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Microinjection of purified ornithine decarboxylase into *Xenopus* oocytes selectively stimulates ribosomal RNA synthesis

(transglutaminase/putrescine/α-difluoromethylornithine/RNA polymerase I)

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ABSTRACT **This study has utilized stage VI oocytes of *Xenopus laevis* which have amplified the rDNA gene 1,000-fold to assess whether the microinjection of ornithine decarboxylase (OrnDCase) would stimulate [α-^{32}P]guanosine incorporation into 45S and 18S/28S RNA selectively. The injection of purified OrnDCase into individual oocytes resulted in a greater than 2-fold increase in the incorporation of [^{32}P]guanosine into 45S RNA and 18S/28S RNA with no increased incorporation into low molecular weight RNA. Further, an irreversible inhibitor of OrnDCase, α-difluoromethylornithine (CHF_2-Orn), rapidly inhibited the endogenous activity of OrnDCase when added to the buffered Hepes solution bathing the oocytes and also inhibited the incorporation of [^{32}P]guanosine into rRNA. The inhibitory effect of CHF_2-Orn could not be reversed totally by addition of 10 μM putrescine to the oocytes. OrnDCase injected into oocytes in the presence of CHF_2-Orn in the media did not stimulate incorporation of [^{32}P]-guanosine label into rRNA. However, when CHF_2-Orn was removed from the buffered medium at the time of the injection of label and enzyme, a 3-fold increase of ^{32}P incorporation into 18S/28S RNA occurred. Therefore, in an *in vivo* model in which amplified extrachromosomal rDNA gene copies are present, the microinjection of OrnDCase was capable of specifically stimulating rRNA synthesis. CHF_2-Orn, a suicide enzyme inactivator of OrnDCase, was able to inhibit rRNA synthesis and, after washout, there was a more marked stimulation of rRNA synthesis than occurred after only the injection of OrnDCase alone. These data suggest further that OrnDCase is the labile protein that regulates the initiation of RNA synthesis.**

Several investigators have indicated that the control of RNA polymerase I (EC 2.7.7.6) activity is not through increased synthesis of the enzyme but rather through modification of the enzyme structure that facilitates the attachment of the enzyme complex to rDNA gene sites (1–5). RNA polymerase I activity appears to be dependent upon the presence of an extremely labile half-life protein that is sensitive to amino acid pool sizes (6, 7). Ornithine decarboxylase (OrnDCase, EC 4.1.1.17), the initial enzyme in the polyamine biosynthetic pathway, has the properties of such a protein (8–11).

Additional evidence for an interrelationship between these two proteins includes: (*i*) the early, rapid increase in OrnDCase activity that immediately precedes increased RNA polymerase I activity after stimulation with any of a wide variety of hormones (12, 13); (*ii*) inhibitor studies that indicate that any attenuation of OrnDCase activity is reflected in a similar attenuation of RNA polymerase I activity (14); (*iii*) OrnDCase activity declines with a half-life of 15 min after treatment with cycloheximide. In the same system, the activity of RNA polymerase I, after a lag period of 15 min, also declines with a half-life identical to that of OrnDCase (12); (*iv*) in the slime mold *Physarum polycephalum*, a nucleolar protein that stimulates rRNA synthesis has been identified as OrnDCase (15–17). A Novikoff hepatoma protein (C-14) that also stimulates rRNA synthesis has an amino acid content nearly identical to that of OrnDCase of *Physarum* (15, 18); (*v*) the transglutaminase-mediated conjugation of putrescine to OrnDCase results in a rapid loss of enzymatic activity and a concomitant increase in its ability to stimulate RNA polymerase I activity when added to isolated rat liver nuclei (19–21). Because both phosphorylation (16, 17) and transamidination (19–21) of OrnDCase have been implicated in its ability to regulate RNA polymerase I activity, we sought an experimental system in which enzymes responsible for modifications of OrnDCase would not be rate-limiting. Oocytes of *Xenopus laevis* appeared to be ideal because they have selectively amplified extrachromosomal copies of the rDNA gene by 1,000-fold and have relatively high levels of rRNA synthesis (22). Further, they are large enough so that microinjection of individual oocytes is possible. Therefore, the ability of OrnDCase microinjected into oocytes to stimulate rRNA synthesis was tested.

