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Source: Zoological Science, 16(3) : 445-451

Published By: Zoological Society of Japan

URL: https://doi.org/10.2108/zsj.16.445

ZOOLOGICAL SCIENCE **16**: 445–451 (1999)

Asymmetrical Distribution of Mitochondrial rRNA into Small Micromeres of Sea Urchin Embryos

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ABSTRACT—Blastomeres of the 16-cell stage embryos of the sea urchin, *Hemicentrotus pulcherrimus*, were separated by an elutriator. By differential display, several RNA species that are enriched in micromeres are detected and their cDNA was cloned. One of the cloned cDNA encodes mt 12S rRNA. cDNA for mt 16S rRNA was also cloned from the cDNA library of unfertilized eggs. Two mt rRNAs contain poly(A) tails in their 3' ends. Both mt rRNAs distribute asymmetrically along a vegetal-animal axis of the 16-cell embryos and are enriched in micromeres, and this is also confirmed by whole mount *in situ* hybridization as well as electron microscopic *in situ* hybridization. As development proceeds, these mt rRNAs become more enriched in small micromeres. Results of electron microscopical *in situ* hybridization reveal both mt rRNAs localize extramitochondrially. Though at present we have no evidence on the role of the extramitochondrial mt rRNAs in sea urchin development, it is speculated considering roles of extramitochondrial mt 16S rRNA in *Drosophila* development that extramitochondrial mt rRNA may be implicated in development of sea urchin embryos.

INTRODUCTION

In a variety of animal groups, molecules localized asymmetrically in egg cytoplasm play an important role in the specification of topologically-restricted cell lineages (Davidson, 1986; Gilbert, 1997). The sea urchin embryo offers a relatively simple model system to study early cell type specification. It was revealed more than a decade ago that the early cleavage planes of sea urchin embryo are invariant with respect to the animal-vegetal axis (Dan, 1979; Dan *et al.*, 1983; Dan, 1987). The subsets of blastomeres aligned perpendicular to the animal-vegetal axis of early embryos acquire distinct cell fates. It has been proposed that molecules distributing in a gradient along animal-vegetal axis provide positional information to define micromeres, and the cell type specification in the entire embryo occurs through cell-cell interaction initiated by the micromeres (Boveri, 1901; Runnström, 1928 and 1975; Hörstadius, 1928 and 1973; Davidson, 1989). To assess how the animal-vegetal polarity is established during embryogenesis, we have focused our study on the molecules asymmetrically localized along the animal-vegetal axis.

It has been reported in the two phylogenetically distinct animals, *Drosophila* and *Xenopus*, that mitochondrial (mt) 16S rRNA is localized in a histologically distinct region of egg cytoplasm, called germ plasm, which contains factors required for germ line establishment (Kobayashi *et al.*, 1994; Kobayashi *et al.*, 1998; Illmensee and Mahowald, 1974, 1976; Ikenishi *et al.*, 1986; Togashi *et al.*, 1986; Smith, 1966; Okada *et al.*, 1974). Indeed, mt 16S rRNA is able to induce germ line progenitors in the UV-irradiated *Drosophila* embryos (Kobayashi and Okada, 1989), and is implicated in pole cell formation (Iida *et al.*, 1998). Ultrastructural studies in *Drosophila* reveals that both mt 16S and mt 12S rRNA are present outside mitochondria in germ plasm during the cleavage stage, and are localized in the distinctive organelles in germ plasm, or in germinal granules (Kobayashi *et al.*, 1993; Amikura *et al.*, 1996; Kashikawa *et al.*, in preparation). Similarly, in *Xenopus* embryos, extramitochondrial mt 16S rRNA is enriched in the germinal granules during discrete stages from 4-cell to blastula stages (Kobayashi *et al.*, 1994; Kobayashi *et al.*, 1998). Here we report that in sea urchin embryos mt 12S and 16S rRNA localize outside mitochondria and distribute asymmetrically among blastomeres along a vegetal-animal axis. As the development proceeds extramitochondrial mt rRNAs become

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enriched in micromeres, and by the 60-cell stage distribution of these RNAs becomes restricted to small micromeres. The present study provides the first report showing that mt rRNAs, germ plasm components in *Drosophila* and in *Xenopus*, are also present in echinoderm embryos and are partitioned into discrete blastomeres.

