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REVIEW ARTICLE

Effects of *S*-adenosylmethionine decarboxylase, polyamines, amino acids, and weak bases (amines and ammonia) on development and ribosomal RNA synthesis in *Xenopus* embryos

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Abstract We have been studying control mechanisms of gene expression in early embryogenesis in a South African clawed toad *Xenopus laevis*, especially during the period of midblastula transition (MBT), or the transition from the phase of active cell division (cleavage stage) to the phase of extensive morphogenesis (post-blastular stages). We first found that ribosomal RNA synthesis is initiated shortly after MBT in *Xenopus* embryos and those weak bases, such as amines and ammonium ion, selectively inhibit the initiation and subsequent activation of rRNA synthesis. We then found that rapidly labeled heterogeneous mRNA-like RNA is synthesized in embryos at pre-MBT stage. We then performed cloning and expression studies of several genes, such as those for activin receptors, follistatin and aldolases, and then reached the studies of *S*-adenosylmethionine decarboxylase (SAMDC), a key enzyme in polyamine metabolism. Here, we cloned a *Xenopus* SAMDC cDNA and performed experiments to overexpress the in vitro-synthesized SAMDC mRNA in *Xenopus* early embryos, and found that the maternally preset program of apoptosis occurs in cleavage stage embryos, which is executed when embryos reach the stage of MBT. In the present article, we first summarize results on SAMDC and the maternal program of apoptosis, and then describe our studies on small-molecular-weight substances like polyamines, amino acids, and amines in *Xenopus* embryos. Finally, we summarize our studies on weak bases, especially on ammonium ion, as the specific inhibitor of ribosomal RNA synthesis in *Xenopus* embryonic cells.

Keywords *S*-adenosylmethionine decarboxylase (SAMDC) · Maternal program of apoptosis · Polyamines · Ornithine · Amino acids · Amines · Ammonium ion · rRNA synthesis · Xenopus embryos

This review is dedicated to the late Prof. Nello Bagni to whom the first and the last author owe very much in their academic activities in polyamine researches.

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Abbreviations

MBT	Midblastula transition
pre-MBT	pre-Midblastula transition
SAMDC	*S*-adenosylmethionine decarboxylase
rDNA	Ribosomal DNA
ODC	Ornithine decarboxylase
SAM	*S*-adenosylmethionine
dcSAM	Decarboxylated SAM
ORF	Open reading frame
EGBG	Ethylglyoxal bis-(guanylhydrazone)
GVBD	Germinal vesicle breakdown
tRNA	Transfer RNA

Introduction

Biochemical embryology in the field of nucleic acid research started on 1962, when Don Brown first analyzed ribosome and protein synthesis in *Rana pipiens* embryos (Brown and Caston 1962). Brown started to use *Xenopus laevis* embryos, and isolated ^{32}P-labeled "undegraded", as opposed to alkali-hydrolyzed, RNA by phenol methods (Brown and Littna 1964). Gurdon and Brown (1965) utilized embryos of anucleolate mutant of *Xenopus laevis*, and showed that nucleoli formation is the cytological manifestation of function of ribosomal RNA genes or ribosomal DNA (rDNA). Dawid (1966) determined the exact amount of nuclear, as opposed to cytoplasmic, DNA of *Xenopus* embryos (6 pg/cell).

In Japan, we first studied ^{32}P-labeled "undegraded" RNA from *Rana japonica* embryos (Shiokawa and Yamana 1965), and then started to use *Xenopus laevis* (Shiokawa and Yamana 1966). We introduced dissociated *Xenopus* embryonic cells as a new experimental system (Shiokawa et al. 1967) to study *Xenopus* embryonic RNA synthesis. Here, we compared patterns of RNA synthesis between $^{14}CO_2$-labeled whole embryos and ^{3}H-uridine-labeled dissociated embryonic cells, and after confirming that initiation and developmental activation of rRNA synthesis (or rDNA transcription) take place similarly in both experimental systems, we concluded that dissociated cells which actively incorporate radioactive precursors provide in fact a good experimental system to study the synthesis of macromolecules in *Xenopus* embryos (Shiokawa and Yamana 1967a). At present, embryos of *Xenopus laevis* are utilized all over the world, and they are now used not only for molecular biological studies but also for studies of morphogenesis, which intend to solve the problem of the mechanism of cell differentiation (Heasman 2006).

In *Xenopus* embryogenesis, fertilized eggs cleave rapidly without G_1 and G_2 phases in the cell cycle. During cleavage stage, mRNAs are translated, but transcription from nuclear genes cannot be detected easily (Shiokawa et al. 1981a, b, 1994, 2008), partly because of the presence of the impermeable surface coat which prevents uptake of radioactive precursors (Shiokawa and Yamana 1967; Shiokawa et al. 1967) and partly because of the occurrence of a small number of nuclei in pre-MBT embryos. After 12 rounds of cell divisions, embryos reach MBT (4,096 cells/embryo), when G_1 and G_2 phases reappear (Heasman 2006) and checkpoint-regulated cell cycles start. After MBT, cell divisions become asynchronous, and cells at the dorsal marginal zone acquire motility (Minoura et al. 1995), and transcription from zygotic nuclei is strongly activated (Shiokawa et al. 1989, 1994; Yang et al. 2002; Nakakura et al. 1987; Shiokawa et al. 1981a, b).

In this article, we first describe cloning and expression studies of *Xenopus* SAMDC, and show the occurrence of the maternally preset program of apoptosis discovered from the study of SAMDC overexpression. We then present results on low-molecular-weight substances in *Xenopus* embryos, including natural polyamines (putrescine, spermidine, and spermine), ornithine and other amino acids, and amines. Then, finally, we show our data on rDNA expression in *Xenopus* development, and propose that weak bases, in particular ammonium ion, which selectively inhibit ribosomal RNA synthesis, serve as a regulator of rDNA expression in early embryonic cells.

