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Putrescine and Spermidine Biosynthesis in the Development of Normal and Anucleolate Mutants of *Xenopus laevis*

DIANE H. RUSSELL

National Institutes of Health, National Cancer Institute, Baltimore Cancer Research Center, Laboratory of Pharmacology, Baltimore, Maryland 21211

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ABSTRACT Ornithine decarboxylase (EC 4.1.1.17), the enzyme that catalyzes the synthesis of putrescine from ornithine, increases dramatically in developing *Xenopus* embryos. Between the 2-cell stage and early blastula stage, activity increases 10-fold, and in swimming tadpoles, the enzyme activity is 100-fold higher than that present in either unfertilized eggs or 2-cell embryos. *S*-adenosyl-L-methionine decarboxylase, an enzyme that catalyzes spermidine synthesis from putrescine and *S*-adenosyl-L-methionine, increases 40-fold in activity during the development of *Xenopus*, but does not increase in activity prior to gastrulation. Concomitant with these enzyme changes, putrescine and spermidine concentrations are elevated during the development of *Xenopus* embryos. Maximal accumulations are present in the swimming tadpole and correspond to maximal enzyme activities. Anucleolate-mutant embryos of *Xenopus*, which do not synthesize new ribosomes, have no detectable *S*-adenosyl-L-methionine decarboxylase activity and do not accumulate spermidine after gastrulation. Ornithine decarboxylase activity is depressed in these mutants and putrescine accumulation is decreased also. The activity of some dehydrogenases that increase in *Xenopus* embryos after gastrulation show normal increases in the anucleolate mutants. Thus, the synthesis of putrescine and spermidine in embryos correlates with the onset of ribosomal-RNA synthesis and the formation of a viable nucleolus in the embryonic cell.

Although the physiological role or roles of putrescine and the polyamines, spermidine and spermine, are not completely understood, there is increasing evidence that these amines may play a role in growth processes by regulating RNA metabolism. These compounds occur ubiquitously in living tissues, but are found in highest concentrations in tissues that actively synthesize protein and have a high RNA content (1). Indeed, there are many parallels that can be cited between the cellular concentrations of RNA and spermidine. Spermidine concentration, for example, decreases with age in rat liver, as does RNA content (2). In regenerating rat liver, increased spermidine concentration is closely correlated with increased RNA concentration (3-6). Maximal spermidine and RNA concentrations also coincide in developing chick embryos (7). Hypophysectomy causes a drop in spermidine and RNA concentrations in the liver; the concentrations can be restored to normal by administration of growth hormone (8).

Another aspect of polyamine physiology that is being actively explored is the apparent "priming" of tissues about to undergo proliferation or rapid growth. This priming takes the form, in various tissues (9-12), of dramatically elevated activity of ornithine decarboxylase (ODC), the enzyme catalyzing the formation of putrescine. In contrast, ODC activity in static or nongrowing tissues is very low. When induced in mammalian liver, ODC activity rises rapidly and remains elevated for several days. As soon as the stimulus is removed, ODC activity returns to the control activity or below, with a half-life of 10-20 min (13). This rapid fluctuation of ODC activity in response to the introduction or withdrawal of stimuli suggests that putrescine synthesis is finely controlled. In addition, ODC has two important characteristics that presuppose an important function in growth processes:
(*1*) ODC exhibits a dramatic early induction after growth-hormone administration (14, 15). *De novo* synthesis is involved since the administration of RNA and protein inhibitors indicate that both protein synthesis and DNA-dependent RNA synthesis are necessary for this elevation to occur (13).
(*2*) ODC has the most rapid turnover rate of any known mammalian enzyme (13).

The early increase of putrescine synthesis correlates well with the several-fold increase in RNA polymerase activity that occurs early in the growth process in a number of organisms. RNA polymerase I (a nucleolar enzyme) increases severalfold 6-12 hr after partial hepatectomy in the rat (16); during this same period, ODC activity increases 10-20 fold (9). RNA polymerase activity can be affected *in vitro* by the addition of putrescine (17, 18).

In bacteria, spermidine is synthesized by two enzymes: *S*-adenosyl-L-methionine (AdMet) is first decarboxylated by *S*-adenosyl-L-methionine decarboxylase AdMet decarboxylase and the decarboxylated AdMet then serves as a propylamine donor for putrescine. A propylamine transferase then completes spermidine synthesis by transferring propylamine from decarboxylated AdMet to putrescine (1). In contrast, a soluble enzyme has been prepared from rat ventral prostate that forms spermidine and 5′-methylthioadenosine from putrescine and AdMet. Decarboxylated AdMet could not be isolated as an intermediate and the reaction required putrescine, indicating that one enzyme, or enzyme complex, may be responsible for both decarboxylation and propylamine transfer (19).