MATERIALS AND METHODS

Dissociation of embryo into blastomeres

Gametes of the sea urchin, *Hemicentrotus pulcherrimus*, were shed by the conventional KCl procedure. Eggs were fertilized and developed in artificial seawater (ASW; Jamarin U, Jamarin laboratory, Japan) at 15°C until the 16-cell stage. The fertilization membrane was removed using 5 mM *p*-aminobenzoic acid (PABA, Nasir *et al.*, 1992). Four-cell embryos were transferred to CFSW (Ca-free seawater) just before the 16-cell stage, then they were transferred to CMFSW (Ca, Mg-free seawater) at 16-cell stage, and blastomeres were dissociated by gently shaking.

Elutriation

Size-dependent separation of blastomeres were performed by the centrifugal elutriation according to Nasir *et al.* (1992) with a slight modification. Segregated blastomeres were fixed with ethanol and stored at 4°C. Fixed embryos were resuspended in autoclaved CMFSW and centrifuged in an elutriator rotor (Beckman JE-5.0 elutriation system standard chamber, Beckman, Tokyo) at 4°C with a flow rate of 24 ml/min at 2,000 rpm. Homogeneity of each blastomere fraction separated by elutriator was about 100%, 100%, and 84% for micromeres, macromeres, and mesomeres, respectively (Fig. 1).

RNA differential display

Differential display was carried out using RNAmap™ (GenHunter corporation, MA) according to the manufacturer's instruction. RNA were extracted from stored blastomeres with ISOGEN (Nippon gene, Japan), digested with DNaseI (Boehringer Mannheim, Germany) for 15 min at 37°C, and extracted by phenol/chloroform followed by ethanol precipitation. cDNA was synthesized by reverse transcription of the total blastomere RNA with MMLV reverse transcriptase for 2 hr at 37°C, and was amplified by PCR using Taq DNA polymerase (Recombinant Taq DNA polymerase, Takara Shuzo, Japan) and ^{35}S-dATP(redivue™ LABELLED NUCLEOTIDES, Amersham, UK) with T_{12}MG (5'-TTTTTTTTTTTTMG-3') and AP-2 (5'-GACCGCTTGT-3') as primers. After electrophoresing the PCR products in 6% DNA sequencing gel, the gel was dried on a filter paper (Whatman 3MM paper, Whatman Int. Ltd., England), and covered with wrap film (Saran Wrap, Asahikasei, Japan) to subject to autoradiography.

DNA extraction from gel bands

cDNA bands enriched in the micromere were excised from the gel, and DNA was extracted by soaking the gel pieces in TE (1 mM EDTA in 10 mM Tris-HCl at pH 8.0) overnight at 4°C followed by 15 min boiling. DNA was ethanol-precipitated from the extract, and reamplified by PCR using the same set of primers. The PCR products were electrophoresed on agarose gel and subcloned.

RT-PCR for isolation of poly(A)$^+$ mt 16S rRNA

RT-PCR was carried out using TaKaRa RNA PCR Kit (AMV) Ver.2 (Takara Shuzo) according to the manufacturer's instruction using the specific primers (described below) designed for each clones.

The presence of poly(A) tails in the PCR products were surveyed using an oligo(dT) primer and a specific forward primer. One μg of total RNA was reverse transcribed with 2.5 μM oligo(dT)$_{20}$-M4 Adaptor primer (5'-GTTTTCCCAGTCACGACTTTTTTTTTTTTTTTT-TTT-3') for 30 min at 50°C. After boiling, the PCR reaction was carried out by addition of the PCR mixture containing 0.2 μM mt 16S rRNA-specific forward primer (16F; 5'-AGTCCTGCCTGCCCAGT-GAC-3'). After initial denaturation at 94°C, 2 min, PCR was performed for 30 cycles using the following parameter, 94°C for 30 sec, 55°C, 30 sec and 72°C, 1.5 min, and the product was electrophoresed on 1.5% agarose gel.

