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Stimulation of RNA polymerase I and II activities by 17β -estradiol receptor on chick liver chromatin

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ABSTRACT

The endogenous transcriptional capacity (RNA polymerase I and II activity) of liver chromatin from chicks treated with 17β-estradiol for 24 h (E 24) was double that of the controls. E 24 chromatin contained estradiol receptor activity while control chromatin did not. Its presence suggested an implication in the enhanced activities of RNA polymerases of E 24 chromatin. When semi-purified estradiol receptor was added to control chromatin, the endogenous transcriptional capacity of this chromatin was greatly increased. Studies with α-amanitin showed that both RNA polymerase I and II were stimulated by the estradiol receptor. This stimulation was observed as long as homology of the system was maintained. Solubilized homologous RNA polymerases were stimulated much less by the hormone complex in the presence of heterologous DNA than with homologous chromatin. Prokaryotic RNA polymerase could not be stimulated by chick liver estradiol receptor in the presence of heterologous DNA.

INTRODUCTION

In 1971 Arnaud et al.[1,2] demonstrated an increased nucleolar RNA polymerase I activity in calf uterus after estrogen stimulation and proposed that it was due to an activation of the enzyme by the estradiol receptor complex. Müller et al.[3] while purifying RNA polymerase I from estrogen-stimulated quail oviduct found that the enzyme copurified with the estradiol receptor. In our laboratory we purified RNA polymerase I and II[4,5] from liver of estrogen-treated chicks and we noted that after solubilizing the RNA polymerases from their template estradiol receptor activity was present in the fraction containing the polymerases. All these findings suggest that the

association of estradiol receptor with RNA polymerases may not be entirely fortuitous but may have an underlying function such as the suggested activation of the RNA polymerases. We tested this hypothesis in the following way: estradiol receptor was semi-purified from liver nuclei of chicks pretreated with estrogen for 24 h and then added to chromatin from control nuclei which contained endogenous RNA polymerases I and II and the activities of these enzymes were measured. Under conditions of chromatin saturation by the estradiol receptor the activities of the two polymerases were increased by approximately 200%. A number of control experiments were included to ascertain that the observed stimulation of the estradiol receptor fraction was indeed due to the receptor itself. This paper describes these experiments.

MATERIALS

Unlabelled nucleotide triphosphates (ATP,CTP,UTP and GTP) were obtained from Boehringer Mannheim GmbH (Mannheim,Germany) and [5-^{3}H]cytidine 5'-triphosphate (spec.act. 17 Ci/mmol) was from The Radiochemical Centre (Amersham, U.K.). Salmon sperm DNA was from Serva Ltd. (Heidelberg, Germany) and α-amanitin from Boehringer Ingelheim GmbH (Ingelheim a.R., Germany). New England Nuclear Corporation (Boston, Mass., U.S.A.) supplied the [6,7-^{3}H(N)] estradiol-17β (spec.act. 48 Ci/mmol). 17β-Estradiol was obtained from CIBA-GEIGY Ltd. (Basel, Switzerland). E. coli polymerase (spec.act. 833 units/mg protein) was from Miles Laboratories Inc. (Kankakee, Illinois, U.S.A.). All other chemicals not mentioned were of analytical grade.

White leghorn chicks of both sexes were supplied locally at the age of 1 week. They were maintained in electrically heated brooders and were used after they had reached a body weight of 100 g. It was previously[6] established that immature chicks of either sex responded to the estrogen treatment. Chicks were starved overnight after being injected with 17β-estradiol (25 mg/kg) dissolved in propylene glycol (1 mg/0.1ml)

and sacrificed 24 hours later. Dose response data were described earlier[6]. Nuclei or chromatin originating from 24 h estrogen treated animals are given the prefix E 24.

METHODS

Preparation of purified liver nuclei

Nuclei were prepared at pH 5.8 as described previously[7] except that they were resuspended in TGMED buffer containing 50 mM Tris-HCl pH 7.9, 25% glycerol, 5 mM $MgCl_2$, 0.1 mM EDTA and 0.5 mM dithiothreitol, at a concentration corresponding to 1 g liver/ml. In this buffer nuclei could be stored at -80°C for about 1 month without measurable loss of endogenous RNA polymerase activities. The protein:DNA:RNA ratio was 1.67± 0.38:1:0.30±0.08 (means of 10 determinations ± S.D.).

