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POSTTRANSLATIONALLY MODIFIED ORNITHINE DECARBOXYLASE MAY REGULATE RNA POLYMERASE I ACTIVITY*

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Abstract—Purified ornithine decarboxylase (EC 4.1.1.17, ODC) transamidated with four putrescine moieties on four glutamine residues through the action of transglutaminase (EC 2.3.2.13, TGase) purified from guinea pig liver, when added to isolated rat liver nuclei, stoichiometrically increased the activity of RNA polymerase I (EC 2.7.7.6). The increase was relative to the pmoles of purified conjugated ODC added to the reaction and could be reinitiated after the reaction had plateaued by the further addition of ODC-putrescine conjugate. The kinetics of the reaction suggest that the ODC-putrescine conjugate was not reused but degraded after each initiation. Otherwise, the rapid plateau would not be observed. The repeated addition of 278 pmoles of purified ODC-putrescine conjugate to rat liver nuclear preparations containing 200 μg total protein consistently stimulated the incorporation of 600–700 pmoles UMP/mg protein. We suggest that ODC transamidated by its product putrescine may be the posttranslationally modified 65,000 M_r protein which has been reported by several laboratories to serve as a labile subunit of RNA polymerase I.

Polyamines are organic cations which are found ubiquitously in living systems [1, 2]. Studies of both mammalian and bacterial cells suggest that the physiological roles of polyamines might be as growth factors [3–7], possibly through their ability to regulate RNA synthesis [2, 7, 8]. Several studies indicate a direct correlation between the amount of spermidine in a cell and the amount of ribosomal RNA (rRNA) that can be accumulated [8–10]. However, significant changes in the accumulation of spermidine and rRNA occur much later [11, 12] than increases in both ornithine decarboxylase (EC 4.1.1.17, ODC), the initial enzyme in the polyamine biosynthetic pathway, and RNA polymerase I (EC 2.7.7.6), a nucleoside triphosphate-RNA nucleotidyltransferase, the enzyme responsible for rRNA synthesis. Both of these enzymes are markedly increased in activity within 4 hr of partial hepatectomy in the rat [5, 13] and, in fact, increase almost simultaneously [5, 14].

RNA polymerase I from vertebrates has been shown to consist of 5 to 8 subunits [15–17] and to be separable on various ion-exchange resins into two forms, RNA polymerase IA and IB [15, 17–19]. Enzyme IB contains a 62,000–65,000 M_r subunit (S3) which is lacking in the IA form. Solubilization and fractionation of RNA polymerase I from isolated rat liver nucleoli by actinomycin D and poly d(A-T) demonstrate that the previously reported chromatin-bound enzyme consists of form IB and that the unengaged enzyme is a mixture of forms IA and IB [16], strongly suggesting that IB, the enzyme with the 62,000–65,000 M_r subunit, is the transcriptionally active form of the enzyme. These results are in agreement with previous studies indicating that a short-lived protein of 62,000–65,000 M_r is required for a normal level of transcription of the nucleolar genes [20, 21]. Further, in Ehrlich ascites cells, amino acids stimulate the synthesis or, possibly, decrease the degradation rate, of this protein [21]. Because of the numerous relationships already reported between polyamines and rRNA, and because ODC is a very labile enzyme with a half-life of 10–20 min and a molecular weight of 65,000 [11, 22], whose half-life is increased in response to amino acids [23, 24], we postulated that ODC might be the labile protein that regulates the activity of RNA polymerase I.

The addition of purified ODC from methylxanthine-stimulated rat liver to the RNA polymerase I assay increased the time of linearity of the assay and, further, restored linearity when added after the reaction had plateaued [25]. Cycloheximide attenuation of the increase in ODC activity resulted in a similar attenuation of RNA polymerase I activity [26]. When the half-life of ODC was estimated in methylxanthine-stimulated rat liver, it declined with an estimated half-life of 15 min [27]. In the case of RNA polymerase I, however, there was a latency period of 15 min after cycloheximide administration in which RNA polymerase I did not decrease in activity [27]. Thereafter, it decreased with a half-life of 15 min. The rapid decline in ODC activity could account for the subsequent decrease in RNA polymerase I activity if it is identical to the labile subunit.

