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Authors: Nakamura, Masahisa, Kawano, Kiichirou, Kondo, Yasuyuki, Miura, Ikuo, Kashiwagi, Akihiko, et al.

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Stage-Dependent Distribution of Calreticulin in Oocytes of the Frog, *Rana rugosa*

Masahisa Nakamura*, Kiichirou Kawano, Yasuyuki Kondo,
Ikuo Miura, Akihiko Kashiwagi and Satoshi Yamamoto

Laboratory for Amphibian Biology, Faculty of Science, Hiroshima University, 1-3-1 Kagamiyama, Higashi-Hiroshima, Hiroshima 739, Japan

ABSTRACT—We studied immunohistologically the distribution of a Ca^{2+}-binding protein, calreticulin (CLT), at different stages of growing oocytes in the frog, *Rana rugosa*. Northern blot analysis showed that the CLT gene expression in gonads of metamorphosing tadpoles was low, but was extremely strong in the ovary, but not in the testis, of 2-month post-metamorphosis frogs, followed by decline to a lower level in adult frogs. On the contrary, the β-actin gene expression did not increase in the same ovary. As for the distribution of CLT protein, a weak immunostaining was observed in indifferent gonads of tadpoles at stage I. The CLT distribution in oocytes from stage A to F was stage-dependent. In addition, Western blot analysis revealed that the CLT level was low in gonads of tadpoles at stage I, but increased at stage XVI. It still increased in the ovary of frogs 2 months after metamorphosis, and then dropped to a lower level in adult frogs. The results indicate that CLT gene expression occurs in the early stage of growing oocytes, and that CLT is synthesized actively in oocytes in the ovary of frogs after metamorphosis. Based on these findings, the role of CLT is discussed.

INTRODUCTION

Many aspects of cellular function are regulated by the concentration of free Ca^{2+} in the cytoplasm. Intracellular organelles play major roles in the regulation of cytosolic free Ca^{2+} levels due to their ability to sequester and release Ca^{2+} following cell activation. The release of Ca^{2+} from stores can occur in response to a first messenger such as neurotransmitters, hormones and sperm (Berridge, 1987; Parrington *et al.*, 1996). In some cases, Ca^{2+} that binds intracellular Ca^{2+}-binding proteins may be released.

Calreticulin (CLT) has a high capacity to bind Ca^{2+} in the endoplasmic reticulum (ER); it binds 20 moles of Ca^{2+} per mole of protein at low affinity (Baksh and Michalak, 1991; Nakamura *et al.*, 1993). Full length amino acid sequences of numerous CLTs have been deduced from cDNA (Fliegel *et al.*, 1989; Smith and Koch, 1989; Michalak *et al.*, 1992; Yamamoto *et al.*, 1996). Recently, CLT (Parys *et al.*, 1994) and inositol 1,4,5-trisphosphate receptor (IP_3R) (Callamaras and Parker, 1994) were found in *X. laevis* eggs. Repetitive Ca^{2+} release is induced by IP_3R activation (Parker and Yao, 1991). In addition, overexpression of CLT inhibits repetitive IP_3-induced Ca^{2+} waves (Camacho and Lechleiter, 1995). In view of these findings, CLT is thought to be one of the key factors in signal transduction of frog eggs. In order to understand its function in eggs, it is urgent to study the gene expression and distribution of CLT in growing oocytes of frogs. Here we report that the CLT gene expression occurs at the early stage of growing oocytes of frogs, and that the CLT distribution is stage-dependent.

* Corresponding author: Tel. +81-824-24-7483;
FAX. +81-824-24-7483.

MATERIALS AND METHODS

Animals

Frogs, *Rana rugosa* were used for all the experiments. The frog was primed 20 hr before obtaining unfertilized eggs by injecting the extract of pituitaries of *Rana catesbeiana* into the body cavity according to Kashiwagi and Kashiwagi (1993). Tadpoles were staged as described elsewhere (Shumway, 1940; Taylor and Kollros, 1946).

