Relationship between RNA polymerase I activity and ornithine decarboxylase in rat liver tissues

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In order to examine the relationship between RNA polymerase I and ornithine decarboxylase (ODC), three lines of experiments were performed, with the following results. (1) The glucocorticoid-induced increase of RNA polymerase I in rat liver nuclei was not abolished by administration of inhibitors of ODC synthesis and activity, namely 1,3-diaminopropane and 2-difluoromethylornithine respectively. (2) Anti-ODC antibody did not cross-react with RNA polymerase I solubilized from rat liver nucleoli, indicating the absence of a common protein sequence in these enzymes. (3) The ODC preparation which was treated with transglutaminase in the presence of putrescine could not stimulate the activity of RNA polymerase I in nuclei of liver and prostate. All these results suggest that the increases in ODC protein or activity are not a prerequisite to the increase in RNA polymerase I after hormonal or physiological stimuli, but rather that the increases in both enzymes are separate responses to the primary stimuli.

INTRODUCTION

Stimulation of polyamine biosynthesis is one of the earliest events occurring during tissue growth, and the polyamines have been implicated in the control of synthesis of both RNA and protein. Increased polyamine synthesis reflects a rapid elevation of ODC activity. Thus there are several papers reporting that ODC activity is induced in a few hours by various agents that affect growth processes, such as glucocorticoids, androgens and oestrogens (Jänne *et al.*, 1978). It is well known that these hormones also stimulate the synthesis of rRNA at early times, but there have been conflicting reports about the role of the induced ODC in rRNA synthesis in nuclei. Kuehn and co-workers, who worked with *Physarum polycephalum*, reported that ODC, after being phosphorylated, is capable of stimulating the transcription of rRNA genes by RNA polymerase I (RNA nucleotidyl-transferase, EC 2.7.7.6) (Kuehn *et al.*, 1979; Daniels *et al.*, 1981; Atmar & Kuehn, 1981). Russell and co-workers (Russell, 1981b; Russell & Manen, 1982) reported, using a rather crude preparation of ODC, that an ODC–putrescine conjugate could stimulate the activity of RNA polymerase I in liver nuclei and suggested that the conjugate is the S3 subunit of RNA polymerase I and the labile protein(s) itself which is supposed to mediate the hormonal regulation of rRNA. On the other hand, Seely *et al.* (1984), who worked with kidney tissue, presented evidence indicating that the stimulation of RNA polymerase I by phosphorylated ODC is not due to the ODC protein itself, but to contaminating proteins. Moreover, our finding (Suzuki *et al.*, 1986) that androgen-induced enhancement of RNA polymerase I activity in Shionogi carcinoma tissue was not abolished by inhibition of ODC activity by DFMO threw doubt on the intimate relation between the two enzymes.

In order to shed some light on the relationship

between the two enzymes, experiments in the following three directions were projected in this paper: (a) whether the suppression of synthesis and activity of ODC by diaminopropane and DFMO, respectively, can diminish the glucocorticoid-induced RNA polymerase I activity in rat liver nuclei; (b) whether RNA polymerase I molecule contains ODC protein as a subunit; (c) whether the activity of RNA polymerase I in rat liver nuclei is stimulated by adding transglutaminase-treated ODC when a more purified preparation of ODC than that used previously is employed.

MATERIALS AND METHODS

Chemicals

[1,4-¹⁴C]Putrescine dihydrochloride (107.1 mCi/mmol) and DL-[1-¹⁴C]ornithine (57.3 mCi/mmol) were purchased from New England Nuclear Corp., Boston, MA, U.S.A. [5,6-³H]UTP (41 Ci/mmol) was from The Radiochemical Centre, Amersham, Bucks., U.K. Pyridoxal phosphate was obtained from Nakarai Chemical Co., Osaka, Japan. 1,3-Diaminopropane was obtained from Wako Pure Chemicals Co., Osaka, Japan, and DFMO was kindly supplied by Merrell Research Center, Merrell Dow Pharmaceuticals Inc., Cincinnati, OH, U.S.A. Calcium phosphate gel was prepared as described previously (Porter, 1955). Protein A–Sepharose CL-4B was purchased from Pharmacia P-L Biochemicals, Uppsala, Sweden. Other chemicals were of reagent grade and were used without further purification.