Isolation and characterization of *Xenopus* SAMDC cDNA and developmental changes in SAMDC mRNA level and enzyme activity

In eukaryotic cells ornithine decarboxylase (ODC), which converts ornithine to putrescine, and SAMDC, which converts *S*-adenosylmethionine (SAM), a methyl donor to decarboxylated SAM (dcSAM) an aminopropyl donor, turn over rapidly and are rate-limiting enzymes in polyamine biosynthesis. *Xenopus* ODC cDNA has been cloned (Osborne et al. 1991), and its mRNA level is known to be low in fertilized eggs but increases from the early gastrula stage (Rosander et al. 1995). We cloned *Xenopus* SAMDC cDNA (1,030 bp in length) (Fig. 1). This open reading frame (ORF) encoding 335 amino acids (M.W., 37KD) is longer than other vertebrate counterparts by one or two amino acids. The homology of *Xenopus* SAMDC proenzyme at amino acid level to human, mouse, rat, and yeast counterparts was 83, 84, 83, and 31%, respectively. The *Xenopus* SAMDC proenzyme contained consensus sequences for the processing of the proenzyme, for the active site, and for the putrescine-dependent stimulation of the enzyme activity (Pajunen et al. 1988).

Using the cDNA as a probe, we examined changing levels of SAMDC mRNA by northern blot analysis in growing oocytes, oocytes during maturation, and developing embryos. SAMDC mRNA (3.5 kb) occurred from the earliest stage of oogenesis at a relatively high level, and the level doubled at stage II, but decreased from stage III to stage VI, to a low level during oocyte maturation. The lowered level was maintained even after fertilization, until it started to increase after tailbud stage (ca. 25 h after fertilization) (Shinga et al. 1996). Thus, the level of SAMDC mRNA increases relatively late during development as compared with ODC mRNA (Rosander et al. 1995). Using the in vitro transcribed mRNA as a reference, we determined the amount of maternal SAMDC mRNA as 0.005 ng or less (Shibata et al. 1998).

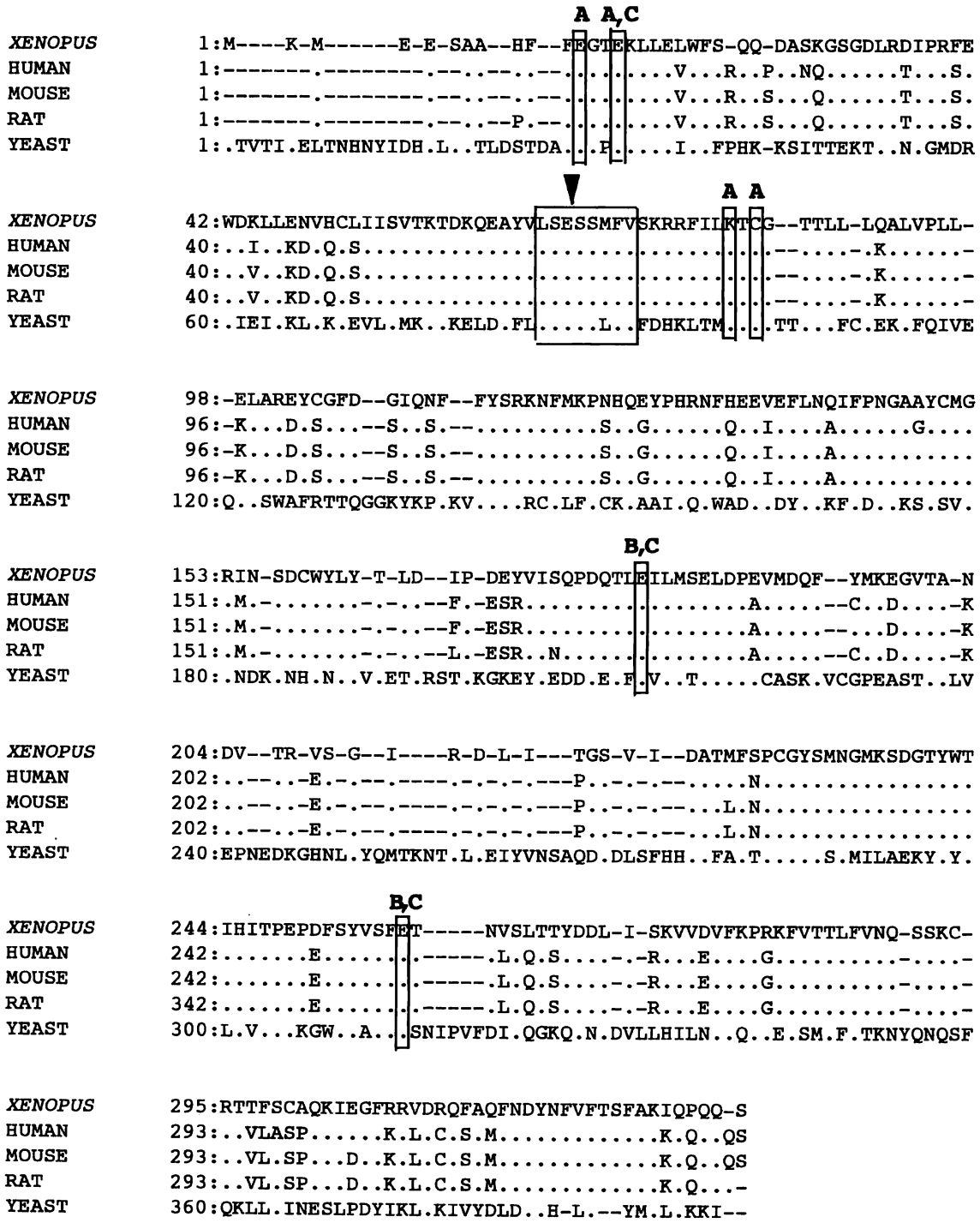

Fig. 1 Comparison of amino acid sequences of SAMDCs. *Dots* are for identical amino acids, and *bars* for gaps or amino acids missing. A *large box* is for proenzyme processing site, and the *arrowhead* for the cleavage site. *A* represents amino acids essential for enzyme activity, *B* represents amino acids involved in proenzyme processing, and *C* represents amino acids responsible for putrescine stimulation of the enzyme activity (from Shinga et al. 1996)

We examined SAMDC enzyme activity at cleavage, early neurula, and early tadpole stages using ^{14}C-SAM as a substrate. The enzyme level was low from the cleavage stage through early neurula stage, and became higher at the tadpole stage (40 h after fertilization). Thus, the changing level of SAMDC mRNA is well reflected in that of SAMDC enzyme activity in *Xenopus* embryogenesis.