However, in ODC purification preparations that utilized a Sephacryl chromatography purification step, we found that the initiation activity was separated from the ODC activity [28]. This can now be

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explained on the basis of a posttranslational modification of ODC.

We reported recently that the transglutaminase-mediated (EC 2.3.2.13, TGase) conjugation of ODC by its product putrescine results in a stoichiometric decrease in catalytic activity [29]. Four putrescine molecules are conjugated to four glutamine residues by purified guinea pig TGase. The modified ODC-putrescine conjugate has a molecular weight of 65,000. We now report that the ODC-putrescine conjugate stoichiometrically regulates RNA polymerase I activity *in vitro*.

MATERIALS AND METHODS

Materials. Dithiothreitol, pyridoxal phosphate, pyruvate kinase, and the hydrochloride salts of putrescine, spermidine and spermine were obtained from Calbiochem (La Jolla, CA). DNA, RNA, GTP, CTP, ATP, UTP, phosphoenol pyruvate, theophylline, aminophylline, actinomycin D, cordycepin, L-ornithine and cycloheximide were obtained from the Sigma Chemical Co. (St. Louis, MO). D,L-[1-^{14}C]Ornithine (7.7 mCi/mmole) and adenosine 5′-triphosphate, tetra (triethylammonium) salt [γ-^{32}P] (8.25 Ci/mmole) were obtained from the New England Nuclear Corp. (Boston, MA). Uridine 5′-triphosphate (19 Ci/mmole), tetrasodium[5-^{3}H], and density gradient grade (ribonuclease free) sucrose were obtained from Schwarz/Mann (Orangeburg, NY). α-Amanitin was purchased from Boehringer-Mannheim (Mannheim, West Germany). DEAE-Sephadex, CNBr-activated Sepharose 4B and Sephacryl S-200 were purchased from Pharmacia Fine Chemicals (Piscataway, NJ). Sprague–Dawley rats (male, 100–125 g) were from the Division of Animal Resources, University of Arizona College of Medicine.

Ornithine decarboxylase assay. ODC activity was determined by measuring the release of $^{14}CO_2$ from D,L-[1-^{14}C]ornithine as previously described by Russell and Snyder [22] with the exception that the buffer used was 0.5 M Na_2HPO_4-KH_2PO_4 phosphate, pH 7.2, containing 1 mM dithiothreitol.

RNA polymerase assay. Nuclei were isolated by a modification of the procedures described by Blobel and Potter [30] and Busch *et al.* [31]. The chilled livers were homogenized in 2 vol. of 0.34 M sucrose and 5 mM MgCl in a glass-Teflon motor-driven homogenizer. The homogenate was filtered through two layers of cheesecloth and diluted with 2 vol. of 2.3 M sucrose. An aliquot (30 ml) was overlaid on 10 ml of 2.3 M sucrose and centrifuged at 95,000 *g* (Beckman SW27 rotor) for 1 hr. The supernatant fraction was discarded, and the nuclear pellet was resuspended in 0.34 M sucrose containing 8 mM KCl, 6 mM NaF and 1.6 mM $MnCl_2$. Nuclei were checked visually for contamination and adjusted to a protein concentration of approximately 200 μg/50 μl before use as the enzyme source in the polymerase assay. In those experiments utilizing disrupted nuclei, an aliquot of the nuclear preparation was sonicated for 30 sec at 0–2° with an ultrasonic cell disrupter equipped with a 4.5-in probe. The resulting homogenates were checked microscopically for whole nuclei before use in the assay.