Animal treatment

Male rats of the Wistar strain, weighing 80–150 g, were used in most of the present study. In some experiments for purification of transglutaminase, male guinea pigs were used. They had free access to conventional

Abbreviations used: ODC, ornithine decarboxylase (L-ornithine carboxy-lyase, EC 4.1.1.17); DFMO, DL-2-difluoromethylornithine; DTT, dithiothreitol.

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laboratory chow and tap water. In the experiment of dexamethasone injection, the animals, which had been adrenalectomized 5–7 days before, were injected intraperitoneally with dexamethasone (50 μ g/0.1 ml per 100 g body wt.) dissolved in 30% ethanol/saline (0.9% NaCl), and killed by decapitation 2, 4 or 6 h after dexamethasone treatment, followed by perfusion of liver with cold saline. 1,3-Diaminopropane in neutralized saline solution (100 μ mol/0.5 ml per 100 g body wt.) was injected every 2 h, starting 30 min before dexamethasone treatment. DFMO dissolved in saline (40 mg/0.5 ml per 100 g body wt.) was administered to rats in the same way. Control animals received vehicle only.

Tissue preparation for the determination of activities of ODC and RNA polymerase I

The tissue preparation was carried out at 0–4 °C. Liver was homogenized in 0.32 M-sucrose/10 mM-MgCl₂ and the homogenate was filtered through a stainless-steel mesh (100 mesh). The filtrate was centrifuged at 700 g for 10 min. To the supernatant, 0.1 vol. of 250 mM-Tris/HCl buffer (pH 7.5) containing 1 mM-EDTA and 10 mM-DTT was added. Then the mixture was re-centrifuged at 105000 g for 60 min, and the resulting supernatant (cytosol) was used for assay of ODC activity. The pellet after the first centrifugation was resuspended in 2.2 M-sucrose/10 mM-MgCl₂ and centrifuged at 52000 g for 60 min and was used for assay of RNA polymerase I.

Assay of ODC activity

ODC was determined by measuring release of ¹⁴CO₂ from DL-[1-¹⁴C]ornithine as described by Noguchi *et al.* (1979), except that bovine serum albumin was omitted. One unit of enzyme activity was defined as the amount forming 1 nmol of CO₂/h.

Assay for RNA polymerase I activity in isolated nuclei

This was done as described previously (Suzuki *et al.*, 1984). When the effect of ODC or its derivatives on the activity of nuclear RNA polymerase I was examined, the activity of RNA polymerase I was measured after nuclei were treated as follows. The mixture, containing 50 mM-Tris/HCl (pH 8.5), 5 mM-CaCl₂, 0.2 mM-putrescine, 10 mM-DTT, ODC and transglutaminase preparation was incubated at 37 °C for 60 min, and then dialysed against 33 mM-Tris/HCl buffer (pH 7.9) containing 2 mM-DTT at 4 °C. A sample (140 μ l) containing 836 units of ODC and 6.2 units of transglutaminase was added to 30 μ l of nuclear suspension (equivalent to 100 μ g of DNA). After preincubation at 30 °C for 5 min, the reaction mixture for RNA polymerase I assay (80 μ l) was added and RNA synthesis was allowed to proceed at 37 °C for 15 min.