If putrescine and spermidine are involved in the synthesis of a particular species of RNA, this might be elucidated by studying the developing embryos of *Xenopus laevis*, the African clawed toad, in which there is some temporal separa-

Abbreviation: ODC, ornithine decarboxylase (EC 4.1.1.17); AdMet, *S*-adenosyl-L-methionine.

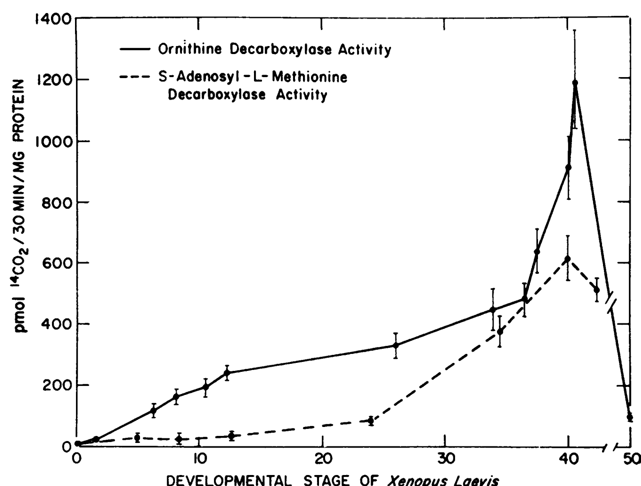

FIG. 1. Ornithine decarboxylase activity and S-adenosyl-L-methionine decarboxylase activity in developing *Xenopus* embryos. The activities of both enzymes were determined by measuring the liberation of [^{14}C]CO_2 from carboxyl-labeled substrates as described in *Methods*. Each point represents the mean ± SE of five or more determinations (50 embryos per determination).

tion between the synthesis of different species of RNA. Brown and coworkers (20) have shown that the oocytes synthesize and accumulate large amounts of ribosomes, most of which are stored in the egg as free monosomes. During early cleavage there is no detectable RNA synthesis. DNA-like RNA synthesis begins at late cleavage (about 8000 cells), 4S RNA synthesis begins at the blastula stage (15,000 cells), and rRNA synthesis is first found at the onset of gastrulation (about 30,000 cells). The relative rates of synthesis of these classes of RNA change during subsequent development until rRNA synthesis predominates.

A further advantage is the availability of a mutant of *Xenopus laevis* in which the structural genes for ribosomal RNA are known to be deleted (21). Embryos homozygous for this deletion do not synthesize any new rRNA but live for a few days utilizing the ribosomes stored in the eggs. There are, therefore, normal periods of development in which no new ribosomes are synthesized (cleavage), periods during which other kinds of RNA are synthesized (late cleavage and blastula), and a discrete period of onset of ribosome synthesis (gastrula) (22). Finally, mutant embryos exist that do not synthesize ribosomes at stages when normal embryos do so.

I therefore studied the synthesis and accumulation of putrescine and spermidine, and the relationship of these polyamines to RNA synthesis during the development of normal embryos and anucleolate mutants of *Xenopus laevis.*

MATERIALS AND METHODS

[1-^{14}C]D,L-ornithine monohydrochloride (2.74 Ci/mol), [1-^{14}C]D,L-methionine (3.54 Ci/mol), and [1,4-^{14}C]putrescine dihydrochloride (11.27 Ci/mol) were obtained from New England Nuclear Corp. Pyridoxal phosphate was obtained from Nutritional Biochemicals. NSD-1055 (4-bromo-3-hydroxy-benzyloxyamine dihydrogen phosphate) was a gift from Smith and Nephew Research, Ltd. *Escherichia coli* B cells were obtained from Grain Processing, Muscatine, Iowa.

Oocytes, unfertilized eggs, fertilized eggs, and anucleolate mutants of *Xenopus laevis* were graciously furnished by Donald D. Brown and Jack Chase, Carnegie Institution of Washington, Baltimore, Md. Embryos were staged according to the table of Nieuwkoop and Faber (23).