Northern blot analysis

At 1 to 4 weeks after tadpoles (stage IV-V) were reared, gonads were excised surgically for isolation of total RNA. For each isolation of total RNA, 150 tadpoles were used. Total RNA was also isolated from testes and ovaries of 2-month post-metamorphosis frogs and adult frogs (Yamamoto *et al.*, 1996). RNA (20 μg/lane) was electrophoresed on a 1% denaturing formaldehyde agarose gel and transferred to nitrocellulose membranes. RNA was then hybridized with the ^{32}P-labeled 487-bp *Kpn*I/*Sau*3AI fragments of CLT cDNA (Yamamoto *et al.*, 1996), or 421-bp *Alu*I/*Alu*I fragments of mouse β-actin cDNA (Nakamura *et al.*, 1993) as a probe, washed at 65°C in 0.2 × SSC and 0.1% SDS, dried and exposed to X-ray films overnight.

Antibodies

Polyclonal antibodies against frog CLT were produced in mice. Ten-week old BALB/C mice were immunized with 5 μg of CLT purified from the frog, *R. rugosa* livers (Yamamoto *et al.*, 1996). The first i.p. injection in complete Freund's adjuvant (Wako) was followed by

boosts after 2 and 4 weeks (5 μg of protein in Freund's adjuvant). Sera were collected and tested 5 days after the second boost on Western blot with the purified frog CLT.

Immunoblot analysis

Gonads in tadpoles at stages VII and XVI, and ovaries of frogs at 2-month and 2-year post-metamorphosis were homogenized in 50 mM Tris-HCl (pH 7.4), mixed with the sample buffer (Laemmli, 1970) and boiled for 5 min. The sample was then subjected to SDS-PAGE (Laemmli, 1970), transferred to nitrocellulose membranes electrophoretically (Towbin *et al.*, 1979) and detected on immunoblot analysis with the antibody of CLT at a dilution 1:500. Protein concentrations were determined by the method of Peterson (1977) using bovine serum albumin as a standard.

Immunohistochemistry

Eggs and ovaries were fixed with 4% paraformaldehyde in 0.075 M phosphate buffer (pH 7.5) at 4°C overnight, dehydrated in graded series of ethanol, embedded in paraffin and sectioned at 5-8 μm thickness. Immunostainings were carried out by the method of Hsu and Soban (1982) with mouse antisera raised against frog CLT (Yamamoto *et al.*, 1996) at a 1:750 dilution. Oocytes were staged according to Grant (1953).

RESULTS

Expression of the CLT gene during differentiation of gonads

The CLT gene expression in gonads of tadpoles of the frog, *R. rugosa* was examined. When tadpoles at stage IV-V were reared, they grew to be mostly at stage XIII-XVI in 4 weeks (Table 1). Using the total RNA prepared from gonads of those tadpoles, and from ovaries and testes of 2-month post-metamorphosis frogs and adult frogs, Northern blot analy-

Table 1. Developmental stages and body weights of tadpoles

Time (weeks)	Body weights (g)	Stages (No. of bodies)														
		IV	V	VI	VII	VIII	IX	X	XI	XII	XIII	XIV	XV	XVI	XVII	XVIII
0	0.198±0.027	30	20													
1	0.397±0.117		3	16	9	10	2									
2	0.915±0.430			2	4	8	6	10	3	6	1					
3	1.093±0.280				2	0	1	3	8	11	5	8	2			
4	1.255±0.207						1	1	0	2	6	10	7	10	3	

Values of body weights are the mean±S.D.