The standard RNA polymerase assay mixture contained in a volume of 125 μl: 2.5 μg pyruvate kinase, 7 μmoles phosphoenol pyruvate, 0.2 μmole 2-mercaptoethanol, 0.075 μmole each of GTP, CTP, and ATP, 0.0125 μmole unlabeled UTP, 0.0005 μmole [^{3}H]UTP, 6.25 μmoles $(NH_4)_2SO_4$, and 50 μl of the nuclear enzyme preparation [32]. Either 1.8 μg/ml or 600 μg/ml (final concentration) of α-amanitin was added to the standard assay in order to determine activities of RNA polymerase II and III [33, 34].

The enzyme was incubated with α-amanitin and salts for 10 min at room temperature in order to assure α-amanitin inhibition. For the *in vitro* nuclear studies, putrescine or ornithine was present during this incubation. Addition of a mixture containing the phosphoenol pyruvate, pyruvate kinase, and triphosphates began the assay. The blanks were stopped immediately with 0.5 ml of 0–4° 10% trichloroacetic acid (TCA). The reaction was allowed to proceed for 6 min at 30° and stopped with 0.5 ml of cold 10% TCA. The precipitate was collected on GF/C (Whatman) filters and washed with 100 ml of cold 10% TCA containing 0.04 M sodium pyrophosphate. The filters were air-dried and counted in 10 ml of a toluene/Omnifluor/NCS scintillation mixture.

Cyclic AMP-dependent protein kinase assay. Cyclic AMP-dependent protein kinase activity was determined by measuring the transfer of the terminal phosphate of [γ-^{32}P]ATP to calf thymus histone in the presence of saturating cyclic AMP [35].

Preparation of RNA polymerase I affinity chromatography. RNA polymerase I was isolated from rat liver nuclei and purified through DEAE-Sephadex chromatography by the methodology of Jänne *et al.* [36]. Those fractions eluted from the DEAE-Sephadex column which exhibited RNA polymerase I activity were pooled and dialyzed for 8 hr against three changes of 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl. This preparation, approximately 25 mg of protein, was added to 5 g of prewashed CNBr-activated Sepharose 4B and mixed overnight at 4° in a wrist-action shaker. The coupled gel was washed with 500 ml of 0.1 M $NaHCO_3$ buffer (pH 8.3) containing 0.5 M NaCl and 1 M glycine. A 2.0 × 5.0 cm column was poured and washed with large amounts of 0.01 M Na_2HPO_4-KH_2PO_4 phosphate buffer (pH 7.2) containing 0.1 mM EDTA, 1 mM dithiothreitol, 5 mM NaF, 1 mM phenylmethylsulfonylfluoride, 0.1 mM ornithine and 3 μM pyridoxal phosphate.

Purification of ornithine decarboxylase. Purification was similar to a previous purification from calf liver [28] except for the use of RNA polymerase I affinity chromatography. ODC utilized in these experiments was isolated and partially purified from the livers of 800 rats, processed in batches of 50 at a time, treated with 3-isobutyl, 1-methylxanthine (50 μmoles/kg, i.p., in 0.9% saline–ethanol, 5:1, v:v) (Table 1). Four hours after treatment, the rats were killed and the livers were quickly removed, chilled and homogenized in 4 vol. of 0.05 M Na_2HPO_4-KH_2PO_4 phosphate buffer, pH 7.2, containing 0.1 mM EDTA, 1.0 mM dithiothreitol, 5 mM NaF, 1 mM phenylmethylsulfonylfluoride, 0.1% polyethylene glycol (M_r 20,000) and 3 μM pyridoxal

