

THE EFFECT OF ESTRADIOL ON THE ACTIVITY OF THE NUCLEOLAR AND NUCLEOPLASMIC RNA POLYMERASES FROM CHICKEN LIVER

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1. Introduction

The synthesis of egg yolk proteins in the liver of birds is controlled by estrogens and can be induced by estrogenic hormones even in immature and male birds [1,2]. RNA synthesis, which is increased after the injection of estradiol [3], seems to be a prerequisite for the induction of this specific protein synthesis [2–4]. An increased synthesis of RNA could be caused by changes of the template activity of the chromatin and/or by modifications of the enzymes (DNA-dependent RNA polymerases). The aim of this investigation was to study the latter possibility. We found an increased activity of both, the nucleolar and the nucleoplasmic RNA polymerases 24 h after estradiol treatment. Furthermore, we could clearly show that this stimulation is not just simulated by a lower RNase activity of estradiol preparations or inactivation of some inhibitor which might normally be present in controls.

2. Materials and methods

2.1. Materials

α -Amanitin was a generous gift from Professor T. Wieland, Max-Planck-Institute, Heidelberg. Estradiol [1,3,5 (10)-estratriene-3,17 β -diol] was obtained from Merck, Darmstadt and [6,7- 3 H]estradiol, specific activity 48 Ci/mmol, from New England Nuclear (Great Britain). [5- 3 H]Uridine-5'-triphosphate (ammonium salt), specific activity 17.7 Ci/mmol, was obtained from the Radiochemical Centre, Amersham. Yeast RNA (8 S) was purchased from Serva, Heidelberg and used only for the RNase assay.

For the RNA polymerase assay yeast RNA from Boeringer, Mannheim was used.

2.2. Animals

4 week old male chickens were used. These chickens which had been obtained as newly hatched chicks from a local supplier were raised in our laboratory and fed a standard diet ad libitum. The animals received an intramuscular injection of estradiol in propylene glycol (25 mg/kg body weight) 24 h before sacrifice.

2.3. Isolation of nuclei and solubilisation of RNA polymerases

Nuclei were isolated following essentially the procedure of Dierks-Ventling and Jost [5]. All steps were performed at 4°C. Chick livers were homogenized in 10 vol (ml/g tissue) of buffer A (0.32 M sucrose, 2 mM KH_2PO_4 , pH 5.8, 3 mM MgCl_2 , 10^{-4} M dithiothreitol, 10^{-4} M EDTA) with 10 strokes of a glass Teflon homogenizer at 1500 rev/min and filtered through 2 layers of gauze. After centrifugation in a Sorvall centrifuge using an SS-34 rotor at 800 g for 10 min, the supernatant fluid was decanted and the crude nuclei were resuspended in 10 vol (ml/g of original tissue) of buffer B (buffer A with 0.1% Triton-X-100 added). This was followed by another centrifugation for 10 min at 800 g. The supernatant fluid was again discarded and the semipurified nuclei were resuspended in 8 vol (ml/g of original tissue) of buffer B. Aliquots of 24 ml were layered over 10 ml of buffer C (1.65 M sucrose, 2 mM KH_2PO_4 , pH 5.8, 1 mM MgCl_2 , 10^{-4} M dithiothreitol). After centrifugation in a Beckman ultracentrifuge using an SW-27 rotor at 16 000 g for 10 min the supernatant fluid was aspirated and the purified nuclei were resuspended in

buffer D (0.25 M sucrose, 5 mM Tris-HCl, pH 7.4) (0.33 ml/g of original tissue).

RNA polymerases were solubilized from liver nuclei and further purified by a method slightly modified from Roeder and Rutter [6]. The nuclear suspension was adjusted to 0.01 M Tris-HCl, pH 7.9, 1.0 M sucrose, 5 mM MgCl₂, 5 mM dithiothreitol. Ammonium sulfate (4 M, adjusted to pH 7.9 with ammonia) was added to bring the concentration to 0.3 M. The viscous solution was sonicated 10 × 10 sec with a Branson S-125 (setting 3). The suspension was then rapidly mixed with 2 vol of TGMED (0.05 M Tris-HCl, pH 7.9, 25% glycerol, 5 mM MgCl₂, 0.1 mM EDTA, 5 mM dithiothreitol). The precipitate was removed by centrifugation for 1 h at 105 000 g and discarded. The clear supernatant was treated with ammonium sulfate first up to 30% saturation and then after centrifugation for 30 min at 105 000 g up to 45% saturation. The precipitate was collected by centrifugation for 1 h at 105 000 g and resuspended in TGMED. After dialysis for 2 h against TGMED containing 0.05 M ammonium sulfate, the dialysate was centrifuged at 160 000 g for 1 h. The supernatant was either stored at -90°C or immediately used for the following assays.