Preparation of chromatin

Chromatin was made rapidly from the above nuclei immediately after thawing if they had been kept at -80°C using the procedure of Paul and Gilmour[8] and suspended in distilled H_2O. The chromatin was mechanically sheared by means of 18 and 23 gauge needles attached to a syringe[9] and diluted to contain 500 µg DNA/ml. One ml nuclei containing 4.8 mg DNA usually yielded 2 ml of chromatin containing 1 mg DNA/ml. Loss of proteins amounted to 50% and loss of RNA to 80%. 45% of the endogenous RNA polymerases of the nuclei were maintained in the chromatin. Since chromatin preparations had to be used immediately to get optimal RNA polymerase activities, DNA was determined by means of the A_{260} nm and proteins according to Ehresmann et al.[10]. The chromatin had a protein:DNA:RNA ratio of 1.44±0.12:1:0.16±0.01 (means of 30 determinations ± S.D.).

Preparation of solubilized RNA polymerases (Sc).

Nuclei suspended in TGMED buffer were stirred rigorously in ice while a saturated $(NH_4)_2SO_4$ solution in 50 mM Tris-HCl pH 7.5 was slowly added until the suspension was 0.24 M in $(NH_4)_2SO_4$. This was followed by sonication (Branson sonifier

B-12, 3-4 times for 15 sec at 70 watt). Addition of the $(NH_4)_2SO_4$ solution was continued until 30% saturation. After centrifugation for 30 min at 100,000 x g the pellet was discarded and the supernatant fluid brought to 45% saturation in $(NH_4)_2SO_4$. The centrifugation step was repeated and the resulting pellet was homogenized gently in TGMED buffer and left overnight stirring in ice. After a final centrifugation at 100,000 x g the supernatant fluid termed fraction Sc contained the solubilized RNA polymerases. The three classes I, II and III were present as measured under optimum conditions for each one of them[11]. A characterization of fraction Sc will appear elsewhere[5].

Preparation of estradiol-receptor fraction

E 24 nuclei were extracted with 0.5 M KCl-10 mM Tris-HCl pH 8.0 after a freezing-thawing step as described previously[12]. The crude extract was adjusted to contain 3-5 mg protein/ml with buffer E (10 mM Tris-HCl pH 8.0, 0.5 M KCl, 1 mM EDTA, 5 mM $CaCl_2$) and brought to 42% saturation with a saturated solution of $(NH_4)_2SO_4$ in 0.1 mM Tris-HCl pH 8.0 under a stream of nitrogen. The resulting precipitate was centrifuged for 10 min at 20,000 x g and the pellet resuspended in buffer E. A further centrifugation for 30 min at 20,000 x g pelleted insoluble material. The supernatant fluid contained usually 120-150 fmoles estradiol receptor/mg protein. Purification was 20 fold. The yield was approximately 3 pmoles estrogen receptor/g original liver. This preparation will be called estradiol receptor for reasons of simplicity.

Biochemical assays

a) RNA polymerases. The following basic incubation mixture for the determination of RNA polymerase I and II activities together[13] was chosen. It consisted of 12% glycerol, 80 mM Tris-HCl pH 7.9, 4 mM thioglycerol, 0.1 mM EDTA, 0.1 mM dithiothreitol, 1 mM each of ATP, GTP and UTP, 0.2 mM CTP, 0.2 µM [^{3}H]CTP (spec.act. 23 Ci/mmol) and 2 mM $MnCl_2$. To 200 µl of this mixture were added either 50 µl chromatin (500 µg DNA/ml) or 50 µl of fraction Sc or 50 µl E. coli

polymerase (0.5 units). In the last two cases, 50 µg DNA as template was also included. Incubation was always for 10 min at 30°C and the reaction was stopped by the addition of 2 ml of 10% CCl_3COOH - 5% $Na_4P_2O_7$, 200 µg BSA was added as a carrier and the precipitates were collected on Whatman GF/C filters which were washed extensively with 5% CCl_3COOH - 0.5% $Na_4P_8O_7$ before being teated with 1 ml 20% NCS in toluene for 1 h at 37°C. ^3H was counted at an efficiency of 33% in an Isocap 300 liquid scintillation counter. Results are expressed as pmoles [^3H]CTP incorporated in 10 min at 30°C or in units whereby 1 unit corresponds to the amount of enzyme which incorporates 1 nanomole [^3H]CTP in 10 min at 30°C.

b) Estradiol receptor. The method of Mester and Baulieu[14] was used for nuclear extracts and for chromatin prepared as above. Chromatin (1 mg DNA/ml) was made 0.5 M in KCl in 10 mM Tris-HCl pH 8.0, pelleted at 4,000 x g for 20 min and the estrogen receptor was estimated in the supernatant fraction just like for nuclear extracts. A Scatchard plot[15] was used to calculate the dissociation constant which was of the order of 10^{-10}M and the number of fmoles of receptor present.