MATERIALS AND METHODS

Microinjection Procedure into Oocytes. Stage VI oocytes (23), from female toads that had not ovulated for at least 2 months, were prepared as described (24) and stored in Hepes-buffered modified Barth's solution (MBS-H) (24) until injected. Oocytes were injected with 50 ± 10 nl of sample by using microforge-sharpened pipettes as described (25). OrnDCase was partially purified from rat liver stimulated for 5 hr with 3-isobutyl-1-methylxanthine [40 μmol/kg intraperitoneally in 0.9% NaCl/ethanol, 5:1 (vol/vol)] to a specific activity of 28 nmol per min per mg of protein by the procedure of Haddox and Russell (26), and 0.015 pmol (1 μg of protein) was injected in 50 nl of 0.05 M sodium/potassium phosphate buffer at pH 7.2, containing 1 mM dithiothreitol, 0.1 mM EDTA, and 0.1 μCi of guanosine 5′-[α-^{32}P]triphosphate, triethylammonium salt (410 Ci/mmol; 1 Ci = 3.7×10^{10} Bq), obtained from the Radiochemical Centre. The injection was positioned near the nucleus (germinal vesicle) but not in the nucleus. Controls were injected with the same amount of protein as in the OrnDCase preparation and eluted from the same column just prior to the elution of OrnDCase activity. DL-α-Difluoromethylornithine (CHF_2-Orn) was a generous gift from Dow-Merrell Research Center (Cincinnati, OH) and certain oocytes in MBS-H solution were exposed to 10 mM CHF_2-Orn for 20 hr prior to the microinjection of protein and radiolabel. RNA was extracted individually from each oocyte 4 hr after the microinjection procedure.

Extraction of RNA from Single Oocytes. A single oocyte contains ≈4 μg of RNA. The RNA of single oocytes was extracted according to the method of Brown and Littna (27) with

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Abbreviations: OrnDCase, ornithine decarboxylase; CHF_2-Orn, α-difluoromethylornithine.

minor modifications. The oocyte was rapidly homogenized in 0.2 ml of 50 mM Tris·HCl, pH 7.5/5 mM EDTA/0.5% NaDodSO$_4$/0.3 M NaCl/2 mg of proteinase K per ml. The aqueous supernatant was extracted with water-saturated phenol/chloroform, 1:1 (vol/vol), and then reextracted, and the RNA in the supernatant was precipitated by the addition of 1 ml of absolute ethanol (RNase-free); the sample was stored for 24 hr at −20°C. The samples then were centrifuged in a Microfuge for 8 min, the ethanol was removed, and the precipitate was dried in a vacuum desiccator. The sample was resuspended in 40 μl of a 1:2 dilution of loading buffer (E buffer, Tris borate buffer, pH 8.19) (28) containing 10% glycerol and 0.025% bromophenol blue as a tracking dye. Radioactivity in a 5-μl aliquot was counted and 30 μl was loaded on a 1.5% agarose gel. The gel was prepared by addition of 1.8 g of high-melting point agarose to 120 ml of E buffer. The solution was heated to 100°C, refluxed, and poured onto a 13 × 23 cm^3 gel-taped glass plate with the proper gel comb suspended vertically. The RNA was chromatographed 4–6 hr at 140 V on a water-cooled electrophoresis plate. After separation, the gel was stained with ethidium bromide, photographed with a Polaroid camera under UV light, and dried, and the bands were cut and the radioactivity was counted.