2.4. RNA polymerase assay

0.5 ml of the reaction mixture for the assay of polymerase A contained: 50 μmol Tris-HCl, pH 7.9, 5 μmol MgCl₂, 10 μmol thioglycerol, 0.5 μmol each of ATP, GTP, CTP, 5 μCi [³H]UTP (specific activity: 17.7 Ci/mmol), 0.4 μg α-amanitin and 100 μg calf thymus DNA.

For the measurement of polymerase B two assays were performed:

(a) 0.5 ml of the reaction mixture contained: 50 μmol Tris-HCl, pH 7.9, 2 μmol MnCl₂, 10 μmol thioglycerol, 10 μmol (NH₄)₂SO₄, 0.5 μmol each of ATP, GTP, CTP, 5 μCi [³H]UTP and 100 μg calf thymus DNA.

(b) like (a) except that 0.4 μg α-amanitin were added.

The difference between assay (a) and assay (b) is a measure for the activity of polymerase B.

The samples were incubated at 37°C for 10 min (polymerase A) or 5 min (polymerase B). The reaction was stopped by cooling to 0°C and adding 50 μl of a solution containing 2 mg/ml yeast RNA, 2 mg/ml

bovine serum albumin and 5 mM UTP in 0.1 M Na₄P₂O₇, pH 7 and 4.5 ml 5% trichloroacetic acid. The precipitate was collected on GF/C microfiber glass filters (Whatman) and washed with 30 ml 5% trichloroacetic acid. The filters were heated in 0.5 ml 5% trichloroacetic acid at 95°C for 30 min. The radioactivity was measured in a Nuclear Chicago Mark II liquid scintillation counter after the addition of 10 ml Bray's solution (60 g naphthalene, 4 g 2,5-diphenyl-oxazole, 0.2 g 1,4-bis-(5-phenyl-oxazole-2-yl)-benzene, 100 ml absolute methanol and 20 ml ethylene glycol in 1 litre dioxane).

2.5. Measurement of the decrease of the *in vitro* synthesized [³H]RNA

Assay (a) for polymerase B was carried out as described above (2.4.) (in this case we measure the sum of the activities of polymerase A and B in the high salt medium). After incubation for various time periods the reaction was stopped by adding 50 μl of a solution containing 2 mg/ml yeast RNA, 2 mg/ml bovine serum albumin and 5 mM UTP in 0.1 M Na₄P₂O₇, pH 7, to diminish the incorporation of [³H]UMP to a level which is no longer measurable. Then the samples were again incubated at 37°C. After various periods of time samples were cooled to 0°C, the RNA precipitated, collected on filters and the radioactivity measured as described above (2.4.). The decrease of the [³H]RNA content is a direct measure for the RNase activity of the polymerase preparation (acting on the *in vitro* synthesized RNA).

2.6. RNase assay

50 μl of a sample were incubated at 37°C for 15 min with 200 μl of a yeast RNA solution (2 mg/ml). The reaction was stopped by the addition of 4 ml 0.4 M perchloric acid. After 15 min at 0°C the precipitated RNA was pelleted by centrifugation at 50 000 g for 30 min. An aliquot of the supernatant was then used to determine the absorption at 258 nm (A₂₅₈). Controls which contained TGMED buffer instead of the polymerase preparation were subtracted. This is a measure of the amount of acid-soluble material resulting from the RNA broken down by RNase and is thus a measure of the RNase activity.

2.7. [^3H] Estradiol-binding assay

For the determination of total binding 100 μl of a sample were incubated at 37°C for 10 min with 100 μl 2 nM [^3H]estradiol in TE (1.5 mM EDTA, 1 mM Tris-HCl, pH 7.4) and 20 μl TE. For the determination of low affinity binding 100 μl of a sample were incubated at 37°C for 10 min with 100 μl 2 nM [^3H]estradiol in TE and 20 μl 10 μM estradiol in TE. Then 20 μl of a charcoal-dextran suspension (2.5% Norit A, 0.25% Dextran T-70 in TE) were added and the mixture kept in ice for 15 min. After centrifugation at 2000 g for 5 min the radioactivity of the supernatant was measured as described for the RNA polymerase assay. The high affinity binding was obtained by subtracting the low affinity from the total binding.