Assay for the activity of solubilized RNA polymerase I

Enzyme activity was essentially determined by the method of Rose *et al.* (1983). The reaction mixture (240 μ l) was as follows: 50 mM-Tris/HCl (pH 8.0), 0.5 mM-ATP, -GTP and -CTP, 25 μ M-[³H]UTP (0.167 Ci/mmol), 3.1 mM-MgCl₂, 1.25 mM-MnCl₂, 0.5 mM-spermine, 2 mM-NaF, 250 μ g of albumin/ml, 0.6 mM-DTT, 60 μ g of calf thymus DNA/ml, 10% (v/v) glycerol, and 150 μ l of the supernatant, from which immune complexes were removed by Protein A-Sepharose CL-4B. Then the reaction mixture was incubated for 30 min

at 30 °C and stopped by the addition of 1 ml of 10% (v/v) trichloroacetic acid. The resulting pellet was transferred to a Whatman GF/C filter and washed five times with 5% trichloroacetic acid containing 1.5% sodium pyrophosphate, and once with ethanol, and then the radioactivity of the pellet was counted. One unit of RNA polymerase I activity was defined as the amount of enzyme that catalysed the incorporation of 1 pmol of UMP into RNA in 30 min under the condition described above.

Preparation of ODC and anti-ODC antibody

Rat liver ODC and anti-ODC antibody were provided by Professor S. Hayashi and Dr. T. Kameji (Kameji *et al.*, 1982). The purity of the enzyme preparation was at least 80%, judged from the specific activity (1.2 $\times$ 10⁶ units/mg of protein). Anti-ODC antibody was obtained by precipitation with 40% -satd. (NH₄)₂SO₄ from antiserum. Control antibody was similarly obtained from a non-immune serum of a rabbit.

Transglutaminase assay

The activity was determined essentially by the method of Russell (1981a,b), with some modification. The reaction mixture (50 μ l) contained 50 mM-Tris/HCl (pH 8.5), 5 mM-CaCl₂, 0.2 mM-putrescine, 0.1 μ Ci of [1,4-¹⁴C]putrescine, 10 mM-DTT and 250 μ g of casein. After preincubation at 37 °C for 5 min without the enzyme, the reaction was initiated by the addition of 10 μ l of transglutaminase preparation, followed by incubation at 37 °C for 10 min. The reaction was stopped by adding 1 ml of 10% trichloroacetic acid. After standing at 4 °C for 20 min, the insoluble materials were trapped on a GF/C filter, washed, dried, and radioactivity was counted. In experiments to examine the effect of pH on the transglutaminase activity, Tris/HCl buffer was replaced by glycine/NaOH buffer in the higher-pH region. One unit represents the incorporation of 1 nmol of putrescine/min.

Purification of transglutaminase

This was done from guinea-pig liver by the method of Russell (1981b). The final preparation gave a single band of protein when tested by SDS/polyacrylamide-gel electrophoresis (Laemmli, 1970), and had a specific activity of 155 units/mg of protein. The enzyme was also isolated from rat liver by the method with modifications. The supernatant obtained by centrifugation from homogenate was treated with calcium phosphate gel, and Ultrogel Aca44 column chromatography was omitted. The specific activity of the final preparation obtained from rat liver was 16.3 units/mg of protein.

Effect of anti-ODC antibody on the activity of RNA polymerase I solubilized from nucleoli

Preparation of liver nucleoli and solubilization of RNA polymerase I were performed as described previously (Matsui *et al.*, 1986). Solubilized RNA polymerase I (60 μ l; 90 units) was mixed with 390 μ l of phosphate-buffered saline (0.15 M-NaCl/50 mM-sodium phosphate, pH 7.4) containing anti-ODC antibody or control antibody, 4.2 mM-MgCl₂, 330 μ g of albumin/ml, 0.6 mM-DTT, 0.5 mM-GTP and 0.625 mM-ATP. After incubation for 15 min at 30 °C, 60 μ l of Protein A-Sepharose CL-4B suspension (1:1 in phosphate-buffered saline) was added and the mixture was further

Fig. 1. Suppression of the activity of ODC by administration of 1,3-diaminopropane or DFMO

Adrenalectomized rats were injected with 1,3-diaminopropane (Δ ; 100 μ mol/100 g body wt.), DFMO ($\bullet$; 40 mg/100 g body wt.) or vehicle ($\circ$; saline) every 2 h. Dexamethasone (50 μ g/100 g body wt.) was injected into the animals at 30 min after the first injection of these inhibitors. Rats were killed at the times indicated, and ODC activity in liver cytosol was assayed as described in the Materials and methods section. Arrows indicate the times when inhibitors were administered.

incubated for 20 min at 30 °C with occasional shaking. After centrifugation (8000 g) for 5 s, the activity of RNA polymerase I in the supernatant was determined by the method described above.