OrnDCase Assay. OrnDCase activity was determined by measuring the liberation of $^{14}CO_2$ from L-[1-^{14}C]ornithine as described previously with minor modifications (29). Oocytes were homogenized in 5 vol of 50 mM sodium/potassium phosphate at pH 7.2, containing 0.1 mM EDTA, 1.0 mM dithiothreitol, 5 mM NaF, 1 mM phenylmethylsulfonyl fluoride, and 0.03 mM pyridoxal phosphate. Either 100- or 200-μl aliquots of a 10,000 × g supernatant from each sample were incubated for 60 min at 37°C with 1 μCi of L-[1-^{14}C]ornithine (59 mCi/mmol) and enough unlabeled ornithine to adjust the substrate concentration to 0.25 mM. The assay reaction was stopped with 0.5 ml of 1 M citric acid, and the $^{14}CO_2$ released was collected on Whatman 3MM filter papers prespotted with 20 μl of 2 M NaOH. Radioactivity in the filter papers was counted in toluene/Omnifluor scintillant. All enzyme activities were corrected for blanks which contained 4-bromo-3-hydroxylbenzyloxamine dihydrogen phosphate in the reaction mixture. The enzyme activity was linear with respect to incubation time and enzyme concentration.

RESULTS

Alteration of Incorporation of [^{32}P]Guanosine into the Acid-Insoluble Fraction by OrnDCase. The microinjection technique resulted in a relatively consistent amount of [^{32}P]guanosine per oocyte (Table 1). In the controls injected with 1 μg of protein, the acid-insoluble cpm were 1.1% of the total cpm per oocyte, whereas in those injected with 1 μg of OrnDCase, the ratio of acid-insoluble cpm/total cpm per oocyte was 2.1%. Incorporation into the acid-insoluble fraction was determined 4 hr after the microinjection of radiolabel and protein.

Effect of Microinjected OrnDCase on [^{32}P]Guanosine Incorporation into RNA and the Effect of CHF$_2$-Orn. The microinjection of 1 μg of the OrnDCase preparation resulted in approximately a 4-fold increase in incorporation of [^{32}P]guanosine into high molecular weight RNA (45 S) and a 2-fold increase in label detected in the 18S/28S RNA bands (Table 2). The increased radiolabel incorporation was specific for rRNA because low molecular weight RNA had identical cpm in the control and OrnDCase-injected oocytes.

Incubation of the oocytes with 10 mM CHF$_2$-Orn (5 mM as L-CHF$_2$-Orn) for 20 hr, injection of oocytes, and extraction 4 hr later, still in the presence of 10 mM CHF$_2$-Orn, inhibited incorporation of [^{32}P]guanosine into control-injected oocytes by 89% of the incorporation into control-injected oocytes not incubated in CHF$_2$-Orn (Table 2). However, removal of the 10 mM CHF$_2$-Orn from the buffered Barth's solution at the time of microinjection of the label and protein resulted in a marked stimulation of incorporation of [^{32}P]guanosine into rRNA by greater than 10-fold that detected in the presence of CHF$_2$-Orn in both control- and OrnDCase-injected oocytes. The highest incorporation of ^{32}P was detected in rRNA after OrnDCase microinjection and CHF$_2$-Orn removal at time of injection.

Table 1. Alteration of incorporation of [^{32}P]guanosine into the acid-insoluble fraction by OrnDCase

Microinjection	cpm Total per oocyte	cpm Acid-insoluble	Acid-insoluble cpm/total cpm per oocyte, %
Control	143,700	1,608	1.1
	152,544	1,492	1.0
	147,887	1,711	1.2
	138,703	1,505	1.1
OrnDCase	165,183	3,648	2.2
	163,167	3,361	2.1
	164,641	3,582	2.2
	152,340	3,200	2.1

Oocytes were injected with 50 ± 10 nl of 1 μg of OrnDCase protein or 1 μg of protein that was eluted from the same column just prior to OrnDCase without detectable OrnDCase activity. The injectate also contained 0.1 μCi of [^{32}P]guanosine. Acid-insoluble cpm were determined by spotting an aliquot on GF/C filters, extensive washing with 5% trichloroacetic acid/70% acidified ethanol, drying, and counting of radioactivity in Aquasol.