3. Results

RNA polymerases were solubilized from liver nuclei and purified up to the dialysis step following the procedure of Roeder and Rutter [6] i.e. before DEAE-Sephadex chromatography. In this fraction nucleolar polymerase A and nucleoplasmic polymerase B are not yet separated but they can be distinguished in the assay because of their different properties with respect to the ionic conditions and the α -amanitin

sensitivity. Polymerase A shows maximal activity in a low salt medium containing Mg-ions. Polymerase B is only active at high ionic strength. The main difference between A and B, however, is their different sensitivities to the inhibitor α -amanitin. While polymerase A is not influenced even by high concentrations of α -amanitin, polymerase B is completely inhibited even by concentrations as low as 0.4 $\mu\text{g/ml}$. Therefore this concentration was used for the assay of polymerase A. Since there is no specific inhibitor for polymerase A we performed two assays in the high salt medium, one (a) without α -amanitin measuring the sum of the activities of polymerase A and B, and the other (b) with α -amanitin (0.4 $\mu\text{g/ml}$) measuring the activity of polymerase A in the high salt medium. Subtracting (b) from (a) we got the activity of polymerase B.

24 h after a single injection of estradiol the RNA polymerases were found to be much more active in synthesizing RNA in a cell-free system with calf thymus DNA as template than the RNA polymerases from controls. The activity of polymerase A as well as polymerase B from estrogenized animals is more than 150% higher than that of the control (table 1), when measured at a concentration where the RNA synthesis increases linearly with an increasing amount of polymerase in the assay (up to 50 μg protein/assay).

Table 1
Enzyme and estradiol-binding activities in solubilized RNA polymerase preparations from estrogen-treated and control chickens

	Control	%	Estradiol (24 h)	%
Polymerase A activity (cpm/mg protein)	87 375	100	231 055	264
Polymerase B activity (cpm/mg protein)	46 584	100	125 485	269
[^3H]estradiol bound (cpm/ A_{280})	198	100	379	191
RNase activity (A_{288}/A_{280})	1.5	100	0.95	63

For each experiment 6 livers were pooled. The various assay systems are described in Materials and methods. The RNase and binding activities were calculated relative to the amount of protein (A_{280}).

The solubilized polymerase preparations were also tested for their content of estradiol-binding sites as well as their RNase activity (table 1). High affinity estrogen-binding sites are present in both enzyme preparations and appear to be increased after estrogen treatment. The binding sites in the latter case might be masked by endogenous estradiol to a high degree. Thus the actual amount of binding sites in the enzyme preparation after estrogen treatment is probably much larger. When we measured the degradation of yeast RNA by polymerase preparations, we found it to be 40% lower with preparations from estrogenized animals than with those from controls (table 1). In another experiment the [^3H] RNA synthesized during the polymerase assay itself was used as an endogenous substrate for RNase. After a certain time of incubation, during which [^3H] RNA had been synthesized, the incorporation of [^3H] UMP was stopped by the addition of an excess of UTP. During subsequent incubations for various times the degradation of the in vitro synthesized [^3H] RNA was measured. In this case we did not find any significant difference in the extent of [^3H] RNA degradation by polymerase preparations from controls and estradiol treated animals (fig. 1a and b). Therefore, we followed another approach to test whether we were dealing with a real increase of polymerase activity rather than

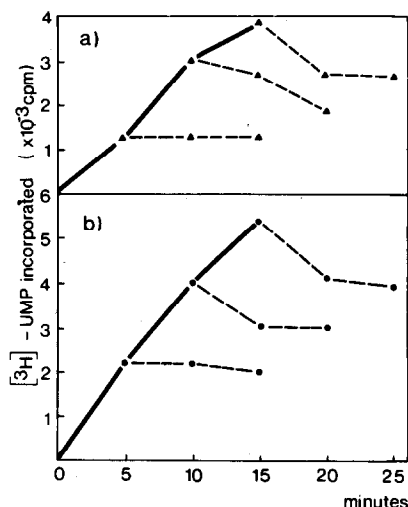


Fig. 1. Degradation of endogenous RNA. (a) Control (\triangle — \triangle) RNA synthesis. (\triangle --- \triangle) RNA degradation. (b) Treated with estradiol. (\bullet — \bullet) RNA synthesis. (\bullet --- \bullet) RNA degradation.