Determination of protein and DNA

Protein was determined by the method of Lowry *et al.* (1951), and DNA was determined by the method of Burton (1956).

RESULTS

Does the injection of 1,3-diaminopropane or DFMO suppress the glucocorticoid-induced increase in RNA polymerase I activity in rat liver nuclei?

As reported by Matsui *et al.* (1986), the administration of dexamethasone to adrenalectomized rats brings about

an increase in the activity of RNA polymerase I in rat liver nuclei without synthesis of the enzyme protein *de novo*. On the other hand, it was reported that the ODC activity in the cytosol of liver cells reached a maximum 3 h after injection of cortisol (Panko & Kenney, 1971) or dexamethasone (Beck *et al.*, 1972) and that ODC molecules are present in nuclei, particularly in the nucleolar region (Emanuelsson & Heby, 1982; Zagon *et al.*, 1983; Persson *et al.*, 1983). A question arises whether the increase of RNA polymerase I is mediated by the increase in ODC activity induced by the hormones.

In order to assess the function of ODC in RNA synthesis, an inhibitor of the synthesis of ODC protein, 1,3-diaminopropane (Pösö & Jänne, 1976), or an inhibitor of ODC activity, DFMO (Metcalf *et al.*, 1978), was injected into dexamethasone-primed adrenalectomized rats. As shown in Fig. 1, the activity of ODC in the cytosol was increased about 10-fold 6 h after the injection of dexamethasone, and this enhancement was indeed abolished by the drugs injected every 2 h. The drugs, however, were unable to suppress the glucocorticoid-induced increase in RNA polymerase I activity in liver nuclei, as shown in Table 1.

Do anti-ODC antibodies bind to RNA polymerase I?

The possibility that RNA polymerase I and ODC have a common subunit or protein sequence was tested by the immunoprecipitation method with polyclonal antibodies to ODC (Fig. 2). In these experiments, RNA polymerase I solubilized from liver nucleoli was incubated with various amounts of anti-ODC antibodies and, after centrifugation in the presence of protein A-Sepharose, the remaining activity of RNA polymerase I in the supernatant was measured (Fig. 2a). For comparison, the cytosol of liver cells which contained ODC was treated similarly with the anti-ODC antibodies (Fig. 2b). The results indicate that the antibodies react with ODC, but not with RNA polymerase I in liver nucleoli.

Effect of transglutaminase-treated ODC on the activity of RNA polymerase I in isolated rat liver nuclei

Russell & Manen (1982) reported that transglutaminase purified from guinea-pig liver cytosol catalyses incorporation of [3 H]putrescine into ODC protein and that the modified ODC is capable of stimulating greatly the activity of RNA polymerase I in rat liver nuclei. Since the purity of the ODC preparation they used was estimated to be no more than 11%, on the basis of its specific activity reported previously (Kameji & Hayashi,

Table 1. Change in RNA polymerase I activity in liver nuclei after administration of dexamethasone, 1,3-diaminopropane and DFMO to adrenalectomized rats

The schedule of administration of these compounds is described in the legend to Fig. 1. Rats were killed 6 h after administration of dexamethasone, and the activity of RNA polymerase I in isolated nuclei was measured as described in the Materials and methods section. Values are means $\pm$ S.E.M. for three experiments.

