows the COLL1α mRNA to be localized. This mechanism has principally been studied in Drosophila and Xenopus and it has been found that in this process the 3′ UTR of the mRNA studied and some proteic factors are involved (17). Recently, a protein of 54 kDa involved in the localization of the maternal bep mRNA in *P. lividus* has been identified (18). It will be of interest to know whether the same protein or others are involved in the spatial distribution of COLL1α mRNA.

The 3′ UTR fragment of COLL1α mRNA has also been employed to investigate the number of COLL1α genes present in the *P. lividus* genome. The presence of a double band, as discussed above, does not explain whether there are one or two COLL1α genes. However, in the case of the presence of two genes, the different rate of transcription, observed during oogenesis and the gastrula embryonal stage, may be explained by different gene organization, whereas, in the case of the presence of only one coding gene, the different rate of transcription may be due to the presence of different transcription factors. Experiments have already been planned to clarify this point.

ACKNOWLEDGMENTS

We are deeply indebted to Professor G. Giudice for his continual encouragement and for critically reading the manuscript.

REFERENCES

1. Horstadius, S. (1935) *Pubbl. Stn. Zool. Napoli* **14,** 251–479.
2. Wilson, E. B. (1925) The Cell in Development and Heredity. McWilliam, New York.
3. Davidson, E. H. (1986) Gene Activity in Early Development. Academic Press, Orlando, FL.
4. St. Johnston, D., and Nusslein-Volhard, C. (1992) *Cell* **68,** 201–211.
5. Melton, D. A. (1987) *Nature London* **328,** 80–82.
6. Di Carlo, M., Romancino, D. P., Montana, G., and Ghersi, G. (1994) *Proc. Natl. Acad. Sci. USA* **91,** 5622–5626.
7. Montana, G., Romancino, D. P., and Di Carlo, M. (1996) *Mol. Rep. Dev.* **44,** 36–39.
8. Vlahou, A., Gonzalez-Rimbau, M., and Flytzanis, C. N. (1996) *Development* **122,** 521–526.
9. D'Alessio, M., Ramirez, F., Suzuki, H. R., Solursh, M., and Gambino, R. (1989) *Proc. Natl. Acad. Sci. USA* **86,** 9303–9307.
10. Esposito, J. Y., D'Alessio, M., Di Liberto, M., and Ramirez, F. (1993) *J. Biol. Chem.* **268,** 5249–5254.
11. Esposito, J. Y., Suzuki, H., Geourijon, C., Garrone, R., Solursh, M., and Ramirez, F. (1994) *J. Biol. Chem.* **269,** 13067–13171.
12. Spoerel, N., Nguyen, H. T., and Kafatos, F. C. (1986) *J. Mol. Biol.* **90,** 29–35.
13. Maniatis, T., Fritsh, E. F., and Sambroo, K. J. (1982) *in* Molecular Cloning: Laboratory Manual, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.
14. Kedes, L. H., Cohn, R. M., Lowry, J. C., Chang, A. C. Y., and Cohen, S. N. (1975) *Cell* **6,** 359–369.
15. Baccetti, B. (1985) *in* Biology of Invertebrate and Lower Vertebrata Collagens. (Bairati, A., and Garrone, R., Eds.), Plenum, New York.
16. Marsden, M., and Burke, R. D. (1997) *Develop. Biol.* **181,** 234–245.
17. St. Johnston, D. (1995) *Cell* **81,** 161–170.
18. Montana, G., Bonura, A., Romancino, D. P., Sbisà, E., and Di Carlo, M. (1997) *Europ. J. Biochem.*, in press.