Inhibition of OrnDCase Activity in *Xenopus laevis* Oocytes by Incubation in 10 mM CHF$_2$-Orn. Incubation of oocytes in 10 mM CHF$_2$-Orn for 4 hr resulted in a 92% inhibition of detectable OrnDCase activity (Table 3). After 24 hr in the presence of 10 mM CHF$_2$-Orn in Hepes-buffered modified Barth's solution, OrnDCase activity was 3% of that detected in the absence of inhibitor.

Addition of 10 μM putrescine to the modified Barth's solution for 4 hr at hour 20 of the CHF$_2$-Orn incubation did not alter the OrnDCase activity from that detected with CHF$_2$-Orn alone for 24 hr. The incubation of oocytes in 10 mM CHF$_2$-Orn and 10 μM putrescine and then removal of these substances from the modified Barth's solution after 20 hr of incubation re-

Table 2. Effect of injection of purified OrnDCase on incorporation of [^{32}P]guanosine into RNA in the presence and absence of CHF$_2$-Orn and putrescine

Microinjection	cpm in RNA High M_r	18S/28S	Low M_r
Control	142 ± 20	1,343 ± 148	72 ± 6
+ CHF$_2$-Orn	16 ± 2	158 ± 9	13 ± 2
+ CHF$_2$-Orn + washout	660 ± 53	2,239 ± 187	78 ± 5
OrnDCase	549 ± 48	3,107 ± 295	71 ± 5
+ CHF$_2$-Orn	21 ± 3	411 ± 38	16 ± 2
+ CHF$_2$-Orn + washout	1,041 ± 98	5,372 ± 480	86 ± 10

RNA was extracted individually from single oocytes, separated on 1.5% agarose gels, and stained with ethidium bromide; the gels then were dried and the radioactivity in the bands was determined. Each value represents the mean ± SEM of 10 oocyte determinations. CHF$_2$-Orn (10 mM) was added to the MBS-H solution of certain oocytes for 20 hr prior to the injection of protein with [^{32}P]guanosine. In washout experiments, new MBS-H solutions without CHF$_2$-Orn was added to the oocytes at the time of microinjection. RNA was extracted from oocytes 4 hr after the microinjection procedure. In the washout experiments each value represents the mean ± SEM of 4–10 oocytes.

Table 3. Effect of CHF_2-Orn, a suicide substrate of OrnDCase, on OrnDCase activity in mature oocytes of *Xenopus laevis*

Oocytes	Incubation time, hr	OrnDCase, pmol/hr per oocyte
Stage VI		27.0 ± 3.0
+ 10 mM CHF_2-Orn	4	2.1 ± 0.2
+ 10 mM CHF_2-Orn	24	0.7 ± 0.05
+ 10 mM CHF_2-Orn + 10 μM putrescine	24 4	0.6 ± 0.04
+ 10 mM CHF_2-Orn + 10 μM putrescine and 4-hr washout	20	68.3 ± 9.1

Oocytes were maintained in Hepes-buffered modified Barth's solution at 25°C (24). Each value represents the mean ± SEM of five determinations on separate oocyte pools. Each pool contained 50 oocytes.

sulted in a marked elevation of OrnDCase 4 hr later to 253% of that detected in stage VI oocytes with no treatment.

Decrease in Putrescine Content in Oocytes Exposed to CHF_2-Orn. Putrescine concentration was decreased significantly after incubation with CHF_2-Orn for 24 hr (Table 4). Therefore, we determined polyamine concentrations in the CHF_2-Orn-treated oocytes after addition of 10 μM putrescine to the buffered Barth's solution after 20 hr of preincubation with CHF_2-Orn. Addition of putrescine for 4 hr to the MBS-H solution resulted in a significant elevation of putrescine and spermidine in the oocytes that had been incubated with CHF_2-Orn for 24 hr.