For enzyme assays, the material was homogenized in five vol of 0.1 M sodium–potassium phosphate buffer, pH 7.2. The homogenate was centrifuged at 20,000 × *g* for 20 min, and the supernatant was assayed. The supernatant after centrifugation at 100 000 × *g* for 90 min gave identical enzyme activities, therefore, the 20,000 × *g* supernatant was used routinely. No activity was detectable in the pellet. Protein was determined by the Lowry method (24) with bovine serum albumin as the standard. Neither enzyme was stimulated by the addition of either 2-mercaptoethanol or dithiothreitol. These compounds seem to be stimulatory to ODC activity only after $(NH_4)_2SO_4$ fractionation or prolonged dialysis (25).

Determination of ODC Activity. ODC activity was determined by measuring the liberation of [^{14}C]CO_2 from carboxyl-labeled substrate as described (9). The substrate concentration (50 μM as L-ornithine) was not saturating. However, in some experiments, excess ornithine (2 mM) was used as substrate, and the same general changes in enzyme activity were obtained. The reaction was linear for at least 30 min; two protein concentrations were run for each determination.

Preparation of Carboxyl-labeled AdMet. Carboxyl-labeled AdMet was prepared from [1-^{14}C]methionine and ATP: L-methionine-S-adenosyltransferase (EC 2.5.1.6) isolated (26) from *E. coli* B cells. The reaction mixture and procedures for the preparation were as described (19) except that the *E. coli* enzyme was used.

Determination of AdMet Decarboxylase Activity. Enzyme activity was determined by measuring the liberation of [^{14}C]CO_2 from [^{14}C]AdMet as described (19). Incubation mixtures consisted of 150 μM [^{14}C]AdMet, 2.5–5 μM sodium–potassium phosphate buffer (pH 7.2), 50 μM pyridoxal phosphate, 2.5 mM putrescine, and 2–6 mg of protein from the 20,000 × *g* supernatant fraction, to make a total volume of 0.2 ml. At least two different protein concentrations were run (in duplicate) for each sample and the measured activity was always proportional to the amount of enzyme supernatant solution added. When excess AdMet was used, the same proportional changes in enzyme activity were obtained.

Determination of [^{14}C]spermidine Formation from [^{14}C]putrescine. To determine if the evolution of [^{14}C]CO_2 from [^{14}C]AdMet was a reliable index of spermidine formation, [^{14}C]putrescine was added to the assay mixture instead of [^{14}C]AdMet. Incubation conditions were the same as described above. However, the reaction was stopped with 0.25 ml of 0.1 N NaOH, and the solution was extracted into *n*-butanol as described (6). Amines were separated by high-voltage electrophoresis and quantitated with an acid ninhydrin stain (27). In all cases, [^{14}C]CO_2 evolution from [^{14}C]AdMet was a reliable index of spermidine formation.

Determination of Putrescine, Spermidine, and Spermine Concentrations. Pools of embryos (0.5 g) were homogenized in 4 vol of 0.1 N HCl and the supernatant solution extracted into *n*-butanol (6). The amines were separated by high-voltage electrophoresis (80 V/cm for 1.5 hr) in a 0.1 M citric acid–NaOH buffer, pH 4.3. Recoveries of amines were corrected by the recoveries determined from samples to which 0.15 μCi of the appropriate [^{14}C]amine had been added prior to homoge-

nization. Amine concentrations were determined by staining the chromatography sheet (Whatman 3MM paper) with a mixture of 1 g ninhydrin, 100 ml acetone, 5 ml concentrated acetic acid, and 100 mg cadmium acetate, drying 90 min at 60°C, eluting the color, and recording the absorbancy at 505 nm (27).

Detection of Anucleolate Mutants. Only 25% of the embryos of a heterozygous mating will be homozygous recessive (anucleolate) mutants (28). Anucleolate mutants were separated, starting at stage 24, by the following procedure: a tiny section of the tail of each embryo was dissected and placed in isotonic salts on a glass slide. A cover slip was placed over this and tapped gently with a pencil to spread the cells. These slides of embryos of stages 24–33 were examined under the phase microscope. If nucleoli were not detectable, the embryos were classified as anucleolate mutants. By stage 41, visible morphological abnormalities appeared in the mutants that made it possible to separate them on the basis of examination under a dissection scope.

RESULTS

ODC activity at various developmental stages

ODC activity was low in unfertilized eggs and in embryos of the two-cell stage (Fig. 1). However, activity was 10-fold higher in early blastulae than in two-cell embryos; it continued to increase, and was 100-fold higher at stage 41 than the activity present in unfertilized eggs. The enhancement of ODC activity prior to gastrulation (stages 10–12) is worthy of special note since very few enzymes increase in activity prior to gastrulation in amphibians (29).