Rats	UMP incorporated (pmol/8 min per 100 μ g of DNA)	Relative activity (%)
Adrenalectomized	35.9 $\pm$ 2.6	100 $\pm$ 7
+ Dexamethasone	60.8 $\pm$ 4.7	169 $\pm$ 13
+ Dexamethasone and 1,3-diaminopropane	60.4 $\pm$ 1.8	168 $\pm$ 5
+ Dexamethasone and DFMO	78.2 $\pm$ 8.4	218 $\pm$ 23

Fig. 2. Effect of anti-ODC antibodies on the activities of RNA polymerase I and ODC

(a) A constant amount of RNA polymerase I (90 units) solubilized from liver nucleoli was mixed with anti-ODC antibodies at various dilutions (●) or non-immune antibodies (○) as described in the Materials and methods section. After incubation at 30 °C for 15 min, immune complexes were precipitated by Protein A–Sepharose and the RNA polymerase I activity in the supernatant obtained was determined. Enzyme activities are plotted against the antibody dilution. (b) A constant amount of cytosol from dexamethasone-primed rat liver was treated in the same manner described above, and the activity of ODC in the supernatant was determined. ▲, Anti-ODC antibody; △, non-immune antibody.

1982), we attempted to examine whether similar results are obtained when our more purified preparation (80% pure) is used. Russell (1981a,b) described that the treatment of their ODC preparation with transglutaminase in the presence of putrescine at pH 10.3 caused complete loss of ODC activity. The treatment of our

Fig. 3. Effect of pH on the activity of transglutaminase

The activity of transglutaminase from rat liver was measured as described in the text, with casein as substrate, except that the pH in the medium was changed as indicated. Each point is the average ($\pm$ S.E.M.) for three experiments. Buffer: ○, Tris/HCl; ●, glycine/NaOH.

ODC preparation with transglutaminase indeed resulted in almost complete loss of ODC activity (Expts. 1 and 4 in Table 2). However, the decline of ODC activity was observed even in the absence of transglutaminase, as shown in Expts. 2 and 3 of Table 2. This suggests that the loss of ODC activity during the incubation with transglutaminase is not due to a direct action of transglutaminase, but to possible inactivation of ODC on incubation at such high pH (10.3). In fact, the activity of ODC in the ODC preparation was not suppressed after treatment with transglutaminase at pH 8.5, where the activity of transglutaminase itself is almost equal to that at pH 10.3 (Fig. 3), in spite of significant incorporation of labelled putrescine into the ODC preparation. Although the ODC activity was not abolished by the action of transglutaminase, we considered that there is a possibility that the modification of ODC or contaminating proteins may stimulate the activity of RNA polymerase I in isolated nuclei. If the activation occurs as described by Russell & Manen (1982), the expected increase in RNA polymerase I in these experiments, where the amount of ODC used in each tube was 836 units, would be 1.6-fold, since they found a 4.7-fold increase with 2480 units of ODC. As

Table 2. Change in ODC activity after treatment with or without transglutaminase at pH 10.3

ODC solution (10 μ l; 60 units) was mixed with 30 μ l of transglutaminase assay medium (Russell & Manen, 1982) which was devoid of transglutaminase, and the mixture was incubated for 0 or 5 min at 37 °C (preincubation). Then 10 μ l of rat liver transglutaminase solution (0.37 unit) in Expt. 4, or buffer alone [Tris/HCl buffer (pH 7.4) containing 2 mM-EDTA] in Expts. 1, 2 and 3 were added and incubated for 0 or 30 min at 37 °C, and ODC activity was determined by the method described in the text.

Expt.	Conditions			ODC activity	
	Preincubation	Incubation		(unit)	(%)
		Transglutaminase	Time		
1	0 min	—	0 min	46.21	100
2	5 min	—	0 min	2.36	5.1
3	5 min	—	30 min	0.55	1.2
4	5 min	+	30 min	0.63	1.4

Table 3. Change in RNA polymerase I activity in isolated nuclei after incubation with ODC

After 50 mM-Tris/HCl buffer (pH 8.5) containing 10 mM-DTT, 5 mM-CaCl₂ and 0.2 mM-putrescine was incubated with or without ODC or transglutaminase from rat liver, the mixture was dialysed for 5 h at 4 °C and added to isolated nuclei from rat liver (Expts. 1 and 2) or nuclei from prostates of castrated rats (Expt. 3). Then RNA polymerase I activity was determined. The details were described in the Materials and methods section. Values are means $\pm$ S.E.M. for three to four experiments.