Overexpression of SAMDC in *Xenopus* early embryos induces execution of the maternal program of apoptosis at MBT

We in vitro transcribed *Xenopus* SAMDC mRNA and microinjected it (1–10 ng/egg) into *Xenopus* fertilized eggs. Since the level of endogenous SAMDC mRNA is 0.005 ng/embryo or less, microinjection of 1 ng of SAMDC mRNA results in more than 200-folds of overexpression of the mRNA. In the mRNA-injected embryos, SAMDC enzyme activity became 40-folds in 1 h and 200-folds at fifth hour (blastula stage) of the control level. The SAMDC-overexpressed embryos cleaved normally up to the early blastula stage, but at MBT, they were suddenly dissociated into cells and completely dissolved (Shibata et al. 1998). When we injected beta-galactosidase and SAMDC mRNAs together into fertilized eggs, only beta-galactosidase-expressing cells were dissociated. When GFP mRNA and SAMDC mRNA were co-injected into one of the two blastomeres at 2-cell stage, only half portion of embryos that expressed GFP was dissociated, not at the early blastula stage (Fig. 2, top two), but at midblastula stage (Fig. 2, bottom two), indicating that only cells that received SAMDC mRNA were dissociated.

SAMDC mRNA was effective at 0.1–100 ng/egg, and in this wide range of dosage, cell dissociation took place constantly at MBT, suggesting the occurrence of some clock mechanism for determination of the timing of the cell dissociation. We dissociated SAMDC mRNA-injected embryos at early to late blastula stages into cells as described in our previous paper (Shiokawa and Yamana 1967), and examined DNA, RNA and protein synthesis by labeling the dissociated cells with ^{3}H-thymidine, ^{3}H-uridine and ^{14}C-leucine, respectively. We found that protein synthesis was the first to be inhibited. When we co-injected 1 ng of SAMDC mRNA and 20 pmol of ethylglyoxal-*bis*(guanylhydrazone) (EGBG), a specific inhibitor of SAMDC, cell dissociation was suppressed. In such embryos, the level of SAMDC was extremely high and that of SAM was extremely low. We then injected SAMDC mRNA (0.1 ng/egg) together with a large amount of SAM (200 pmol/egg). In such embryos, the level of SAM was recovered to the normal level, and cell dissociation did not take place. These results show that overexpression of SAMDC induces SAM deficiency, and this in turn induces massive cell dissociation. Electron microscopic analyses revealed that nuclei of such dissociated cells were fragmented, and in such embryos a large number of cells became TUNEL-positive, and DNA extracted therefrom formed "ladders" (Kai et al. 2000, 2001). When fertilized eggs were injected with SAMDC mRNA, and at 2-cell stage only one blastomere was further injected with a mixture of GFP mRNA and mRNA of Bcl-2, an anti-apoptotic factor, only the Bcl-2 mRNA-injected (GFP-expressing) half of the embryo was rescued from the dissociation (Kai et al. 2000). Furthermore, when fertilized eggs were co-injected with SAMDC and Bcl-2 mRNAs, a significantly large percentage (60–80%) of the injected

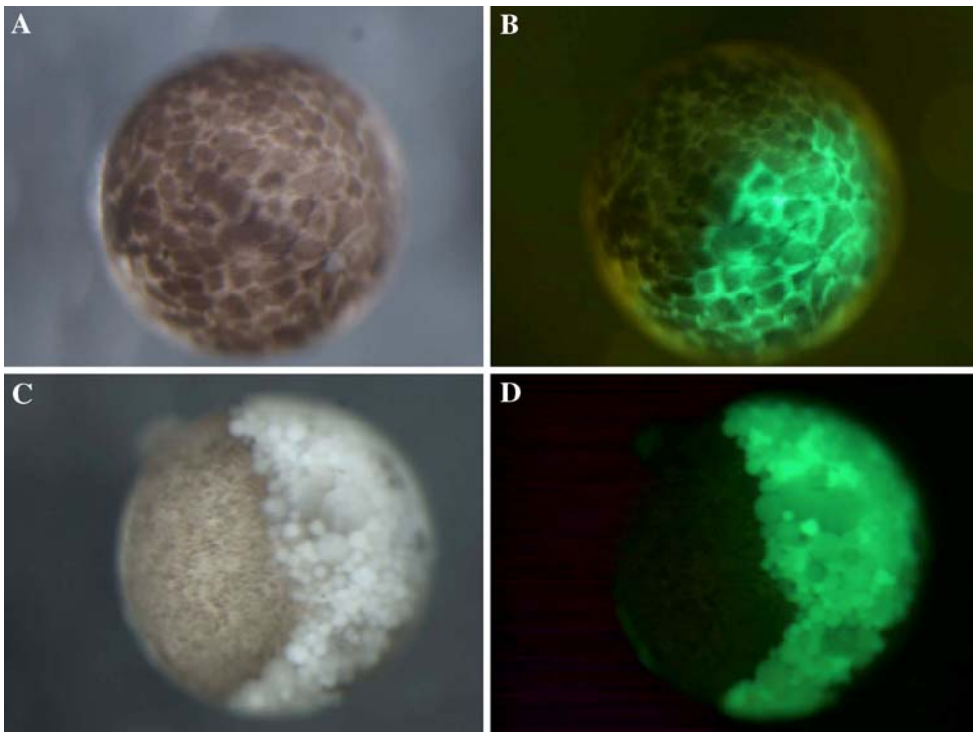

Fig. 2 Development of SAMDC mRNA-injected embryos. SAMDC mRNA (1 ng/egg) and GFP mRNA (100 pg/egg) were co-injected into a blastomere of a 2-celled embryo. The embryo was filmed at early blastula (**a**, **b**) and early gastrula (**c**, **d**) stages under the visible light (**a**, **c**) and UV light (**b**, **d**). In **b**, cells expressing GFP, hence overexpressed with SAMDC, cleaved normally, but at MBT apoptosis was executed in GFP-expressing (**d**) cells (**c**) (from Shiokawa et al. 2008)