ODC activity of the anucleolate mutant

The pattern of ODC activity was strikingly different in the anucleolate mutant (Table 1). At hatching (about stage 24), the earliest time that anucleolate mutants could be separated reliably from normal embryos, ODC activity was 70% of that found in normal embryos; activity was 40% of controls by stage 33, and activity was only 6% of that found in normal embryos by stage 41. It is important to note that the ODC activity detectable at stage 24 in the anucleolate mutant could be accounted for by the enzyme present before gastrulation. Since protein concentration and weight do not change from fertilization to about stage 41, the decreased activity could be due to inactivation of ODC. When the supernatant from normal and anucleolate mutants is mixed, the activity is always additive, and is comparable to the amounts of the supernatants assayed separately.

AdMet decarboxylase activity in developing embryos

AdMet decarboxylase activity was low in unfertilized eggs; in contrast to the pattern with ODC, it did not increase prior to gastrulation (Fig. 1). However, there was a 5- to 6-fold increase in activity by stage 24; thereafter, the activity rose rapidly and was 40-fold higher than in unfertilized eggs by stage 40.

AdMet decarboxylase activity in anucleolate mutants

The activity in anucleolate mutants of stage 24 was similar to that found in unfertilized eggs (Table 1), and at all stages prior to gastrulation. In contrast to the considerable AdMet decarboxylase activity detectable in normal embryos at both stages 33 and 41, AdMet decarboxylase was undetectable in anucleolate mutants (Table 1). The activity present at stage 24 could be accounted for by the amount of enzyme present before gastrulation. These data indicate that there is no induction of AdMet decarboxylase in anucleolate mutants. Also, when the supernatant from normal and anucleolate mutants is mixed, the activity is always additive and is comparable to that of the supernatants assayed separately.

TABLE 1. *Putrescine and spermidine biosynthesis in Xenopus laevis*

Developmental stage	Ornithine decarboxylase		S-adenosyl-L-methionine decarboxylase	
	Anucleolate mutants	Controls	Anucleolate mutants	Controls
Stage 24	226 ± 27	321 ± 36	14 ± 3.3	87 ± 82
33	184 ± 29	463 ± 57	<2.0	285 ± 32
41	94 ± 15	1450 ± 200	<2.0	673 ± 81
42	58 ± 9.1	880 ± 120	<2.0	692 ± 83

Each value represents the mean ± SE enzyme activity (nmol $[^{14}C]CO_2$ per 30 min per mg of protein) for five pools, each containing five embryos. Embryos were staged according to the table of Nieuwkoop and Faber (23).

Activity of certain dehydrogenases as measured in normal and anucleolate mutants

Since both ODC and AdMet decarboxylase activities were low or absent during the development of the anucleolate mutant, the question arose as to whether all enzymes might be depressed in anucleolate mutants. Several enzymes known to increase in amphibians (29) after hatching were examined in normal embryos and in anucleolate mutants. The enzymes studied (29) were lactate dehydrogenase (EC 1.1.1.27), malate dehydrogenase (EC 1.1.1.37), $NADPH_2$-cytochrome *c* reductase (EC 1.6.2.3), glucose-6-phosphate dehydrogenase (EC 1.1.1.49), and 6-phosphogluconate dehydrogenase (EC 1.1.1.43). In all cases, the activities of these enzymes measured at stage 41 were comparable in normal and in mutant *Xenopus*. All of the enzymes measured increase in activity after hatching (29).

Putrescine and polyamine content at various developmental stages

Oocytes, unfertilized eggs, and embryos contain considerable amounts of putrescine and spermidine and a small amount of spermine (Table 2). Putrescine concentration has increased by early gastrulation and increases rapidly after hatching. On the other hand, spermidine concentration does not increase before gastrulation, but doubles between hatching and stages 37–38. These increases in putrescine and spermidine correlate well with the increases in ODC and AdMet decarboxylase activity, respectively (Fig. 1, Table 2).