Quantitative RT-PCR

Mt rRNA from each three types of blastomere was quantified by quantitative RT-PCR using following primers. For mt 12S rRNA; 5'-GGGACTTACTGCTGAATCCAATTTC-3' (reverse,12R) and 5'-GACCGCTTGTATACCATCGTCG-3' (forward, 12F1), and for mt 16S rRNA; 5'-CGGTCTGAACTCAGATCAGGTAG-3' (reverse, 16R) and 5'-AGTCCTGCCTGCCCAGTGAC-3' (Forward, 16F).

cDNA screening

PCR products were labelled with ^{32}P-dCTP (NEN, USA) by the random-labelling procedure. Using these oligonucleotides as the probe, cDNA library of the unfertilized egg was screened at a high stringency (hybridization in 5×SSPE, 1% SDS, 5% ICL at 65°C; washing with 0.1×SSC, 1% SDS at 65°C).

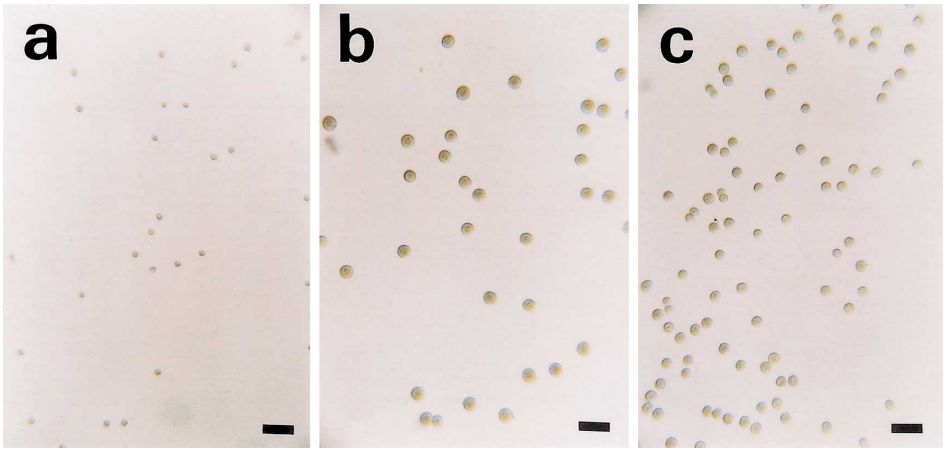

Fig. 1. Blastomeres isolated from 16-cell stage sea urchin embryos by centrifugal elutriation. Purity of micromere (a), macromere (b), mesomere (c) was about 100%, 100%, 84%, respectively. Bars=100 μm

Northern hybridization

Two μg each of the total RNA and poly(A)$^+$ RNA from the 16-cell stage embryos was electrophoresed in 1% agarose gel containing formaldehyde, and RNA bands were transferred to nylon filter (NY 13N NYTRAN: Schleicher & Schuell, Germany). The membranes were prehybridized for 3 hr at 65°C in 5×SSPE containing 1% SDS, 5% ICL and 100 μg/ml salmon sperm DNA, hybridized with the probe DNA overnight at 65°C, and washed under a high stringency condition. The random-primed ^{32}P-labelled DNA probe for mt 12S rRNA was prepared by PCR-labelling method using both 12R reverse primer and 12F2 forward primer (5'-CCAGGATTAGATACCCTGTTATAC-3'). Primers used to prepare the probe for mt 16S rRNA were 16R and 16F.

Whole mount *in situ* hybridization

Whole mount *in situ* hybridization was performed basically as described by Ransick *et al.* (1993). The 16-cell stage embryos freed from fertilization membranes were fixed in S.T.F.(Streck Tissue Fixation : Streck Laboratories, Inc, USA) for several hours at 4°C and then for 2 days at 4°C. Fixed embryos were washed successively with ASW, a series of graded ethanol, xylene, and ethanol and stored at –80°C until use.

The medium was gradually replaced by PBST(phosphate-buffered saline containing 0.1% Tween 20), and the specimen was prehybridized in the hybridization buffer (50% formamide containing10% PEG#6000, 0.6 M NaCl, 5 mM EDTA, 20 mM Tris-HCl pH 7.5, 500 μg/ml yeast RNA, 2×Denhardt's and 0.1% Tween 20) for 3 hr at 46°C, and hybridization with the digoxigenin(DIG)-labelled antisense RNA probe (0.2 μg/ml) for about 18 hr at 46°C. The DIG-labelled probes were synthesized with MEGA script™ T3 or T7 kit (AMBION, USA) and digoxigenin-11-2'-deoxyuridine-5'-triphosphate (Boehringer Mannheim) according to manufacturer's instruction. The antisense probe for mt 16S rRNA was alkaline hydrolyzed to a length of about 500 bp before use. After hybridization, the sample was washed once with PBST, three times with 1×SSC-0.1% Tween 20 at 60°C, and non-hybridized RNA was digested with 50 μg/ml RNase A for 30 min at 37°C. DIG signals were visualized using anti-digoxigenin conjugated alkaline phosphatase (Boehringer Mannheim) and observed under an optical microscope.