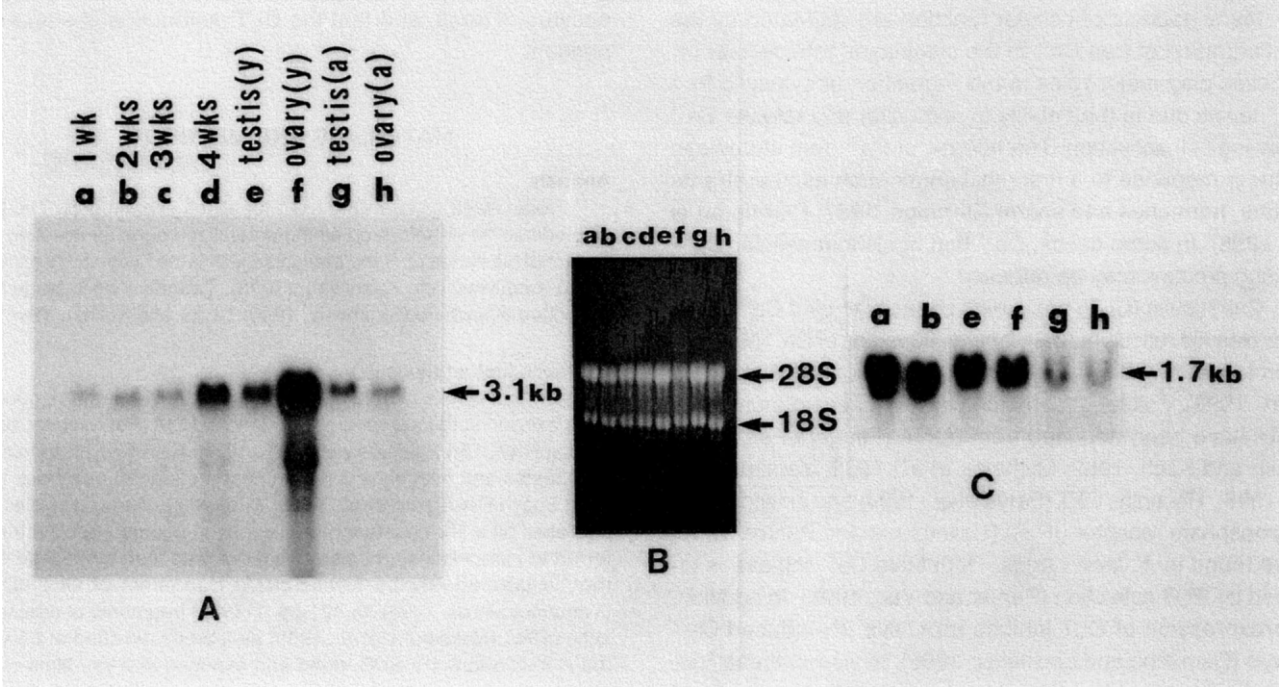


Fig. 1. Expression of the CLT mRNA in gonads. Total RNA was isolated from gonads which were excised surgically one (a), two (b), three (c) and four (d) weeks after tadpoles at stage IV-V were reared. Stages of tadpoles in each group are shown in Table 1. Total RNA was also isolated from testes (e) and ovaries (f) of 2-month post-metamorphosis young frogs, and from testes (g) and ovaries (h) of 2-year old adult frogs. Expressions of the CLT and β-actin genes are shown in the panels **A** and **C**, respectively. Total RNA stained with ethidium bromide was shown in the panel **B**. Alphabetical letters (a-h) on the top of panels **A** and **C** correspond to those in the panel **B**. The number on the right side of panels **A** and **C** indicates the size of a transcript. Arrows in the panel **B** indicate the position of the 28S and the18S ribosomal subunits.

sis was carried out with CLT cDNA. The CLT gene expression with a 3.1-kb transcript increased gradually in gonads as tadpoles developed (Fig. 1A). The ovary of 2-month post-metamorphosis frogs produced an extremely strong signal of the CLT gene expression (Fig. 1A), while the β-actin gene expression with a 1.7-kb trabscript did not increase in the ovary before and after metamorphosis (Fig. 1C). Both CLT and β-actin gene expressions in the ovary of adult frogs declined to the level lower than those in the testis of adult frogs (Fig. 1A, C).