phosphate. The homogenate was centrifuged for 10 min at 50,000 *g*, the pellet was discarded, and the supernatant fraction was adjusted to pH 4.3 by the addition of 2 M acetic acid. After centrifugation at 50,000 *g* for 10 min, the supernatant fraction was readjusted to pH 7.2 by the addition of 2 M dibasic sodium phosphate and recentrifuged. A final concentration of 0.1 mM cyclic AMP was added to the supernatant fraction before it was applied to a DEAE-cellulose column (1.5 × 30 cm). This concentration of cyclic AMP was sufficient to completely dissociate the regulatory-catalytic subunits of cyclic AMP-dependent protein kinase and, consequently, they were eluted in the void volume of the column. There was no detectable cyclic AMP-dependent protein kinase activity after this purification step. The column was washed with large amounts of 0.01 M Na_2HPO_4-KH_2PO_4 phosphate buffer (pH 7.2) containing EDTA, dithiothreitol, NaF, and phenylmethylsulfonylfluoride, and 3-ml fractions were eluted with a linear gradient of 0.1 to 0.4 M NaCl in the above buffer. Rapid concentration of the active ODC fractions after each procedure led to enhanced stability. Fractions were filtered through Amicon YM-10 Diaflow membranes on an Amicon model 202 stirred ultrafiltration cell. Filtration was conducted at 4° by using N_2 at 60 psi. Dialysis also was conducted on the above apparatus after it was connected to an Amicon reservoir containing the appropriate low ionic strength buffer. Those fractions (9–15) which exhibited ODC activity were pooled and applied to the RNA polymerase I affinity column. The column was washed with 0.1 M ornithine in the homogenizing buffer, and the flow was stopped for 1 hr. ODC activity was eluted in the void volume when flow was reinitiated.

The samples containing ODC activity were concentrated, dialyzed and then applied to a 2.5 × 60 cm Sephacryl column pre-equilibrated in homogenization buffer plus 100 mM NaCl. ODC eluted as a single peak between fractions containing bovine serum albumin (BSA) and ovalbumin as standards. The estimated M_r was 55,000. This procedure resulted in an ODC preparation that was free of cyclic AMP-dependent protein kinase and that had been purified over 45,000-fold (sp. act. = 2700 nmoles per min per mg protein). A representative purification procedure starting with 86.4 g of liver from twelve rats is shown in Table 1.

Those fractions which eluted from the Sephacryl column without detectable ODC activity were pooled. This preparation was adjusted to a protein concentration similar to that of the final ODC preparation and used as the control.

Analytical gel electrophoresis of the purified ornithine decarboxylase preparation after affinity chromatography. Analytical polyacrylamide gel electrophoresis was performed on the ODC preparation after Sephacryl chromatography in order to estimate the purity of this fraction. The enzyme was dialyzed against 100 vol. of 50 mM EDTA (pH 7 to 7.5 at 5°) for 2 hr. An aliquot (5 μg protein) was then applied to 5-cm 7.5% acrylamide gels. The enzyme was electrophoresed for 1.5 hr at 3 mA/gel as described by Friedman *et al.* [37]. Gels were sliced into 1-mm sections, homogenized in the assay buffer and assayed for enzyme activity, or fixed, stained with Coomassie blue and scanned for absorbance. The final ODC preparation exhibited one major protein band, estimated to be more than 98% of the total protein, and this band was enzymatically active (Fig. 1).

Transamidation of ornithine decarboxylase by putrescine. TGase was isolated from guinea pig liver as previously described [29]. This included DEAE-cellulose chromatography and Sephacryl chromatography. Fractions having activity greater than 75 nmoles per min per mg protein were used in the assays. Ornithine decarboxylase purified over 45,000-fold from rat liver (Table 1) was used as protein substrate for the Ca^{2+}-dependent incorporation of putrescine through the action of TGase [29]. Since conjugation results in a stoichiometric decrease in enzymatic activity, the reaction was stopped after the preparation no longer exhibited enzymatic activity. The use of [^{3}H]putrescine in the assay resulted in a radiolabeled conjugate. The labeled ODC-putrescine conjugate migrated on gel electrophoresis with an estimated M_r of 65,000 and was tested as the factor which stimulates RNA polymerase I activity. The ODC activity in the TGase assay was not altered by TGase minus Ca^{2+} plus putrescine in the reaction nor by TGase plus Ca^{2+} in the absence of the primary amine. This suggested that the decrease in ODC activity was a result of the conjugation of putrescine through the catalytic action of TGase.