RESULTS

Characteristics of the endogenous transcriptional capacity of chick liver chromatin

The endogenous RNA polymerase activities were compared in chromatin from normal and E 24 chicks. Incubation conditions were such that RNA polymerase I and II activities were measured together, which was in the presence of 2 mM Mn^{2+} [11]. In the presence of α-amanitin (4 µg/ml) a 50% inhibition was observed indicating a 1:1 participation of the RNA polymerases I and II under our conditions. Fig. 1 shows that the endogenous transcriptional activity of both control chromatin and E 24 chromatin tapered off after 10 min of incubation at 30°C, a

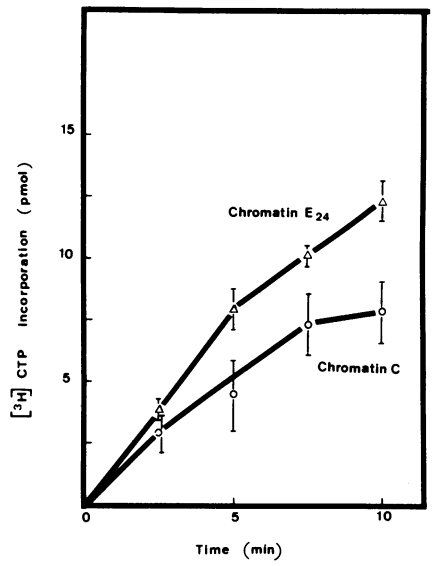

Fig. 1

Endogenous RNA polymerase activities in chick liver chromatin as a function of time of incubation
Chromatin preparation and incubation conditions are described in the experimental section. Chromatin (25 µg DNA) from control chicks (o——o) or from E 24 chicks (Δ——Δ) was incubated as such at 30°C. Horizontal lines indicate standard deviations based on 15 experiments.

plateau was maintained in both up to 60 min (unpublished observation). Routinely, all incubations were therefore terminated at 10 min.

At this time RNA synthesis by E 24 chromatin was almost double that of control chromatin. Incorporation of labelled CTP was indeed into RNA since actinomycin D (Calbiochem) inhibited the reaction completely (Table I). To find out whether the tapering off seen at 10 min of incubation was due to ribonuclease activity inherent in the chromatin preparation, the following tests were carried out: 1) addition of cold excess CTP: this did not result in a decrease of counts (Table I), 2) addition of chromatin (25 µg) to sodium boro-[^{3}H]hydride-labelled purified tRNA from chick liver (courtesy of Dr. J.P. Jost) followed by an incubation under standard conditions for RNA polymerase produced only 73 dpm of acid soluble radioactivity. (The internal control done with 0.5 units pankreatic ribonuclease (Sigma) produced 5195 dpm under these conditions). A ribonuclease activity of our chromatin preparation was therefore ruled out and the formation of a plateau at 10 min is a sign of completion of the reaction rather than of destruction of the product.

Table I: The endogenous transcriptional capacity of chromatin

	^{3}H CTP incorporated into RNA pmol	Estradiol receptor fmol/μg DNA
Control chromatin: complete system*	6.3 ± 1.1	0
+ actinomycin D (20 μg/ml)		
+ 100 fold excess cold CTP added at 10 min, reaction continued until 60 min	7.0	
E 24 chromatin	13.8 ± 2.5	0.25

* containing all 4 nucleotides, 2 mM Mn^{2+} and chromatin (25 μg DNA) as described in the experimental section. Incubation was 10 min at 30°C. Average of 10 experiments ±SD.