Localization of CLT in oocytes and eggs

As shown in Fig. 1A, the CLT gene expression was extremely strong in the gonad of 2-month post-metamorphosis frogs. Then, the localization of CLT in the ovary was examined immunohistologically with the antibody of CLT. For this purpose, its quality was tested first by Western blot analysis. As shown in Fig. 2, a single band with Mr=52 kDa, estimated by SDS-PAGE, was detected with the antibody. The molecular weight of 52 kDa obtained in this study was the same when the antibody of rat CLT raised against rabbits was used (see Yamamoto *et al.*, 1996). Thus, this antibody was used for immunohistological studies.

A faint, but significant staining was seen in the gonad of tadpoles at stage I (Fig. 3a-1), but no staining was observed with non-immune serum (Fig. 3a-2). In the ovary of tadpoles at stage XX, CLT was seen in the peripheral and perinuclear regions in oocytes at stage A (Fig. 3b-1), but there was no significant staining with non-immune sera (Fig. 3b-2). Oocytes at stage B in the ovary of 2-month post-metamorphosis frogs, as indicated with an arrow, showed intense staining in the whole cytoplasm (Fig. 3c). In an oocyte at stage C, a staining was evident in the thin rim of the cytoplasm and in the reticular network structure (Fig. 3d-1, d-2). Again, there was no significant staining in oocytes in the same ovary when non-immune serum was used (Fig. 3d-3). Furthermore, enriched localization of CLT was found in the cytoplasm free from yolk platelets of oocytes at stage F (Fig. 3e).

Finally, the CLT level in the gonad and ovary was examined by Western blot analysis. As shown in Fig. 4A, it was low in gonads at stage VII where oocytes at stage A were not found and the formation of ovarian cavities was still poor (Fig. 4B-1). However, the CLT level increased significantly in gonads at stage XVI where oocytes at stage A were seen (Fig. 4B-2). It was the highest in the ovary of 2-month post-metamorphosis frogs containing oocytes at stage B (Fig. 4B-3), and then declined to a lower level in adult frogs (Fig. 4A).

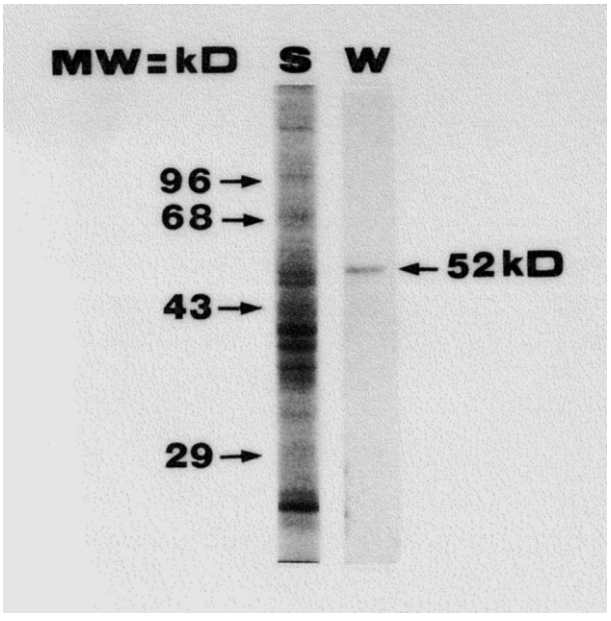


Fig. 2. The quality of an antibody of CLT. Ovaries of 2-month post-metamorphosis frogs were homogenized in 50 mM Tris-HCl (pH 7.4), mixed with the sample buffer (Laemmli, 1970) and boiled for 5 min. The sample was then subjected to SDS-PAGE. Fifty μg of protein were loaded on a lane and transferred to nitrocellulose membranes electrophoretically. Then, proteins were reacted for anti-CLT staining, as described in MATERIALS AND METHODS. S, Coomassie blue staining; W, immunostaining.