Putrescine and polyamine concentrations in anucleolate mutants

The most obvious difference between normal embryos and anucleolate mutants is the difference in their spermidine contents (Table 3). Whereas control embryos double their spermidine content during development, the spermidine content of mutant embryos remains unchanged. Further, putrescine accumulation is depressed and there is no detect-

TABLE 2. *Putrescine and polyamine concentration of Xenopus laevis embryos*

	Developmental morphology	nmol/mg protein		
		Putrescine	Spermidine	Spermine
Oocytes		5.7 ± 0.08	4.6 ± 0.10	1.3 ± 0.05
Unfertilized eggs		5.6 ± 0.10	4.5 ± 0.12	1.2 ± 0.06
Stages 2–7		5.9 ± 0.09	4.7 ± 0.19	1.4 ± 0.03
Stages 10–10.5	Early gastrula	8.0 ± 0.12	4.5 ± 0.11	1.4 ± 0.15
Stage 12.5	Late gastrula	6.9 ± 0.32	4.1 ± 0.11	1.3 ± 0.12
Stages 23–25	Post hatching	8.8 ± 0.15	4.5 ± 0.06	1.6 ± 0.10
Stages 37–38	Swimming tadpole	11.2 ± 0.94*	8.5 ± 0.96†	2.1 ± 0.38
Stage 47	Feeding tadpole	14.4 ± 0.40*	12.1 ± 0.49‡	1.5 ± 0.09

Each value represents the mean ± SE for 3 pools, each containing 500 embryos.
* Differs from other values ($P < 0.001$).
† Differs from values for earlier stages ($P < 0.001$).
‡ Differs from values for stages 2–25 ($P < 0.001$), and from values for stages 37–38 ($P < 0.05$).

able spermine. Again, these changes correlate well with the decreased ODC and AdMet decarboxylase activities of anucleolate mutants (Table 1).

DISCUSSION

Putrescine and spermidine concentrations are high in both the eggs and embryos of *Xenopus*, whereas spermine concentration is low (Table 2) (11). This is similar to the pattern found in microorganisms and in rapidly growing cells in general (1, 30). For instance, in regenerating rat liver, the putrescine pool increases first, followed by an increase in the spermidine concentration and a marked decrease in the spermine concentration (30).

The temporal activity patterns of polyamine biosynthetic enzymes during development are indeed interesting. ODC, the putrescine synthetic enzyme, increases in activity well before gastrulation. In fact, between the 2-cell stage and the early blastula, there is a 10-fold increase in ODC activity (Fig. 1). This is unusual, as very few enzymes increase in activity prior to gastrulation in amphibians (29). Another enzyme known to increase in activity in amphibians prior to gastrulation is RNA polymerase (31). There is, therefore, a very close temporal relationship between putrescine synthesis (ODC activity) and RNA-polymerase activity. After hatching of the *Xenopus*, both ODC and AdMet decarboxylase increase dramatically, to activities 100-fold and 40-fold (respectively) higher than those found in unfertilized eggs. New rRNA synthesis begins at gastrulation; it becomes most apparent after hatching (22), coincident with ODC and AdMet decarboxylase increases.

TABLE 3. *Putrescine and polyamine content of Xenopus laevis: comparison of normal and anucleolate mutants*

	nmol/mg protein		
	Putrescine	Spermidine	Spermine
Stage 41			
Normals	12.8 ± 1.1	10.3 ± 0.99	1.3 ± 0.12
Mutants	9.7 ± 0.83	4.2 ± 0.37	<0.3

Each value represents the mean ± SE for 5 pools, each containing 50 embryos.

Polyamine biosynthesis and accumulation is strikingly different in the anucleolate mutant of *Xenopus laevis.* This mutant does not synthesize any detectable rRNA after gastrulation. The mutants are arrested morphologically at an early tadpole stage, and die at a time when the normal tadpoles from the same mating are at about stage 45 (28). These mutants do not synthesize nor accumulate spermidine and there is no AdMet decarboxylase activity after gastrulation. ODC activity and putrescine synthesis after gastrulation is also markedly depressed (Tables 1 and 3).

Since both rRNA and spermidine synthesis are completely absent in anucleolate mutants of *Xenopus laevis*, it is no longer feasible to think that the absence of a nucleolus in a cell means only the specific absence of rRNA synthesis. Further studies of nucleolar functions might help elaborate the series of controls involved in rRNA metabolism.

Another recent study indicates a relationship between the polyamines and rRNA synthesis. When liver and kidney cells of *Xenopus* in culture are pulse-labeled with [^{3}H]putrescine, autoradiographs show that the label accumulates in the nucleus of the cell with the highest density around the nucleolus, the site of rRNA synthesis. The tritium then slowly moves into the cytoplasm, a pattern that parallels that of [6-^{3}H]uridine movement in these cells (32).

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