Electron microscopic *in situ* hybridization

The specimen was hybridized by the post-embedding method (Kobayashi *et al.*, in press). The 16-cell stage embryos were fixed for 1 hr with 2.5% glutaraldehyde in the 0.3 M NaCl-0.2 M cacodylate buffer, embedded in Lowacryl HM20 resin and sectioned. The sections were hybridized with DIG-labelled double strand DNA probe (1 μg/μl) in the hybridization buffer (50% formamide, 5×SSC, 100 μg/ml Sonicated salmon sperm DNA, 50 μg/ml heparin, 0,1% Tween 20) at 45°C for 5 hr, and then incubated with a drop of 10 nm-gold-conjugated anti-digoxigenin antibody (BioCell, UK, 200-fold diluted in TBST) for 1hr at room temperature. DIG-labelled sections were fixed with 0.5% glutaraldehyde and stained with uranyl acetate-lead citrate.

RESULTS

mt 12S rRNA and mt 16S rRNA are unequally distributed in 16-cell embryos

To find RNA species enriched in micromeres of the 16-cell sea urchin embryos, each blastomeres (micromeres, macromeres and mesomeres) were collected separately from the embryos using an elutriator, and the total RNA from three distinct blastomeres was compared by RNA differential display reactions (Fig. 2). Among several RNA species enriched in micromere fraction, we cloned cDNA of the most distinct band (indicated by an arrowhead in Fig. 2) from the cDNA library of unfertilized sea urchin eggs. Its nucleotide sequence (EMBL access. no. AJ130797) was 95% similar to that of mitochondrial 12S ribosomal RNA (mt 12S rRNA) of other sea urchin species, *Strongylocentrotus drobachiensis* and *S. intermedius*, indicating that the cloned cDNA encodes mt 12S rRNA of *H. pulcherrimus*.

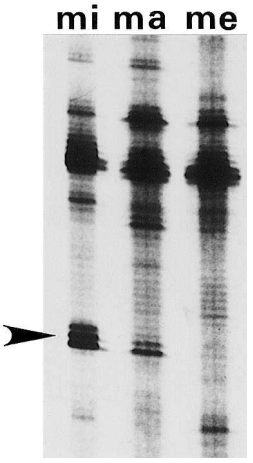

Fig. 2. Differential display of RNA from blastomeres of 16-cell stage embryos. Total RNAs from three types of blastomeres were subjected to differential display analysis using T_{12}MG primer and AP-2 primer as described in MATERIALS AND METHODS. An arrowhead indicates a band corresponding to mt 12S rRNA. mi; micromere, ma; macromere, me; mesomere.

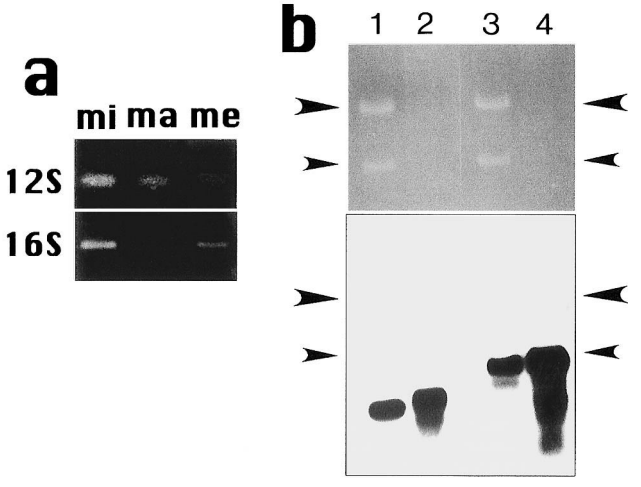

Fig. 3. (a) Quantitative RT-PCR of mt 12S and 16S rRNA in three different blastomeres. Total RNA from micromere (mi), macromere (ma) and mesomere (me) fractions was used as the template in PCR. Conditions for RT-PCR are described in MATERIALS AND METHODS. 12S; mt 12S rRNA, 16S; mt 16S rRNA. (b) Enrichment of mt rRNA in poly(A)$^+$RNA fraction of the 16-cell embryos. Procedures for electrophoresis and Northern blotting are described in MATERIALS AND METHODS. Upper panel: RNA staining by ethidium bromide. Lower panel: Northern blotting probed with cDNAs of mt 12S rRNA (lanes 1 and 2) and of mt 16S rRNA (lanes 3 and 4). Total RNA; lanes 1 and 3, poly(A)$^+$RNA; lanes 2 and 4.