Tube	Treatment		RNA polymerase I activity (pmol of UMP incorporated/15 min per 100 μ g of DNA)		
	ODC	Transglutaminase	Expt. 1 Liver	Expt. 2 Liver	Expt. 3 Prostate
1	+	+	73.3 $\pm$ 1.5	76.5 $\pm$ 1.0	29.3 $\pm$ 0.4
2	—	+	72.0 $\pm$ 1.3	71.6 $\pm$ 1.5	29.5 $\pm$ 0.5
3	—	—	71.4 $\pm$ 1.6	67.6 $\pm$ 0.7	23.0 $\pm$ 0.5

shown by the data of Expts. 1 and 2 of Table 3, slight increases (1.8% and 6.8% respectively) in RNA polymerase I activity were observed on addition of transglutaminase-treated ODC, but these were definitely small as compared with the expected stimulation (60%). Expt. 3 in Table 3 is concerned with nuclei from prostates of castrated rats, which were tested because they are expected to be more sensitive to the putative activators, in view of the experimental results described previously (Suzuki *et al.*, 1984). However, the results were similar to those of Expts. 1 and 2, revealing no significant activation of the enzyme. In the experiments mentioned above, transglutaminase isolated from rat liver was used. Similar results were obtained with transglutaminase from guinea-pig liver, which was at a more purified stage as judged from SDS/polyacrylamide-gel electrophoresis (results not shown).

DISCUSSION

Several workers have presented evidence indicating that the hormonal regulation for rRNA synthesis is mediated by a short-lived protein(s) (Nicolette & Babler, 1974; Zoncheddu *et al.*, 1977; Hosoya *et al.*, 1978; Suzuki *et al.*, 1984), but the nature and function of such protein(s) remain to be determined. A difficulty in the experiments using isolated nuclei is that incorporation of labelled triphosphate *in vitro* represents only elongation of RNA chains initiated *in vivo*, i.e. new initiation does not occur under the conditions. In this connection the findings by Russell and co-workers are noteworthy, that ODC-putrescine conjugate was capable of stimulating the incorporation of labelled triphosphate into nuclei, probably because of causing re-initiation (Russell, 1981b; Russell & Manen, 1982). From these and other results, they presented the idea that the ODC-putrescine conjugate is the labile protein(s) itself, and probably the S3 subunit of RNA polymerase I, which is lacking in the IA form.

However, our results do not support their view, since (a) ODC was inactivated without transglutaminase under the conditions (pH) used by Russell & Manen (1982), but not inactivated with transglutaminase and putrescine at physiological pH (8.5); (b) ODC treated with transglutaminase at pH 8.5 in the presence of putrescine could not stimulate the activity of RNA polymerase I in nuclei of liver and prostate; (c) anti-ODC

antibodies did not cross-react with RNA polymerase I solubilized from liver nucleoli, suggesting that ODC is not a subunit of RNA polymerase I. The reason for the discrepancy is not clear, but it may be attributable to the difference in purity of the ODC preparation used; calculation from the data of Russell (1981a,b) reveals that the purity of their preparation is no more than 11%, compared with ours (80%). Seely *et al.* (1984) also reported that stimulation by phosphorylated ODC of rRNA synthesis in kidney tissue is ascribed not to ODC but to contaminated proteins. They also showed the absence of any antigenic cross-reactivity between purified RNA polymerase I and ODC, in agreement with our present observations.

Ample evidence has now been obtained indicating that induction of ODC activity by hormonal and physiological stimuli is ascribed to a large rise in the amount of ODC protein, which is due partly to an increase in ODC mRNA (Pegg, 1986). Repression of ODC protein by diaminopropane, however, did not affect the enhancement of RNA polymerase I by the hormone (Table 1). Inhibition of ODC activity by DFMO also gave similar results (Table 1). These results are not compatible with the view that ODC protein or its derivative would have a function in nucleoli to regulate the activity of RNA polymerase I.

In conclusion, our experiments do not support the concept that the enhancement of ODC activity or modification of ODC protein is a prerequisite to RNA polymerase I stimulation. It is likely that both increases are separate responses to the primary stimuli by hormones.

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