The finding that the transcriptional capacity of E 24 chromatin was increased over that from controls and the fact that E 24 chromatin contained estradiol receptor but control chromatin did not (Table I) led to the assumption that the receptor may be responsible for the difference in endogenous RNA polymerase activities. We set out to test this with a sort of "reconstituted" E 24 chromatin. For this purpose estradiol receptor was to be added to control chromatin and its transcriptional capacity to be tested before and after. Thus, from E 24 nuclei a semi-purified estradiol receptor preparation was made as described in the experimental section. Before it could be added to chromatin, it was necessary to test whether the estradiol receptor preparation contained RNA polymerase itself (which would have an additional effect) or DNA (which could provide more template) or DNAse (which could cause nicking of the chromatin and thus produce more initiation sites) all of which would lead to false-positive results. These tests are summarized in Table II.

Table II: Possible factors contained in the estradiol receptor fraction which could stimulate RNA polymerase

Suspected factor	Assay conditions	Acid insoluble radioactivity dpm	Conclusions
RNA polymerase	50 µg DNA plus estradiol receptor	23	absent
DNA	50 µl RNA polymerase fraction Sc plus estradiol receptor	no counts above blank	absent
	Internal control: 50 µl RNA polymerase fraction Sc plus 50 µg DNA	2521	
		Acid soluble radioactivity dpm	
DNAse	50 µg ^{3}H-chromatin plus estradiol receptor	no counts above blank	not active
	Internal control: 50 µg ^{3}H-chromatin plus 0.05 µg DNAse I (Worthington)	7259	

For all these tests the standard incubation mixture for RNA polymerase was used which contained in addition the items mentioned above. Estradiol receptor was always added in amounts corresponding to 70 and 140 fmoles, whereby buffer E was added to constant ion concentration. Incubation was for 10 min at 30°C. For the DNAse tests, a 100 g chick was injected with 1 mCi of [6-^{3}H]thymidine (spec. radioactivity 20,000 mCi/mmole) and 30 min later liver nuclei were prepared as the starting material for chromatin, described in details in the experimental section; the incubation mixture contained labelled chromatin instead of labelled nucleotide.

It is evident that none of the suspected factors was present or active under our conditions. (Another, negative factor which needed clarifying was the possible presence of ribo-

nuclease activity in the estradiol receptor fraction: the test was done as with chromatin and from the labelled tRNA 55 dpm were liberated per 10 fmoles of receptor. Since we planned to use up to 100 fmoles, this result had to be borne in mind).

Effect of estradiol receptor on the transcriptional capacity of chromatin

Increasing amounts of estradiol receptor were now added to chromatin from untreated chicks. The effect on the endogenous transcriptional capacity of this chromatin is shown in Fig. 2. A peak value corresponding to 5.6 fmoles estradiol receptor per μg DNA was obtained. This was more than we had determined as present in chromatin preparations from treated animals (see Table I); since the methodology for chromatin preparation was optimized for endogenous RNA polymerase activity and not for estradiol receptor activity, it is possible that a large portion of the estradiol receptor was lost during the preparation. This would also explain why in these "reconstitution" experiments the increase in endogenous transcriptional capacity obtained was much greater than the difference between control and E 24 chromatin (Table I).

When estradiol receptor was added in amounts greater than 5.6 fmoles/μg DNA, then the stimulatory effect diminished. A likely explanation would be that once saturation is obtained, the ribonucleases present in the estradiol receptor fraction are activated. The experiment with 5.6 fmoles receptor/μg DNA was also done in the presence of α-amanitin and demonstrated that the stimulation by estradiol receptor was not linked to one RNA polymerase but affected both RNA polymerase I and II (unpublished observation). Treatment of chromatin with 17β-estradiol alone did not result in any increase in RNA synthesis whatsoever (unpublished observation). When additional homologous RNA polymerase fraction Sc was added to control chromatin to occupy some of the free sites, then the effect by the estrogen receptor on the transcriptional capacity