Since it is known in *Drosophila* that mt 16S rRNA as well as mt 12S rRNA exist extramitochondrially and are components of germ plasm localized in the posterior pole region of the cleavage embryos (Kobayashi *et al.*, 1993; Kashikawa and Kobayashi, unpublished), we cloned mt 16S rRNA from cDNA library of unfertilized *H. pulcherrimus* eggs. The nucleotide sequence of Hp mt 16S rRNA (EMBL access. no. AJ130798) was 90% homologous to that of *S. purpuratus*.

Quantitative RT-PCR analysis confirms that both mt 12S rRNA and mt 16S rRNA are enriched in micromeres of the 16-cell embryos (Fig. 3a).

Nucleotide sequence analysis and Northern blotting of poly(A)$^+$RNA from the 16-cell embryos (Fig. 3b) show that sea urchin mt 12S and 16S rRNAs contain poly(A) tracts at their 3' ends as in the case of several animal species (Wolstenholme, 1992).

Developmental changes in the distribution of mt 12S rRNA and mt 16S rRNA in the embryos

The temporal and spatial distribution of mt 12S and 16S rRNAs was determined by whole mount *in situ* hybridization using antisense mt rRNA probes (Fig. 4, a, b c and d for mt 12S rRNA; e, f, g and h for mt 16S rRNA). Two mt rRNAs showed a similar pattern of spatial distribution in the early embryos. Asymmetrical distribution of two mt rRNAs was observed in uncleaved fertilized eggs (Fig. 4a and e) as well as in the 8-cell embryos (Fig. 4b and f). Asymmetrical distribution of these rRNAs became more evident at 16-cell stages in which mt 16S rRNA is localized specifically in micromeres alone (Fig. 4g) while mt 12S rRNA distributed in a gradient along a vegetal-animal axis (Fig. 4c). As development proceeds up to the 60-cell stage, distribution of mt rRNAs became clearly restricted to small micromeres, the descendants of micromeres (Fig. 4d and h). After the 60-cell stage, any intense signal was no longer detectable. A probe synthesized from pBluescript vector failed to generate signals in the 16-cell embryo (data not shown).

We found that the distribution of mt rRNAs did not simply reflect that of mitochondria in the embryos. The distribution of mitochondria among blastomeres monitored by $DiOC_2(3)$, a fluorescent dye for vital staining of mitochondria (Zalokar and Sardet, 1984), was significantly different from that of the mt rRNAs (Fig. 4i to l). The $DiOC_2(3)$ signal was distributed almost evenly throughout the cytoplasm of the fertilized eggs indicating even distributions of mitochondria among blastomeres. Thus up to 60-cell stage embryos are found no difference among blastomeres in the intensity of $DiOC_2(3)$ staining. Validity of this observation can be assessed by that