embryos developed into tadpoles. We, therefore, concluded that the SAMDC-induced cell dissociation was due to the execution of apoptosis (Kai et al. 2000; Shiokawa et al. 2000). While we were studying the SAMDC-induced apoptosis, Jim Maller and others reported that cleavage stage embryos treated with various toxic agents, such as gamma-ray, hydroxyurea, cycloheximide, and alpha-amanitin all underwent apoptosis at the MBT (Anderson et al. 1997). In all of these experiments, however, injection of Bcl-2 mRNA only postponed the onset of cell dissociation by 2–3 h, but never rescued the embryos.

In the SAMDC-overexpressed embryos, we analyzed caspases, a cysteine protease enzyme family. We found here that caspases 8 and 9, and probably 3 are involved in this order in the execution of the apoptosis (Takayama et al. 2004; Shiokawa et al. 2005, 2008). Furthermore, we found that while mRNAs for caspases 9 and 3 occur as maternal mRNAs, caspase 8 mRNA is newly transcribed in the SAMDC-overexpressed embryos before MBT (Shiokawa et al. 2005). We performed experiments to follow the fate of one blastomere that received GFP and SAMDC mRNAs at 8–32 cell stages, we concluded that the execution of the apoptosis in *Xenopus* early embryos serves as a fail-safe mechanism of early embryogenesis to eliminate metabolically damaged cells at MBT to save the rest of the embryo for subsequent development (Kai et al. 2003; Shiokawa et al. 2008). We, therefore, proposed a model to explain how early embryogenesis proceeds in *Xenopus* (Fig. 3).

Changes in the level of polyamines during oocyte growth and maturation, and embryogenesis in *Xenopus laevis*

Three natural polyamines (putrescine, spermidine, and spermine) occur ubiquitously in eukaryotic cells, and polyamine biosynthesis is strictly regulated by various factors, including polyamines themselves. Developmental studies on polyamines have been performed using oocytes and embryos of mouse, chick, sea urchin, polychaete, and *Drosophila*. In amphibians, including *Xenopus laevis*, polyamines have also been studied in oocytes and embryos (Rosander et al. 1995). Polyamine compositions have not been examined throughout oogenesis, oocyte maturation, and embryogenesis (Shinga et al. 1996). We found that levels of putrescine, spermidine, and spermine per oocyte increase during oogenesis, but during oocyte maturation, the level of spermine greatly decreases to reach a background level (less than 0.1 nmol/embryo), and the lowered level was maintained from fertilization through early tadpole stages. By contrast, both putrescine and spermidine maintained high levels throughout development; the level of putrescine being twice or three times that of spermidine throughout the different developmental stages (Shinga et al. 1996). The high level of putrescine may be important in oocyte maturation or germinal vesicle breakdown (GVBD) and in early embryogenesis. The remarkable decrease in the level of spermine during oocyte maturation may be correlated to the drastic change in physiological states from an oocyte to an egg.

We followed the changes in the levels of polyamines also in SAMDC-overexpressed embryos. In spite of our expectation, SAMDC overexpression did not induce a large change: with 1 ng/egg of SAMDC mRNA, the level of putrescine decreased by ca. 25%, and that of spermidine increased also by ca. 25%, with little change in the level of spermine. Since the putrescine/spermidine ratio was lowered from its normal value (ca. 2.0–3.0) to 1.0 in SAMDC-overexpressed embryos, we co-injected putrescine (1 nmol/egg) and SAMDC mRNA (1 ng/egg) and resumed the normal putrescine/spermidine ratio in SAMDC mRNA-injected embryos (Shibata et al. 1998). However, the

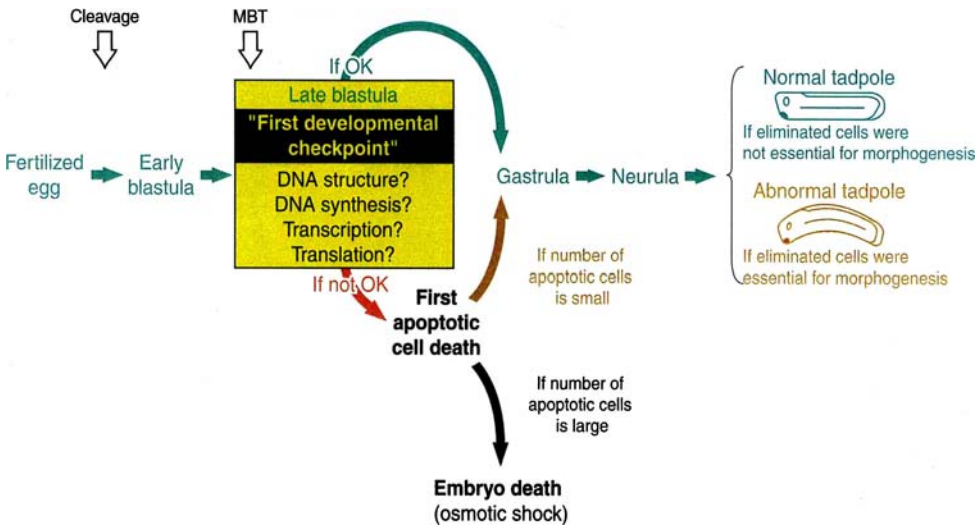

Fig. 3 A model showing how early development proceeds. Fertilized eggs cleave rapidly until MBT, when the "first developmental checkpoint" appears. This check mechanism determines if a cell continues or stops development by execution of apoptosis. When the number of apoptotic cells is small, the embryo continues to develop (from Shiokawa et al. 2008)

recovery of the normal putrescine/spermidine ratio did not rescue the embryos from the execution of apoptosis induced by SAMDC overexpression.