RESULTS

Stoichiometric increase of RNA polymerase I activity in isolated rat liver nuclei by the addition of ODC-putrescine conjugate. Purified ODC-putrescine conjugate (1.2 mg) was concentrated to 18.5

Table 1. Purification of ornithine decarboxylase from rat liver*

Fraction	Volume (ml)	Protein (mg)	Total activity (nmoles/min)	Specific activity (nmoles/mg protein)	Purification (-fold)	Recovery (%)
Supernatant	198	4,039	239	0.059		
Acetic acid ppt	63	277	153	0.552	9	64
DEAE-cellulose	18	74	78	1.05	18	33
Affinity chromatography	0.2	0.1	72	720	12,203	30
Sephacryl S-200	0.1	0.02	54	2,700	45,760	23

* Enzyme activity was measured in triplicate as described in Materials and Methods.

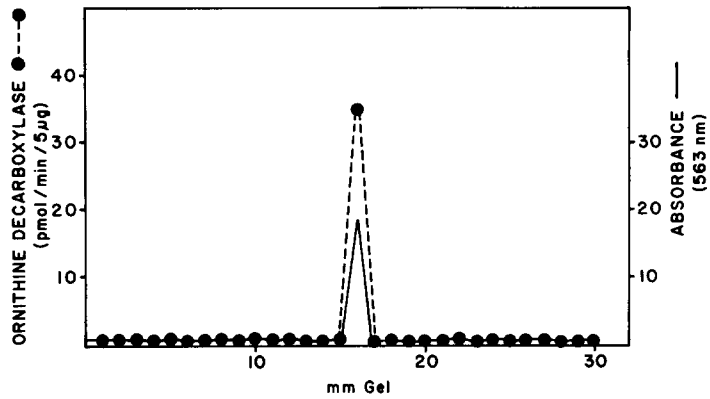

Fig. 1. Analytical polyacrylamide gel electrophoresis of ODC purified over 45,000-fold from rat liver (see Materials and Methods). Paired gels were either fixed, stained with Coomassie blue, and scanned for absorbance at 563 nm (———) or sliced into 1-mm sections and assayed for enzyme activity (●——●).

nmoles/ml. Figure 2 indicates that, without the addition of the ODC-putrescine conjugate, RNA polymerase I activity in nuclei isolated from rats treated for 4 hr with 3-isobutyl, 1-methylxanthine (50 μmoles/kg, i.p., in 0.9% saline–ethanol, 5:1, v:v) was linear for 6 min and then plateaued at an activity of approximately 150 pmoles UMP incorporated/mg protein. In assays in which 15 μl of the ODC-putrescine conjugate was added (278 pmoles ODC-putrescine protein), 692 pmoles UMP was incorporated per mg protein in the same 6-min period. A similar protein-containing fraction without ODC-putrescine was added to the controls to correct for any protein effect. A second 15-μl aliquot of ODC-putrescine preparation was added at 9 min. This resulted in a further increase in incorporation to a total of 1382 pmoles/mg protein. Purified TGase alone added to the RNA polymerase I assay had no effect on activity.