DISCUSSION

We reported previously that the CLT gene was expressed in embryos at stage 1 through 25 (Yamamoto *et al.*, 1996). In this study, the CLT gene expression was shown to be at the moderate level in the gonad of metamorphosing tadpoles, and at the extremely high level in the ovary of 2-month post-metamorphosis frogs. The CLT mRNA level, then, decined to a much less extent in the ovary of adult frogs. As for CLT protein, its distribution in oocytes was stage-dependent. Immunostainings were observed in gonads of tadpoles at stage I, but was not restricted to any specific type of cells. We could not identify the type of cells because they were all indifferent. In oocytes at stage A, a weak staining was observed in the peripheral and perinuclear regions. However, intense staining was observed in the whole cytoplsm of oocytes at stage B. As oocytes grow, immunostaining was observed in the thin rim of cytoplasm and perinuclear regions, and in the reticular network structure in oocytes, in particular at stage D. The staining perhaps depends on the localization of the cytoplasm free from yolk platelets.

Western blottings revealed that the CLT level was low in the gonad of tadpoles at stage VII where no oocytes at stage A were found. However, its level increased significantly in the gonad containing oocytes at stage A (see Fig. 4B), and was higher in the ovary containing oocytes at stage B. These results suggest that the CLT synthesis starts in the early stage of growing oocytes. It becomes active in oocytes at stage A, and more active at stage B, and then less active in adult frogs. Since strong immunostaining was observed in the cytoplasm of oocytes at stage B, but not at stage D, the CLT synthesis may be the highest in oocytes at stage B to C. To prove this, it is necessary to purify oocytes in different stages, and to examine which oocytes can synthesize CLT at the highest level. Unfortunately, the method for purification of frog oocytes in different stages is not available at the present time because