Developmental changes in the amounts of ornithine and other amino acids during oogenesis, oocyte maturation, and early embryogenesis of *Xenopus laevis*

A frog egg is a unique cell in that a number of cellular components are stored as maternal stockpiles. A *Xenopus* egg contains large amount of yolk granules and lipid droplets as nutrient sources and other cytoplasmic constituents, such as mitochondria (approx. 5×10^5/egg), ribosomes (approx. 10^{12}/egg), and maternal mRNAs like mRNAs for caspases (Takayama et al. 2004; Shiokawa et al. 2005), activin receptor (Kondo et al. 1991), and aldolases (Kajita et al. 2000).

We have been interested also in low-molecular-weight stockpiles, such as ornithine, the starting material to produce putrescine, and other amino acids and amines, and analyzed acid-soluble fractions of oocytes, unfertilized eggs, and embryos of *Xenopus laevis* (Shiokawa et al. 1986c). In the amino acid profiles obtained from full-grown oocytes, matured oocytes, unfertilized eggs, and swimming tadpoles, the number of ninhydrine-reacting components was the same (ca. 20). In all the profiles, aspartic acid and glutamic acid were the fast-moving, largest components, and ammonia was as a slow-moving component, whose amount was large in oocytes and unfertilized eggs but was much smaller in tadpoles.

We repeated the amino acid analyses, and summarized the results in Figs. 4 (for oogenesis) and 5 (for embryogenesis). The amount of ornithine was relatively small and did not change greatly from oocyte maturation (Fig. 4) through swimming tadpole stage (Fig. 5). Amounts of both glutamic acid and aspartic acid increased early during oocyte growth (stage III) and the elevated levels were maintained until the end of oogenesis. The amount of lysine increased once from stage III to stage V, but

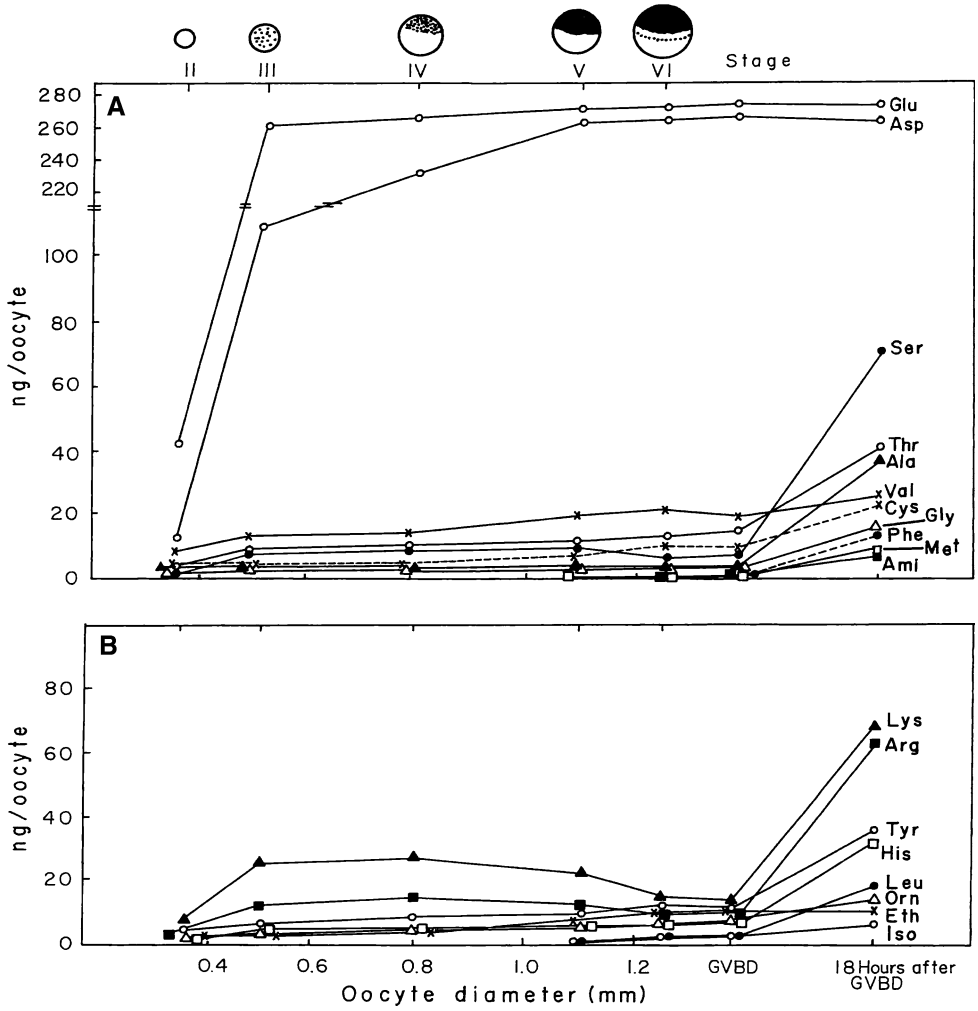

Fig. 4 Changes in amino acid contents during oogenesis and oocyte maturation in *Xenopus laevis*. Amino acids are indicated by three *letters*. The grouping into **a** and **b** is arbitrary (from Shiokawa et al. 1986c)