Effect of Putrescine on [^{32}P]Guanosine Incorporation into RNA in the Presence and Absence of CHF_2-Orn. In the presence and absence of CHF_2-Orn, a consistent effect of 10 μM putrescine was a greater than 2-fold increase in incorporation of ^{32}P into low molecular weight RNA (Table 5). In the presence of CHF_2-Orn and putrescine there also was increased label incorporated into rRNA, which restored it toward control microinjection alone. The same incorporation pattern was detected in the group exposed to CHF_2-Orn and putrescine in the OrnDCase microinjection. After CHF_2-Orn washout, putrescine increased the amount of label in total rRNA (10,006 cpm vs. 6,499 cpm) in the ornithine-injected oocytes as well as in the control-injected oocytes (3,703 vs. 2,977 cpm).

DISCUSSION

These data provide further substantiation in an *in vivo* experimental model that OrnDCase specifically stimulates rRNA synthesis. Certain other studies have implicated increased initiation as the mechanism by which it increases RNA synthesis (21, 31, 32).

It seems likely that the OrnDCase molecule which serves as an initiation factor for RNA polymerase I may be posttranslationally modified by both transglutaminase and, possibly, a polyamine-stimulated protein kinase (15, 17, 19, 20). Protein-conjugated putrescine has been isolated from the nucleolus of regenerating rat liver (33), and the amount of the protein-conjugated putrescine paralleled the increased nuclear transglutaminase activity and accumulated simultaneously with the rapid disappearance of OrnDCase activity at 8 hr. Further, purified OrnDCase can serve as an acceptor protein for putrescine in the presence of transglutaminase purified from guinea pig liver (19, 20). Covalent binding of putrescine to OrnDCase results in a linear decrease in OrnDCase activity. The K_m for putrescine of the transglutaminase reaction is 0.4 mM, and the K_i of the inhibition of OrnDCase activity by putrescine is 0.4 mM. These data suggested that the posttranslational inhibition of OrnDCase, which has been reported in various physiological systems, is related to transglutaminase-mediated transamidination of OrnDCase by its product putrescine.

Transamidinated OrnDCase has been shown to stimulate RNA polymerase I activity. Addition of OrnDCase–putrescine conjugate to methylxanthine-stimulated rat liver nuclei resulted in a 6- to 7-fold increase in RNA polymerase I activity within the first 6 min (21). After the reaction plateaued, addition of a similar amount of OrnDCase–putrescine resulted in reinitiation of the reaction with linearity for 6 min and stimulated a similar incorporation of UMP, as did the original addition of OrnDCase–putrescine conjugate. These data suggested that each molecule of OrnDCase–putrescine conjugate is utilized only once. Otherwise, long periods of linear enzyme activity should have been produced. These data also would be in line with the observation that continual protein synthesis is required for a normal level of transcription of the nucleolar rDNA genes (6, 7) and thus must be required for the regulation of the activity of RNA polymerase I.

The possible identification of a putrescine–protein conjugate assists in the interpretation of some early studies of [^{3}H]putrescine incorporation into *Xenopus* liver and kidney cells in culture (34). When liver and kidney cells of *Xenopus* in culture were pulse-labeled with [^{3}H]putrescine, autoradiographs showed that label accumulated in the cell nucleus with the highest density around the nucleolus, the site of rRNA synthesis. The ^{3}H then moved slowly into the cytoplasm, a pattern paralleling [6-^{3}H]uridine movement in these cells. Because the cells were fixed with 2.5% buffered glutaraldehyde, only putrescine that was

Table 4. Polyamine concentrations in *Xenopus laevis* oocytes in the presence and absence of 10 mM CHF_2-Orn

Oocytes	Polyamine, nmol/mg of protein		
	Putrescine	Spermidine	Spermine
Control	6.2 ± 0.09	4.3 ± 0.11	1.2 ± 0.04
CHF_2-Orn treatment for 24 hr	2.7 ± 0.04*	4.1 ± 0.08	1.3 ± 0.05
CHF_2-Orn treatment + 10 μM putrescine†	8.9 ± 0.14*	5.8 ± 0.06*	1.3 ± 0.04

Each value represents the mean ± SEM for six pools, each containing 100 oocytes. Polyamines were separated and analyzed on a Durrum D-500 amino acid analyzer as described (30).
* Data differ from control values ($P < 0.001$).
† Putrescine was added after 20 hr of preincubation with CHF_2-Orn and polyamine concentrations were assessed 4 hr later.