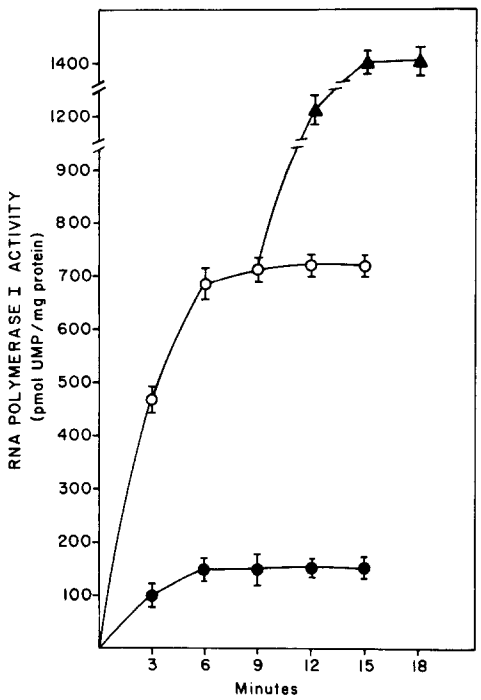

Fig. 2. RNA polymerase I activity in isolated rat liver nuclear preparations with and without the addition of ODC-putrescine conjugate. RNA polymerase I activity was measured in isolated, methylxanthine-stimulated rat liver nuclei as described in Materials and Methods with the exception that the assay also contained either 278 pmoles of the ODC-putrescine conjugate (○——○) or an equivalent amount of protein derived from those fractions displaying no ODC activity after Sephacryl chromatography (●——●). At 9 min, 278 pmoles of ODC-putrescine conjugate again was added to the plateaued reaction (▲——▲).

Purification of ornithine decarboxylase by RNA polymerase I affinity chromatography. ODC binds to the RNA polymerase I-enriched protein fraction coupled to the Sepharose [38]. Extensive washing, up to 500 times the bed volume, failed to elute any ODC activity. Elution of ODC was accomplished by adding 0.1 M ornithine to the buffer (see Materials and Methods) and allowing the column to stand for 1 hr; thereafter, ODC was eluted in the void volume. The utilization of RNA polymerase I affinity chromatography resulted in a 1000-fold purification of ODC with a recovery of approximately 98% of the activity applied to the column (Table 1). The fraction by itself did not contain assayable RNA polymerase I activity. This suggests a physiological interaction between ODC and RNA polymerase I.

Effect of various concentrations of ornithine and putrescine on RNA polymerase I activity. In an effort to assess whether unconjugated putrescine might affect RNA polymerase I activity, the nuclei isolated from non-methylxanthine-stimulated liver were incubated in various concentrations of putrescine, including physiological concentration, for 10 min before the assay was started. There was no effect of putrescine addition on intact or sonicated nuclear preparations. Isolating the nuclei with concentrations of putrescine from 0.01 to 20 mM had no effect on RNA polymerase I activity in either intact or sonicated preparations (Table 2). Preincubation with ornithine concentrations from 0 to 0.5 mM also had no effect on RNA polymerase I activity (Table 3).

Table 2. RNA polymerase I activity of nuclei isolated with putrescine*

Magnesium (mM)	Putrescine (mM)	RNA polymerase I activity (pmoles UMP/mg protein)	
		Intact	Sonicated
5		97.5 ± 13.0	82.3 ± 11.2
5	0.01	74.9 ± 4.4	81.8 ± 4.7
5	0.1	78.4 ± 5.1	90.3 ± 13.4
5	1	86.5 ± 2.6	73.9 ± 2.3
1	5	102.9 ± 4.2	73.0 ± 1.2
1	20	75.6 ± 7.3	72.7 ± 2.1

* Nuclei were isolated with various concentrations of magnesium and putrescine. A portion of each preparation was sonicated for 30 sec with an ultrasonic cell disrupter. Both preparations were then assayed for RNA polymerase I activity as described in Materials and Methods. Each point is the mean ± S.E.M. for five determinations.

This range of concentrations was tested since 0.1 mM ornithine in the affinity chromatography step could have a stimulatory effect on the RNA polymerase I activity.

DISCUSSION

The ability of posttranslationally modified ODC to increase RNA polymerase I activity stoichiometrically (Fig. 2) *in vitro* suggests that ODC-putrescine is synonymous with subunit S3 of RNA polymerase IB. Both S3 and ODC-putrescine have a reported 65,000 M_r, and the lability of S3 can now be related to the lability of ODC prior to conjugation.