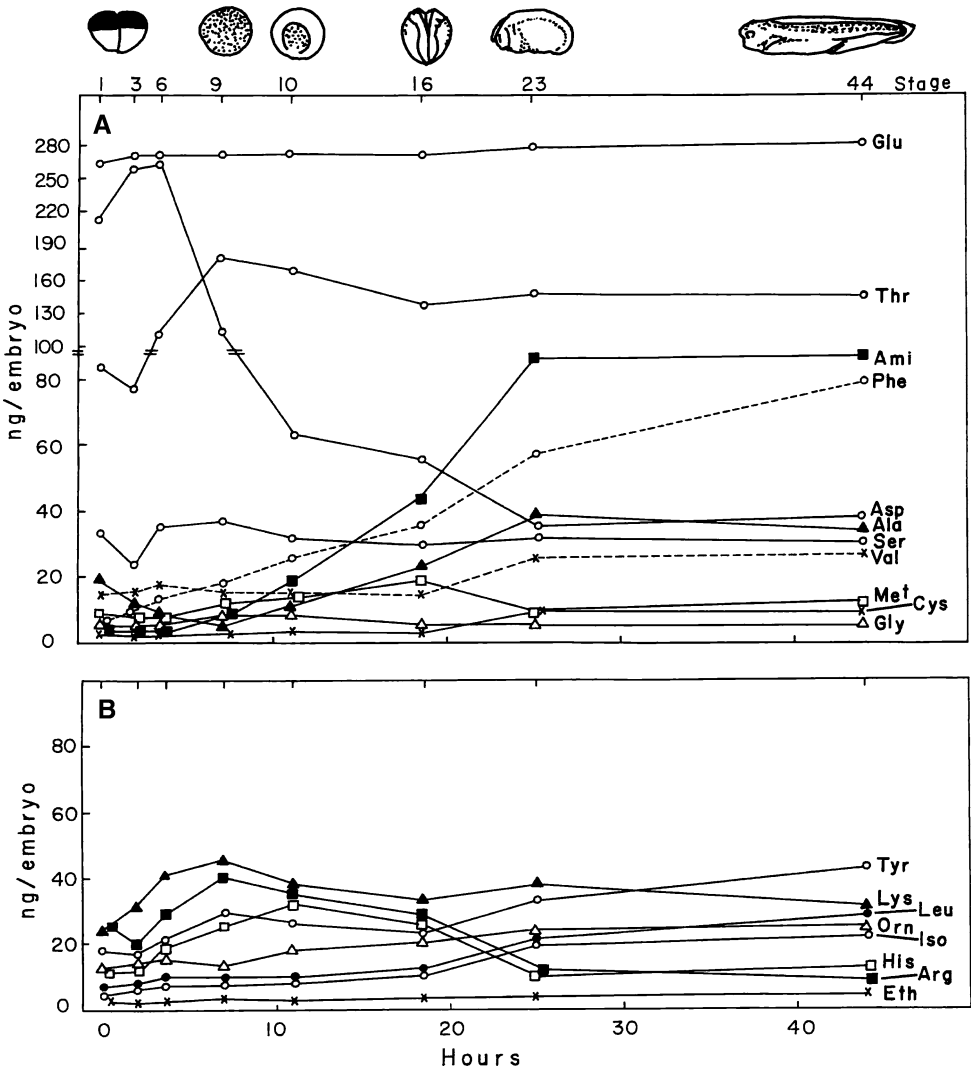

Fig. 5 Changes in amino acid contents during embryogenesis of *Xenopus laevis*. The grouping into **a** and **b** is arbitrary (from Shiokawa et al. 1986c)

decreased at stage VI. The treatment of full-grown oocytes with progesterone induces oocyte maturation, but this exerted little change in the levels of aspartic acid, glutamic acid, and other amino acids, although phenylalanine and gamma-aminobutyric acid became detectable and amounts of threonine, serine, and lysine considerably increased (Fig. 4). During embryogenesis, the amount of gamma-aminobutyric acid increased sharply from neurula stage to tailbud stage, probably reflecting the development of neural tissues.

We also analyzed oocytes and embryos of *Xenopus borealis*, and obtained results, which paralleled those of *Xenopus laevis* (Table 1). It is apparent that an egg of both *Xenopus laevis* and *Xenopus borealis* contains much the same amount of amino acids as a tailbud embryo. This indicates that amino acids are also stockpiles which are accumulated abundantly (more than 10,000-folds of a cell of a tailbud embryo) in the egg for later use during development.

Weak bases selectively inhibit ribosomal RNA synthesis in *Xenopus laevis* neurula cells

An egg of *Xenopus laevis* contains at least several thousand-folds of ribosomes present in an adult cell, which is enough to support protein synthesis in an embryo composed of more than 100,000 cells (Gurdon and Brown 1965). In the development of *Xenopus laevis*, rRNA synthesis is regulated in such a way that it commences at the late blastula stage or shortly after MBT (Shiokawa et al. 1981a, b). As for the mechanism of such developmental regulation, the nuclear transplantation experiment by Gurdon and Brown (1965) strongly suggested the involvement of a cytoplasmic factor.

Shiokawa and Yamana (1967) prepared dissociated blastula and neurula cells and cultured these cells together or separately and reported that *Xenopus* blastula cells release a low-molecular-weight substance that selectively inhibits rRNA synthesis in neurula cells (Shiokawa and

Table 1 Amino acid composition of eggs and embryos of *Xenopus borealis* and *Xenopus laevis*