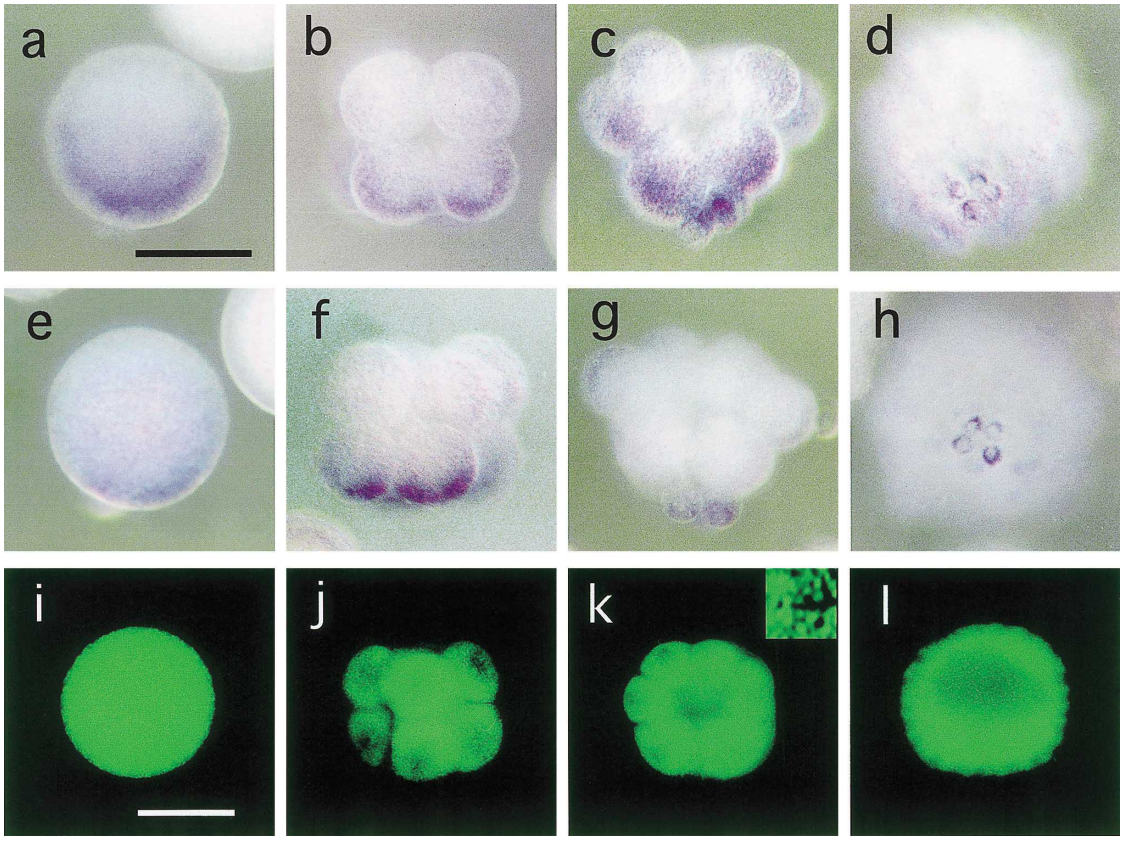

Fig. 4. Whole mount *in situ* hybridization of fertilized eggs and the embryos with mt 12S rRNA (a, b, c, d) and with mt 16S rRNA probes (e, f, g, h). i, j, k and l; stained with $DiOC_2(3)$. a, e and i; fertilized egg, b, f and j; 8-cell stage, c, g and k; 16-cell stage, d, h and l; 60-cell stage. Inset in k; a higher magnification of the surface of embryo shown in k. Bars=50 μm