Table 5. Effect of putrescine on [^{32}P]guanosine incorporation in RNA in the presence and absence of CHF_2-Orn

Microinjection	cpm in RNA		
	High M_r	18S/28S	Low M_r
Control	142 ± 20	1,343 ± 148	72 ± 6
+ putrescine	167 ± 24	1,672 ± 217	152 ± 14
CHF_2-Orn + putrescine	95 ± 6	754 ± 69	183 ± 55
CHF_2-Orn + putrescine + washout	695 ± 44	2,834 ± 310	174 ± 32
OrnDCase	549 ± 48	3,107 ± 295	71 ± 5
+ putrescine	578 ± 73	3,617 ± 350	167 ± 18
CHF_2-Orn + putrescine	90 ± 4	758 ± 71	172 ± 46
CHF_2-Orn + putrescine + washout	1,581 ± 266	8,227 ± 623	198 ± 20

The experiments were conducted as described in the legend to Table 2. In the washout experiments, oocytes were exposed to 10 μM putrescine for 20 hr prior to removal by replacing the modified Barth's solution, and radiolabel incorporation into RNA was assessed 4 hr later. In CHF_2-Orn and putrescine experiments, radiolabel was assessed 24 hr after incubation.

covalently attached to cellular molecules would have been retained for the autoradiograph.

Another study with *Xenopus laevis* provides evidence for tight linkage of polyamine biosynthesis and accumulation and rRNA synthesis (35). Polyamine biosynthesis and accumulation during the development of the anucleolate mutant of *Xenopus laevis* appeared to be totally lacking after gastrulation. This mutant does not synthesize any detectable rRNA after gastrulation, and the mutants arrest morphologically in early tadpole stage and die at a time when the normal tadpoles from the same mating are at about stage 45 (36). These mutants do not synthesize or accumulate spermidine, and there is no adenosylmethionine decarboxylase activity, the enzyme responsible for spermidine synthesis, after gastrulation. The increase in OrnDCase activity that occurs prior to gastrulation in the anucleolate mutant and, presumably, is related to expression of maternal genes rapidly decreases in the anucleolate mutant after gastrulation, suggesting that the genes for OrnDCase, adenosylmethionine decarboxylase, and rRNA all remained unexpressed after gastrulation in the anucleolate mutant.

The present study presents evidence that a suicide enzyme inactivator of OrnDCase, CHF_2-Orn (37, 38), is able not only to inhibit OrnDCase activity but also to inhibit the incorporation of [^{32}P]guanosine into rRNA. CHF_2-Orn also is able to inhibit the stimulatory effects of microinjected OrnDCase on the incorporation of label into rRNA. Of further interest, the inhibitory effect of CHF_2-Orn cannot be overcome totally by addition of putrescine to the oocytes. However, the highest incorporation rate of [^{32}P]guanosine occurred after CHF_2-Orn washout in the presence of putrescine in the buffered Barth's solution with the microinjection of OrnDCase. These data are compatible with a requirement for OrnDCase and putrescine for conjugation to produce a stimulatory protein for rRNA synthesis. Transglutaminase is known to be present in *Xenopus laevis* oocytes because it recently has been implicated in the uptake of vitellogenin by oocytes (39).

In summary, it appears likely from this and other studies that a posttranslationally modified OrnDCase molecule is an initiation factor for the RNA polymerase I enzyme complex to transcribe rDNA gene sites in the nucleolus. Posttranslational modification also may explain the widely varied specific activity of the purified OrnDCase molecule (26).

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