Amino acids	Stage					
	Unfertilized egg		Blastula (ng/egg or embryo)		Gastrula	
	borealis	*laevis*	*borealis*	*laevis*	*borealis*	*laevis*
Aspartic acid	102.7	211	67.0	114	48.0	62.9
Threonine	98.5	80.5	103	117	89.4	164
Serine	27.9	33.7	26.9	37.8	22.8	31.4
Glutamic acid	192	262	220	262	150	262
Glycine	1.54	5.95	2.15	7.01	5.77	9.01
Alanine	12.3	19.3	7.47	6.88	5.20	12.9
Cysteine	3.71	3.33	4.33	2.53	3.77	2.77
Valine	18.6	14.3	15.8	14.4	10.2	13.0
Methionine	1.66	7.15	1.34	15.5	3.04	13.4
Isoleucine	9.71	4.79	6.89	7.21	6.01	8.35
Leucine	14.7	6.80	9.79	9.21	8.79	8.28
Tyrosine	23.7	18.4	21.6	29.9	26.9	25.7
Phenylalanine	11.2	7.89	10.9	18.6	16.4	26.4
γ-Aminobutyric acid	8.31	5.98	7.73	8.39	6.59	19.5
Ornithine	16.0	13.0	16.3	13.4	12.4	18.2
Lysine	19.3	23.4	16.1	46.5	19.1	38.7
Ethanolamine	0.66	3.87	0.60	3.39	0.70	2.51
Histidine	13.0	13.7	11.3	26.1	10.5	33.1
Arginine	12.1	24.8	8.72	40.5	11.1	37.1

From Shiokawa et al. (1986c). Each value is the mean of two independent determinations

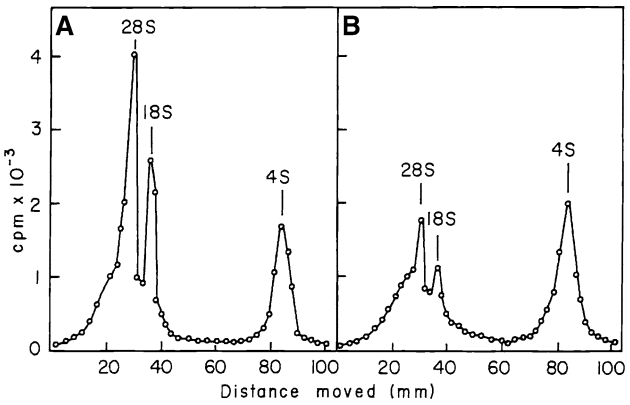

Fig. 6 Patterns of ^{3}H-uridine-labeled RNAs. Neurula cells were labeled for 5 h with ^{3}H-uridine in the presence (**b**) or absence (**a**) of the chloroform–methanol extract. RNAs were extracted and electrophoresed on agarose–polyacrylamide gels (from Shiokawa et al. 1985)

Yamana 1967b). It appeared that the rRNA synthesis-inhibiting activity in blastula cell-conditioned medium or blastula cell extract could be adsorbed on a charcoal column and eluted from the column by ammonia–alcohol (Laskey et al. 1973; Shiokawa et al. 1985). We attempted to purify the active component from the charcoal extract, and after many tries and errors, we finally found that a specific rRNA synthesis-inhibiting component could be extracted by chloroform–methanol from the ammonia–alcohol eluate. Figure 6 shows a typical result showing the specific inhibition of rRNA synthesis by the chloroform–methanol extract. From the chloroform–methanol extract, we finally purified ammonium perchlorate as the active substance, which strongly and selectively inhibited rRNA synthesis in *Xenopus* neurula cells (Shiokawa et al. 1985). Based on this result, we tested the effects of various inorganic and organic ammonium salts on rRNA synthesis in *Xenopus* neurula cells.

We found here that ammonium chloride, ammonium sulfate, ammonium dihydrogenphosphates, ammonium monohydrogenphosphates, ammonium phosphates, ammonium acetate, ammonium aspartate, and ammonium salts of 5′-AMP, 5′-GMP, 5′-UMP, and 5′-CMP all selectively inhibit rRNA synthesis in neurula cells at 2.5 mM (Shiokawa et al. 1985, 1986a, b). We also tested the effect of potassium dihydrogenphosphates, potassium monohydrogenphosphates, potassium phosphates, and potassium salt of aspartic acid, but none of these inhibited rRNA synthesis even at 5–10 mM. We further tested the effect of amines, and found that monomethylamine hydroperchloride, dimethylamine hydroperchloride, trimethylamine hydrochloride, and three kinds of corresponding

ethylamines were all active as selective inhibitors of rRNA synthesis at 2.5 mM. We similarly tested polyamines, such as putrescine, spermidine and spermine, and amino acids like threonine and ornithine, and in addition, alpha-methylornithine that has been claimed to be a direct stimulator of rRNA synthesis in oocytes, but none of these were effective as a selective inhibitor of rRNA synthesis even at 10 mM.

When we analyzed acid-soluble fractions of *Xenopus* neurula cells which had been exposed to 2.5 mM of ammonium chloride, or three kinds of ammonium phosphates, we found that the amount of ammonia became quite large even after 1 h of treatment. In the extract from cells exposed to monomethylamine hydroperchloride, dimethylamine hydroperchloride, or trimethylamine hydrochloride, a large amount of monomethylamine, dimethylamine, or trimethylamine was detected. Therefore, we concluded that ammonium ion and amines selectively inhibit rRNA synthesis in *Xenopus* neurula cells after being uptaken into the cytoplasm.

Ammonium ion is a strong candidate of the regulator that controls the commencement of rRNA synthesis at MBT in *Xenopus* embryogenesis

When we examined ninhydrin-positive materials in the acid-soluble fraction of *Xenopus* early embryos, there was a sizable amount of ammonia but not amines. The level of ammonia in an egg was 55–60 ng/egg before fertilization, which corresponds to ca. 3 mM of ammonia as an intra-egg concentration, since the volume of an egg is ca. 1 µl. During cleavage, the level of ammonia did not change greatly, but after MBT it sharply decreased to ca. 20 ng/embryo, and further to 10–15 ng/egg at the tailbud stage (Shiokawa et al. 1986a).