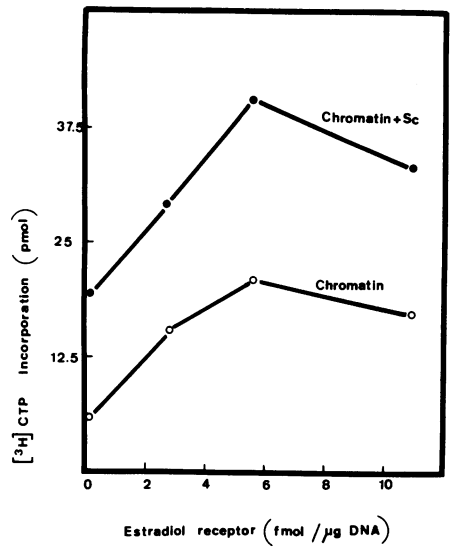

Fig. 2:
Activation of the endogenous transcriptional capacity of chick liver chromatin by estradiol receptor.
To control chromatin (12.5 µg DNA) were added increasing amounts of estradiol receptor fraction in the absence (o——o) or in the presence of additional 0.05 units of solubilized RNA polymerase fraction Sc (●——●). Since the estradiol receptor fraction contained 0.5 M KCl and 5 mM $CaCl_2$, care was taken that all samples contained the same ionic strangth by the addition of buffer E to constant volume. Thus all samples contained 0.2 M KCl and 2 mM $CaCl_2$ in addition to the ions present in the incubation mixture. Incubation conditions (10 min at 30°C) are described in details in the experimental section.

was further enhanced (Fig. 2, upper curve). The peak value of 5.6 fmoles/µg DNA was used to check the activity of the RNA polymerases as a function of time (Fig. 3) and it can be seen that the pattern did not vary from the one obtained with chromatin only (Fig. 1).

All the above experiments were carried out under conditions of constant ion concentration of the incubation mixture, the reason being the fact that the estradiol receptor was in buffer E which contains 0.5 M KCl and 5 mM $CaCl_2$. Whenever increasing amounts of receptor were tested, buffer E was added to the incubation mixtures to give a constant final concentration of 0.2 M K^+ and 2 mM Ca^{++}. Controls with only

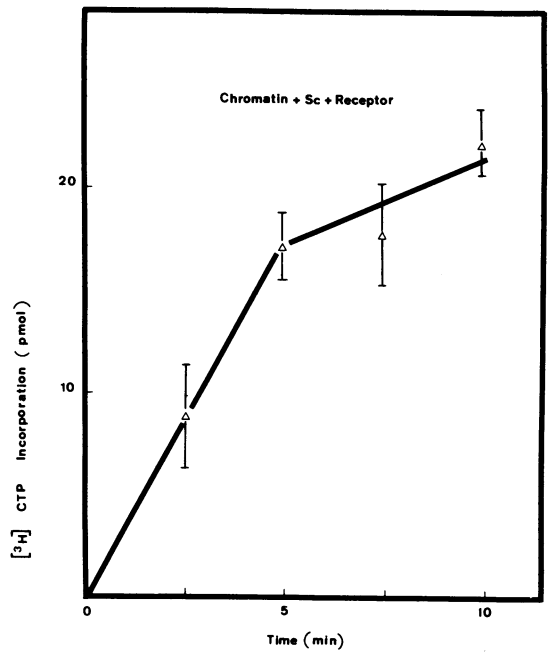

Fig. 3:
Transcriptional capacity of chromatin saturated with estradiol receptor as a function of time of incubation. Control chromatin (12.5 µg DNA) to which were added 0.05 units of RNA polymerase fraction Sc and 72.4 fmoles estradiol receptor (saturating amount), was incubated at 30°C for the time given under standard conditions. Horizontal lines indicate standard deviations based on 6 experiments.

buffer E at these ionic concentrations showed no stimulation of chromatin polymerase activity.

How specific is the estradiol receptor effect on the stimulation of RNA polymerase activity ?

As the estradiol receptor fraction was enriched but not highly purified, a number of parameters needed investigating in order to confirm that the effect seen on the transcriptional capacity of chromatin could indeed be ascribed to a specific stimulation. On chromatin the endogenous as well as the added homologous RNA polymerases were effectively being stimulated, however, it became also necessary to investigate whether their activity would be increased with naked DNA as a template. 0.05 units of solubilized RNA polymerase fraction Sc were thus incubated under standard conditions with increasing amounts of estradiol receptor in the presence of 50 µg salmon sperm DNA as a template. Fig. 4 shows a small increase with estradiol receptor up to 1.5 fmoles/µg DNA, but increasing amounts of estradiol receptor caused increasing inhibition of the reaction. As the estradiol receptor fraction contained