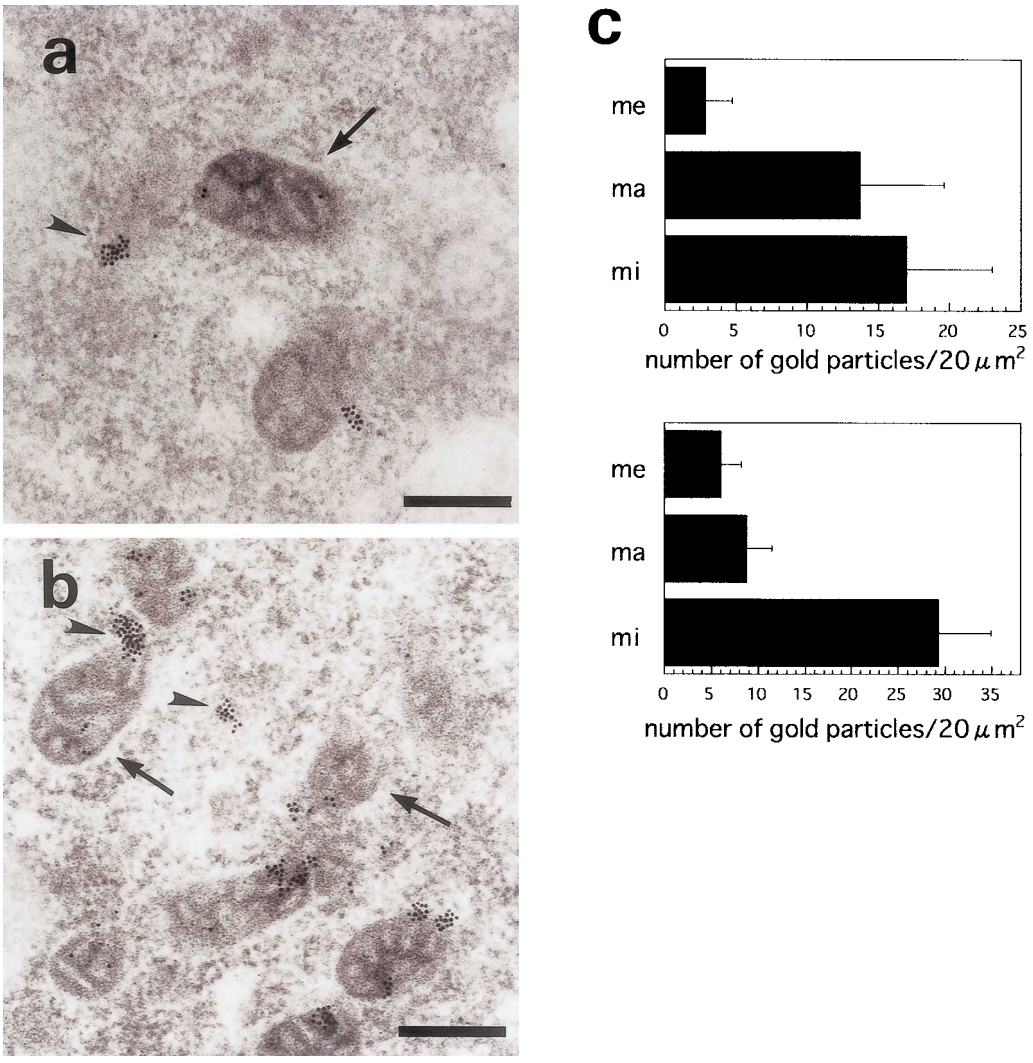

Fig. 5. Electron microscopic *in situ* hybridization of mt 12S rRNA (a) and mt 16S rRNA (b). Arrows indicate mitochondria, and arrowheads indicate the signals outside mitochondria. Bars=200 nm. (c) Distribution of mt 12S and 16S rRNA outside mitochondria in three types of blastomeres of the 16-cell stage embryos. The number of gold particles in the total area of 100-180 μm^2 in each blastomeres (1–3 embryos) were counted and the results expressed by the average numbers of gold particles per 20 μm^2. Bars indicate the standard error.

$DiOC_2(3)$ stained granular structures similar in size to mitochondria (inset in Fig. 4k). This result suggests that the mt rRNA signal we detected by whole mount *in situ* hybridization did not result from intramitochondrial signals but from extramitochondrial molecules.

In *Drosophila*, mt 16S and 12S rRNA are present outside mitochondria only in posterior polar plasm of cleavage embryos (Kashikawa *et al.*, unpublished). To confirm if mt rRNAs are really localized outside mitochondria in blastomeres of sea urchin embryos as they are in *Drosophila* embryos, intracellular distribution of mt rRNAs were surveyed by electron microscopical *in situ* hybridization using cDNA probes for mt rRNA, and localization of these RNA was visualized by gold particles (Fig. 5). Significant amounts of mt 12S rRNA (Fig. 5a) and mt 16S rRNA (Fig. 5b) were localized extra-mitochondrially. Densities of extramitochondrial mt rRNA in blastomeres of 16-cell stage embryos were estimated by counting the number of gold particles per 20 μm^2 of the cytoplasmic area (Fig 5c). The result shows that extramitochondrial 12S and 16S mt rRNAs distribute asymmetrically among blasto-meres enriching in the vegetal-most region of the 16-cell embryos, while intramitochondrial mt rRNA distributes evenly among three types of blastomeres (data not shown). The asymmetrical distribution is much clear for mt 16S rRNA than for mt 12S rRNA. Electron micrograms show that extramitochondrial mt rRNA are not associated with cytoplasmic organelles (Fig. 5a and b).