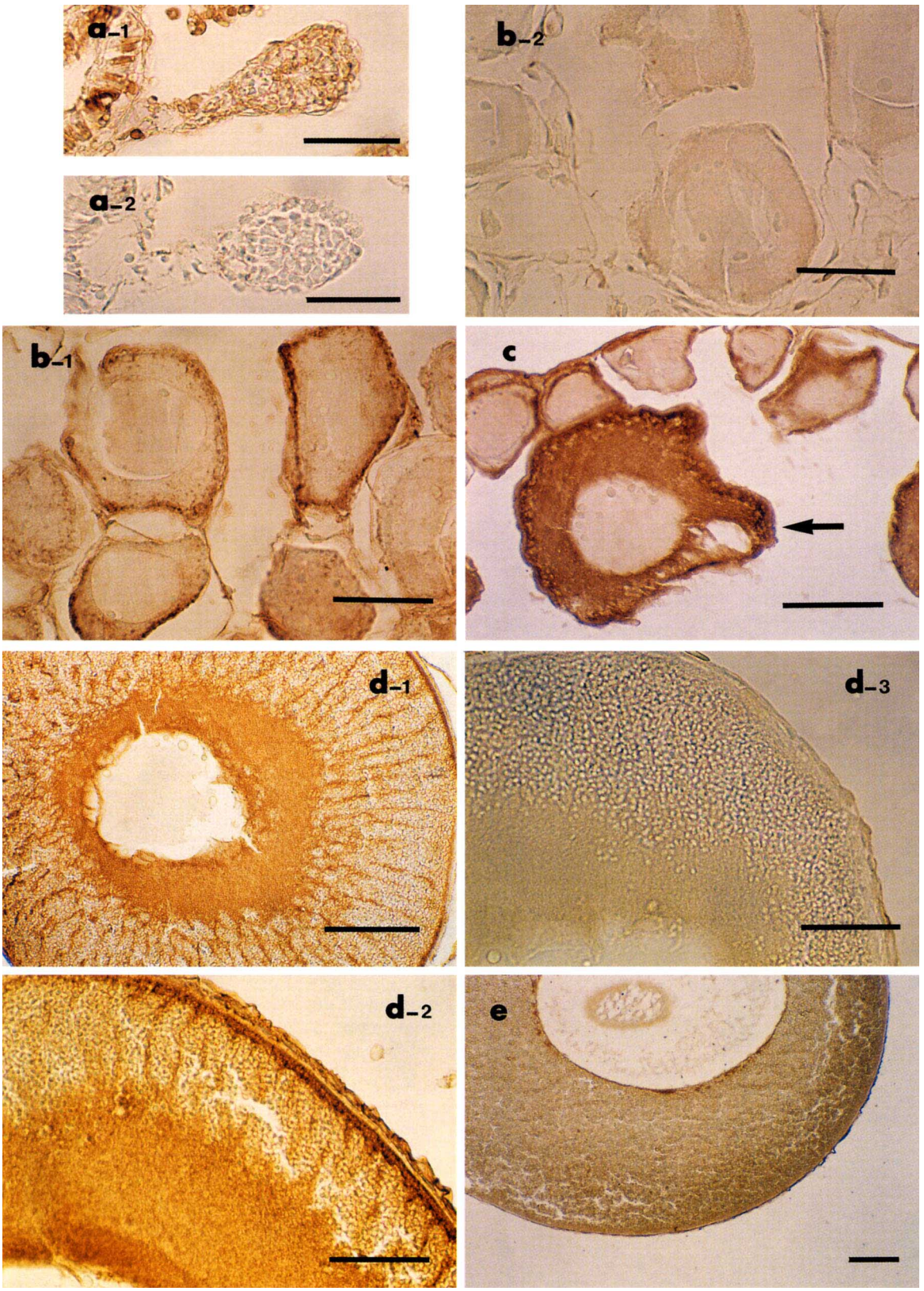
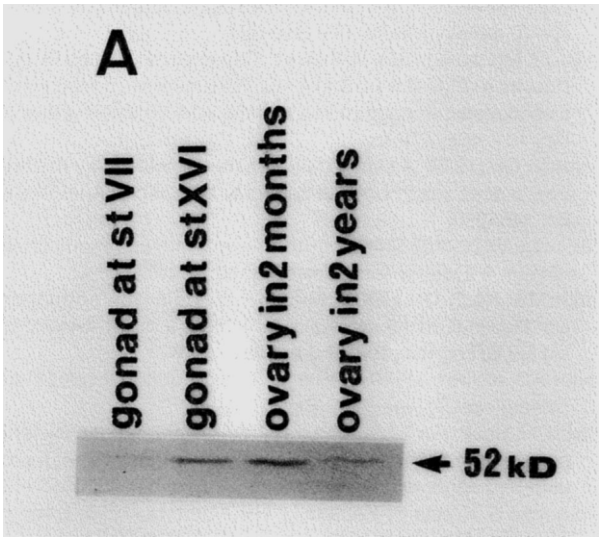
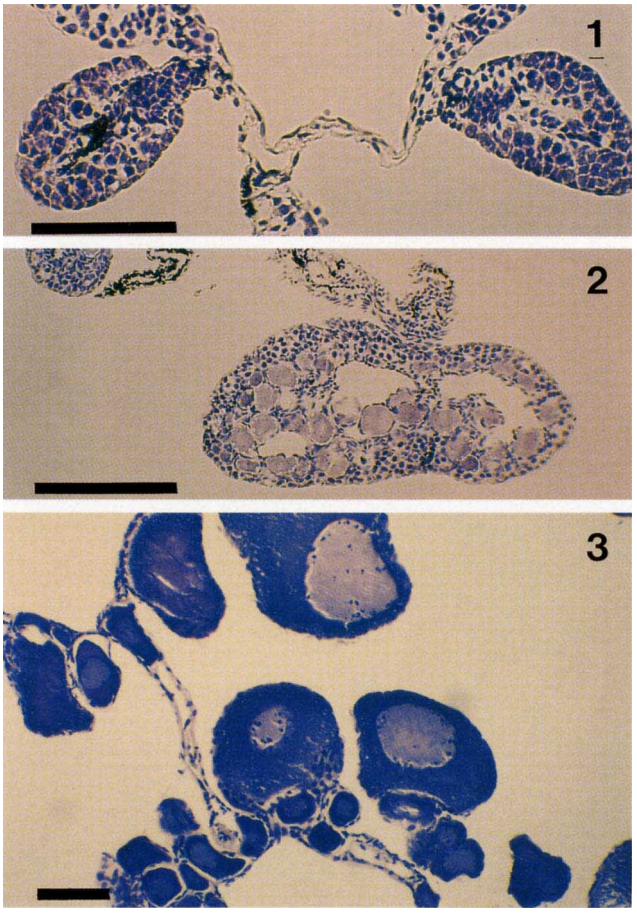