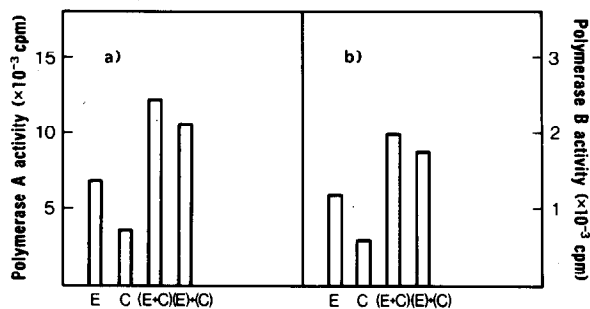


Fig. 2. Mixed incubations. (E) Polymerase preparation from estrogenized animals. (C) polymerase preparation from controls. (E + C) Mixed incubation containing simultaneously E and C. ((E) + (C)) sum of the single polymerase activities E and C. (a) RNA polymerase A activity. (b) RNA polymerase B activity.

an increase simulated by a lower RNase activity. Mixed incubations were carried out which contained simultaneously at a 1:1 ratio (with respect to the amount of protein) polymerase preparations from control and estrogenized animals. In these experiments the activity of the mixed incubation is equal to the sum of the single polymerase activities. This holds for polymerase A as well as polymerase B (fig. 2a and b). In addition kinetics of RNA synthesis in the cell free system showed that the stimulation is already measurable after a very short incubation time (2 min) and remains nearly constant during longer incubation periods (fig. 3a and b).

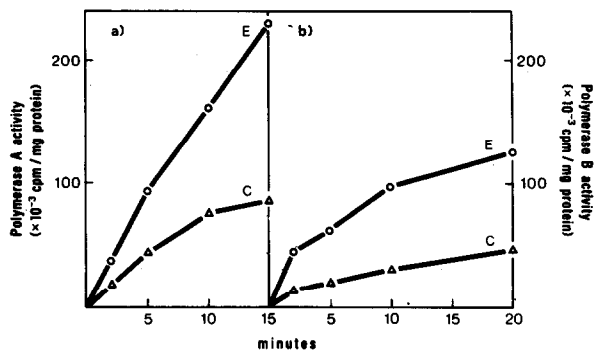


Fig. 3. Kinetics of RNA synthesis. (\triangle — \triangle) Control (\circ — \circ) Treated with estradiol. (a) RNA polymerase A activity. (b) RNA polymerase B activity.

4. Discussion

24 h after the injection of estradiol we found a stimulation of both solubilized RNA polymerase activities in chicken liver that of the nucleolar as well as that of the nucleoplasmic polymerase. This is in agreement with results of Bieri and Dierks-Ventling ([7] and personal communication) who tested RNA polymerase activities in isolated liver nuclei. Berg et al. [8], however reported a selective enhancement of the nucleolar RNA polymerase activity after DEAE-Sephadex chromatography. We do not know if these authors also tested their polymerase preparation before chromatography. Together with the polymerase activities the dialyzed fraction contains estrogen-binding sites which are increased several fold after estrogen treatment. They may be identical with Baulieu's soluble nuclear receptor [9] and/or the estrogen-binding sites on the chromatin [10]. Their binding characteristics, however, have yet to be determined.

Measuring the degradation of yeast RNA by polymerase preparations we found that this was decreased by about 40% after estradiol treatment of the animals, indicating a lower RNase activity. Therefore it became necessary to test whether the increased amount of the RNA synthesized in vitro using polymerase preparations from estrogenized animals was due only to this lower RNase activity.

A much better test for that RNase activity, which acts on the RNA synthesized during the polymerase assay, is to measure directly the degradation of this endogenous RNA. Performing such a test we could not detect any significant difference between preparations from controls and estrogenized animals. This endogenous RNA may be better protected against attack by RNase than exogenous yeast RNA. The RNA synthesized during short incubation times (5 min, see fig.1) seems to be especially well protected, since no degradation at all is detectable. Even at these short incubation times, however, the difference between polymerase preparations from controls and estrogenized animals with respect to the amount of RNA synthesized is very distinct (see fig.3). It is very unlikely that a difference in RNase activities can be responsible for this effect. Last, but not least, in mixed incubations containing the polymerase preparations from controls and estrogenized animals in the same

test tube the amount of RNA synthesized was equal to that calculated by adding the data of both single incubations. An RNase more active in the control preparation should have also degraded the RNA synthesized by the 'estradiol preparation' during the mixed incubation resulting in a difference of the theoretically calculated and the experimentally determined amounts of synthesized RNA. The same holds for an inhibitor of RNA synthesis.

Taking these results together we may conclude that the increased synthesis of RNA by polymerase preparations from estrogenized chickens is actually due to a stimulated RNA polymerase activity, which is not just simulated by a lower RNase activity or the inactivation of some inhibitor of RNA synthesis.

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