In most growth-stimulated systems, continual synthesis of ODC is required for the maintenance of a high level of ODC activity. Further, the administration of cycloheximide results in a rapid decline in ODC activity with an estimated half-life of 15 min [22]. The enzyme activity can be stabilized by the addition of amino acids [23, 24] and, therefore, fulfils the criteria to date for the labile protein which regulates nucleolar rDNA transcription [11, 22–24].

The ODC-putrescine conjugate can be formed *in vitro* using purified or partially purified ODC as a protein acceptor for putrescine in the presence of purified guinea pig liver TGase [29]. The conjugation of putrescine to the ODC molecule results in a stoichiometric decrease in the catalytic activity of the protein. Concomitantly, it results in a stoichiometric increase in the ability of the conjugate to stimulate RNA polymerase I activity in isolated methylxanthine-stimulated rat liver nuclei (Fig. 2). Putrescine-protein conjugates have been identified in the nucleus in the 600 mM NaCl-extractable fraction and also tightly bound to the nucleolus [39]. The tightly bound chromatin fraction requires 3 M NaCl and 7 M urea for extraction. The putrescine-protein conjugate has been identified as ODC-putrescine in regenerating rat liver [40], and the increase in the amount of the conjugate parallels the increase in nuclear TGase activity in regenerating rat liver [41]. We were able to label the pool of putrescine conjugate with [^{14}C]putrescine and isolate the protein complex, and we found that it chromatographed through DEAE chromatography and Sephacryl chromatography in a pattern similar to authentic ODC-putrescine conjugate generated in a test tube [41]. Gel electrophoresis of the putrescine-protein conjugate in regenerating rat liver suggested a molecular weight of 65,000.

Table 3. Effect of ornithine concentration on RNA polymerase I activity of intact and sonicated nuclear preparations*

Ornithine concn (mM)	RNA polymerase I activity (pmoles UMP/mg protein)	
	Intact	Sonicated
0	90.6 ± 10.3	88.3 ± 1.6
0.01	90.1 ± 8.7	92.4 ± 4.3
0.05	93.8 ± 6.6	94.1 ± 3.7
0.1	95.0 ± 5.9	88.6 ± 9.8
0.2	92.3 ± 4.2	90.8 ± 4.5
0.5	91.0 ± 6.1	91.6 ± 6.1

* RNA polymerase I activity was assayed as described in Materials and Methods. Nuclei were preincubated for 10 min with the indicated concentration of ornithine. Each point is the mean ± S.E.M. for five determinations.

Data from several laboratories related to the ratio of RNA polymerase IA to RNA polymerase IB in tissues as a function of growth state can be related to the continual generation and turnover of ODC-putrescine molecules. Ornithine decarboxylase is universally induced in response to an alteration in growth state by a variety of hormones and growth factors [25]. Transglutaminase appears to be universally present in tissues and either undergoes an activation process or is synthesized by the cell [42, 43]. The ability of TGase to conjugate ODC to a primary amine is related to the concentration of free primary amines within the cell. It appears that this concentration is very low until ODC is induced. The K_m for primary amines for TGase in rat liver is 0.4 mM. It is interesting that, in those tissues in which ODC has been shown to be inhibited by the addition of primary amines, the K_i of the inhibition is also 0.4 mM [29]. On the basis of these data, we have postulated that all of the effects of primary amines to inhibit ODC are due to competitive inhibition of the conjugation of putrescine to ODC by TGase.

It is not known at this time whether the increase in activity of RNA polymerase I in response to the addition of ODC-putrescine conjugate is related to new initiation of rRNA chains or whether chain elongation is involved. The ability to restore the initial linear incorporation rate of [^{3}H]uridine into acid-precipitable protein by repeated additions of the ODC-putrescine conjugate is suggestive of an initiation process. Also, initiation is not blocked by heparin, again suggestive of identity with the authentic *in vivo* initiation factor [44]. At the present time, we are attempting to answer this question using mercury-agarose affinity chromatography after incubation with γ-thio ATP [45, 46].

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