We attempted to isolate ammonia (and amines if any) from cleavage stage embryos. Starting from 25,000 cleavage embryos, we obtained ca. 10 mg of residual materials after evaporating the hydrochloric acid solution used to capture the cellular volatile components. Mass spectrometric analysis revealed the presence of ammonium chloride but not amines. Finally, 10 mg of ammonium chloride was obtained, which corresponded to ca. 4 mg of ammonia. When we cultured neurula cells in the medium which contained 1.0 or 5.0 mM of the embryo-derived ammonium chloride, rRNA synthesis was inhibited by 60 or 90%, respectively, with only a slight inhibition (less than 10%) in 4S RNA (tRNA) synthesis.

We then tested if the obtained ammonium salt inhibits the commencement of rRNA synthesis in late blastula cells. When blastula cells were labeled for 5 h, a small amount of incorporation was detected in 28S and 18S rRNA in both untreated and 2.5 mM potassium chloride-treated cells, but not in 2.5 mM ammonium chloride-treated cells. Much clearer inhibition of rRNA synthesis was obtained when we treated blastula cells for 5 h with 2.5 mM of ammonium chloride and then labeled the cells for 5 h in the continued presence of ammonium chloride. The results were the same also when we labeled cells for 5 h after 10 h of pre-treatment with the ammonium chloride. These results indicate that ammonium salt actually inhibits both the commencement and subsequent activation of rRNA synthesis in *Xenopus* early embryos. In this experiment, the synthesis of 4S RNA, the main portion of which is transfer RNA (tRNA), and heterogeneous nuclear RNA, and in addition, the synthesis of DNA was not inhibited. In addition, protein synthesis, cell division, and cellular reaggregation were not inhibited. Furthermore, when ammonium ion was eliminated by repeated medium change, the activity to synthesize rRNA was restored, indicating that the inhibitory effect was reversible. We, therefore, concluded that ammonium ion is a strong candidate for the regulator of rRNA synthesis in *Xenopus* embryos (Shiokawa et al. 1986a).

Weak bases inhibit rRNA synthesis at the transcription level and the inhibition is realized most probably via slight pH elevation within the cell

To know the mechanism of the inhibition of rRNA synthesis by weak bases, *Xenopus* neurula cells were first inhibited for 2.5 h by 5 mM of ammonium chloride or monomethylamine hydroperchloride then pulse-labeled for 2.5 h in the continued presence of the weak bases. Both ammonium chloride and monomethylamine hydroperchloride inhibited 40S pre-rRNA synthesis (Shiokawa et al. 1979), in addition to the inhibition of 18S and 28S mature rRNA synthesis. These results indicated that the inhibition is at the transcriptional level. We then selected conditions in which ammonium chloride and trimethylammonium perchloride inhibit rRNA synthesis partially (by ca. 60%). When we treated neurula cells under such conditions (2.6 mM ammonium chloride or 1 mM trimethylammonium perchloride for 2.5 h) and labeled the cells for only 1 h, similar extents of inhibition were obtained in the labeling of 40s pre-rRNA, 30s rRNA intermediates and 18S rRNA (60, 59, and 70%, respectively). These results suggested that the weak bases did not inhibit the processing of rRNA primary transcript to the mature rRNAs. We further tested the effect of these weak bases on the stability of mature 18S and 28S rRNA by chasing the label that had been incorporated into these RNA using a high dose of actinomycin D (10 µg/ml). We found that the weak bases used here did not instabilize 18S and 28S mature rRNAs.

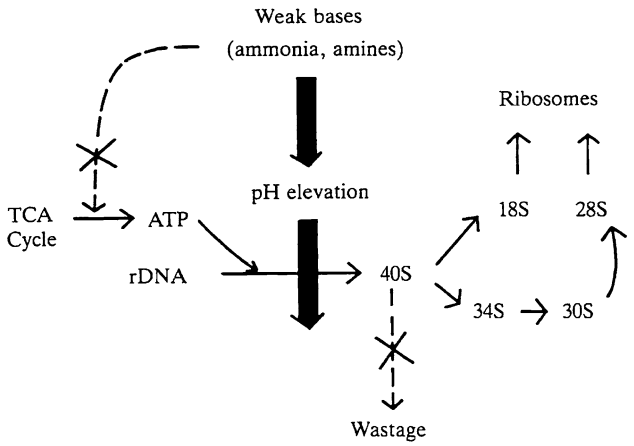

Fig. 7 A working hypothesis of the mode of action of weak bases on rRNA synthesis in *Xenopus* embryonic cells. Weak bases neither interfere with ATP generation (*upper cross symbol*), nor induce degradation (*wastage*) of 40S pre-rRNA (*lower cross symbol*), but inhibit 40S pre-rRNA formation (*lower solid arrow*), probably at the transcription level via a slight elevation of intracellular pH (*upper solid arrow*) (from Shiokawa et al. 1987)

When we tested the effect of 3 mM ammonium chloride and 1.5 mM trimethylammonium perchloride in neurula cells in a medium in which all the Na^+ was replaced by choline ions, the inhibitory effects of both of these weak bases were completely abolished, suggesting strongly that the inhibition observed is realized by a slight increase in the intracellular pH. Finally, we measured the level of ATP and other ribonucleotide triphosphates in the acid-soluble fraction of neurula cells after treating them for 3 h with 3 mM ammonium chloride or 0.5 mM trimethylammonium perchloride. The results obtained showed that the level of ATP as well as other ribonucleotide triphosphates remained unchanged. Based on these results, we proposed a working hypothesis that weak bases suppress rDNA transcription probably via a process that involves a slight increase in the intracellular pH (Fig. 7).

Conclusion

We demonstrated the following results: (1) the amino acid sequence of *Xenopus* SAMDC, (2) the occurrence of the maternal program of apoptosis in *Xenopus* eggs, (3) developmental changes in polyamine composition, (4) developmental changes in the levels of ornithine and other amino acids, (6) ammonium ion is a strong candidate for the regulated initiation and activation of ribosomal RNA synthesis which takes place not before MBT but shortly after MBT in *Xenopus* embryogenesis, and (7) weak bases inhibit rRNA synthesis at the transcriptional level probably via slight increase in the intracellular pH.

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