Fig. 3. Distribution of CLT in oocytes. Gonad of tadpoles at stage I (**a**). Oocytes at stage A (**b**), stage B (**c**), stage C (**d**), and stage F (**e**). Bars represent 50 (**a**, **b**, **d-2**, **d-3**) and 100 (**c**, **d-1**, **e**) μm, respectively.

of small sizes of gonads in tadpoles and 2-month post-metamorphosis frogs. It should also be mentioned that CLT is actively synthesized during the growth of oocytes in frogs, suggesting that CLT may play an important role(s) in growing oocytes. But, its function is unclear presently. As far as we know, however, this is the first report showing the CLT distribution in growing oocytes in animals.





In a wide variety of eggs such as medaka fish (Gilkey *et al.*, 1978), frog (Kubota *et al.*, 1987) and hamster (Miyazaki, 1988), a transient increase in Ca^{2+} concentration occurs at fertilization and propagates as Ca^{2+} waves. Microinjection of IP_3 into eggs activates eggs and induces early developmental events (Busa *et al.*, 1985). However, the IP_3-induced Ca^{2+} release was blocked, when an IP_3 antibody was injected into eggs (Miyazaki *et al.*, 1992), or when overexpression of CLT was induced as described earier. Since CLT is an intraluminal Ca^{2+}-binding protein of the endoplasmic reticulum (ER) and can bind a large amount of Ca^{2+}, CLT might play an important role in the release of Ca^{2+} from the inside of the ER at fertilization. In addition, Fluck *et al.* (1991) recently studied the relationship of calcium to contractile band in the cleaving medaka fish eggs. They found two successive Ca^{2+} waves in those eggs; the first wave accompanies furrow elongation in each of the early cleavages of medaka eggs and comes from a high calcium region within the assembling contractile band, while the second one accompanies the slow zipping together of the separating cells. Coppolino *et al.* (1997) also showed that CLT is an essential modulator for calcium signalling and cell adhesion in embryonic stem cells. Taking together with these findings, CLT may be related to cell division and signal transduction in frog cells. To prove these, further investigations must be required. Nevertheless, it is very interesting to note that CLT is synthesized in growing oocytes and distributed in a stage-dependent manner.

ACKNOWLEDGMENT

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Fig. 4. Immunoblot analysis (**A**) and histological aspects (**B**) of gonads and ovaries. (**A**) homogenates of gonads of tadpoles at stages VII and XVI, and ovaries of 2-months post-metamorphosis frogs and 2-year old frogs were run on SDS-PAGE. Then, nitrocellulose filters used for the detection of CLT were prepared as described in MATERIALS AND METHODS. One hundred μg of protein were loaded on each lane. (**B**) gonads and ovaries were fixed in a paraformaldehyde solution, embedded in paraffin and serially sectioned as described in MATERIALS AND METHODS. Sections were then stained with Mayer's hematoxylin (Merck). Typical histological features of ovaries of tadpoles at stage VII (**1**) and at stage XVI (**2**), and frogs 2 months after metamorphosis (**3**) are shown. Bars represent 50 μm.

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