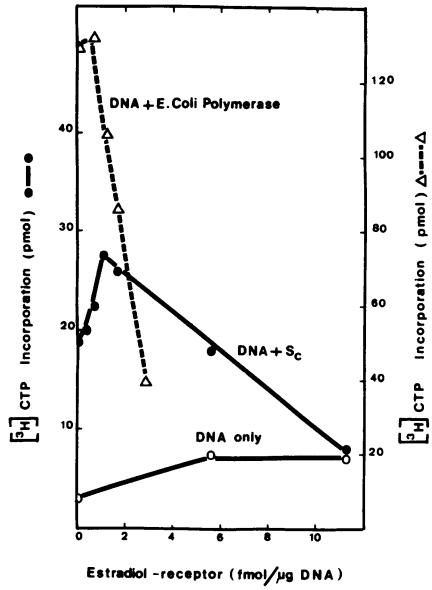

Fig. 4:

Effect of estradiol receptor on homologous and heterologous RNA polymerase in the presence of salmon sperm DNA. To salmon sperm DNA (50 µg) were added increasing amounts of estradiol receptor fraction. This was incubated (10 min at 30°C) as such (o—o) or with 0.5 units of E. coli polymerase (Δ---Δ) or with 0.05 units of chick liver RNA polymerase fraction Sc (●——●). Notice the different scale for E. coli polymerase.

some ribonuclease activity, a possible explanation was that these RNAses became increasingly active as more free nascent RNA chains were formed. With E. coli polymerase no stimulation was obtained and increasing amounts of estradiol receptor caused increasing inhibition of the reaction. Clearly, the estradiol receptor fraction was not capable to activate a prokaryotic RNA polymerase on naked DNA under our conditions.

DISCUSSION

Our results demonstrate that 1) the transcriptional capacity of E 24 chromatin is greatly increased over that of control chromatin, 2) E 24 chromatin contained estradiol receptor but the control chromatin did not and 3) a "reconstituted E 24" chromatin can be made by the addition of estradiol receptor to control chromatin. Our experimental approach was based in part on our findings that the estradiol receptor copurified with RNA polymerases I and II in the initial steps at least[4,5] if

liver nuclei from estradiol treated animals were the starting material. Furthermore, there are several reports in the literature which propose that the hormone receptor may be associated with or even activate RNA polymerases which implies an alteration of the conformation of the genetic structure of the chromatin in order to allow the specific transcription of the gene[1,2,3]. While these models were based on the mere presence of receptor activity in one of the RNA polymerase fractions tested, O'Malley's group studied the correlation between estradiol receptor of the nucleus and initiation sites on chromatin[16,17] and very recently showed that purified progesterone receptor complex increased the initiation sites on chick oviduct chromatin for E. coli polymerase[18] and that this could be attributed to one of the two subunits of the receptor[19]. Our approach was similar except that we concentrated on the homologous system. By adding estradiol receptor complex to chick liver chromatin and then studying, under appropriately controlled conditions, the endogenous transcriptional capacity of this chromatin, we found that there occurred an increase in the RNA synthetic activity which was even greater than in chromatin from E 24 chicks (Fig. 2). E 24 chromatin was found to contain hormone receptor complex, control chromatin did not (Table I). Interestingly enough, control nuclei by contrast do contain a small amount of receptor[12,20] which during the purification of chromatin apparently is lost. Subjecting control chromatin to estradiol receptor complex - but not to 17β-estradiol alone - thus resulted in a sort of "reconstitution" of chromatin from estrogen-treated chicks. The validity of this experiment was enhanced by controlling possible false-positive effects, such as RNA polymerase activity, DNA and DNAse in the receptor fraction itself none of which were present (Table II). The activation of the RNA polymerases was also tested with template-free solubilized RNA polymerases from chick liver: the estrogen receptor did indeed activate these solubilized polymerases but much less so in the presence of DNA than in the presence of chromatin (Fig. 4). This

finding indicated to us that possibly a particular component of chromatin may be responsible for binding the complex to the RNA polymerases. Spelsberg et al.[21,22] have shown earlier in reconstitution experiments using homologous as well as heterologous chromatin that the level of binding of the receptor hormone complex appears to be established by the source of the non-histone proteins, e.g. only non-histone proteins from target tissues were capable to bind the complex. We could not increase the activity of E. coli polymerase on naked DNA by estradiol receptor; O'Malley showed a stimulation of prokaryotic polymerase on chick oviduct chromatin in the presence of progesterone receptor[18,19]. This establishes the importance of the presence of chromatin in this process[17]. However, the exact mechanism as to how the homologous RNA polymerase activity is increased by estradiol receptor remains to be solved.

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