DISCUSSION

Mt 12S and 16S rRNAs are known to be transcribed from a mitochondrial genome and become components of ribosomes within mitochondria. However, asymmetrical distribution of mt rRNAs among blastomeres in early embryos does

not coincide with distribution of mitochondria themselves that are evenly distributed among blastomeres. The result of electron microscopical *in situ* hybridization shows that a significant fraction of mt rRNAs are localized outside mitochondria (Fig. 5) distributing asymmetrically along a vegetal-animal axis of 16-cell embryos (Fig. 5). Asymmetrical distribution of mt rRNA is also detected by whole mount *in situ* hybridization (Fig. 4), suggesting that the present whole mount *in situ* hybridization protocol detects extramitochondrial mt RNAs alone. This is probably because the riboprobes hardly penetrate inner mitochondrial membranes as reported by Kobayashi *et al*. (1993) and Amikura *et al*. (1996).

In *Drosophila*, mt 16S rRNA has been identified as a factor that induces the germ line progenitors in UV-irradiated embryos (Kobayashi *et al*., 1989). Ultrastructural analysis reveals that extramitochondrial mt 16S rRNA is tightly associated with germinal granules, distinctive organelles of germ plasm, during a short period from oviposition to pole cell formation (Kobayashi *et al*., 1993; Amikura *et al*., 1996). In *Xenopus* embryos, germ plasm is localized in the vegetal pole region of the early embryos and contains germinal granules that are very similar to polar granules in *Drosophila* (Mahowald and Hennen, 1971; Czolowska, 1972; Beams and Kessel, 1974; Ikenishi *et al*., 1974; Eddy, 1975; Ikenishi and Kotani, 1975). The extramitochondrial mt 16S rRNA is present in the germinal granules in *Xenopus* embryo from 4-cell to blastula stage (Kobayashi *et al*., 1994; Kobayashi *et al*., unpublished). Thus, the extramitochondrial mt 16S rRNA is a common component of germ plasm in *Drosophila* and *Xenopus* (Kobayashi *et al*., 1994). As in the case of *Xenopus*, extramitochondrial mt 16S rRNA is enriched predominantly in the vegetal pole region of the early sea urchin embryos (Fig. 4). In addition, the mt rRNA is partitioned into four blastomeres locating in the vegetal-most position of the embryos as development proceeds, while germ plasm is partitioned into four blastomeres by the first 2 cleavage division in *Xenopus*. Although these four blastomeres of *Xenopus* embryos continue to divide 10 more times during the rest of the cleavage mitosis, the number of the germ plasm-bearing cells remains four because germ plasm is included only in one of the two daughter cells (Whitington and Dixon, 1975), and the extramitochondrial mt 16S rRNA distribution coincides with the distribution of germ plasm. A similar situation is observed in sea urchin embryos. Unequal cleavage of four micromeres produces four large micromeres and four small micromeres (Dan, 1979, 1987; Dan *et al*., 1983), and mt 16S rRNA enriched in four micromeres is partitioned into four small micromeres with only a trace in four remaining large micromeres (Fig. 4d and h).

Developmental fate of small micromeres has been partly described (Endo, 1966; Pehrson and Cohen, 1986; Amemiya, 1989) and four small micromeres divide once into 8 cells by hatching and remain quiescent at the tip of the invaginating archenteron of gastrulae. In *Strongylocentrotus* plutei, the descendants of small micromeres are incorporated in the coelomic sac (Pehrson and Cohen, 1986) that is known to produce an echinus rudiment which forms adult body. Therefore, it is likely that extramitochondrial mt rRNA enriched in small micromeres may play an important role for further differentiation of these micromeres. Recently Oka *et al*. found that mt large rRNA localizes in myoplasm of early ascidian embryos, though its localization inside or outside mitochondria is not known (personal communication). It is of interest that rRNA of mitochondrial type plays a role in early embryogenesis of a wide range of animals with different pattern of development.

ACKNOWLEDGMENTS

This work was supported in part by Grants-in-Aid for Scientific Research given to H. S. and K. A. from the Ministry of Education, Science, Sports and Culture, Japan. This research was also supported in part by a Grant Pioneering Research Project in Biotechnology given to H. S. by the Ministry of Agriculture, Forestry and Fisheries, Japan, by Tsukuba Advanced Research Alliance (TARA) Kobayashi Project and by a Research Project for Future Program from Japan Society for the Promotion of Science given to S.K. Thanks are also due to the staffs of the Center for Gene Research of Hiroshima University for access to their facilities.

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(Received January 13, 1999 / Accepted January 27, 1999)