Effect of Estradiol on Rat Uterus DNA-dependent RNA Polymerases

STUDIES WITH WHOLE NUCLEI

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A systematic approach to the in vitro measurement of DNA-dependent RNA polymerase activities in nuclei from immature rat uterus has been undertaken in order to evaluate the early effects of estradiol. The experimental conditions include excess of nucleoside 5'-triphosphate and short time assays (6 min) at low temperature (25°C) in order to obtain the maximal velocity of the reaction and to minimize RNase activity. Nucleotide incorporation has been measured in the absence (low ionic strength medium) or in the presence (high ionic strength medium) of 0.25 mM ammonium sulfate under various divalent cation concentrations, either in the presence or absence of α-amanitin.

The α-amanitin-resistant RNA polymerase activity of nuclei is identical under three conditions, low ionic strength with either Mg²⁺ or Mn²⁺ (4 mM) and high ionic strength in the presence of 4 mM Mn²⁺. α-Amanitin-sensitive activity measured at high ionic strength is about 10 times greater than the activity measured at low ionic strength in the presence of 4 mM Mn²⁺. Under all experimental conditions used in these studies, in vitro initiation of new RNA chains is negligible.

Estradiol administration leads to an early (1 to 2 h) increase (50%) in α-amanitin-resistant activity under all experimental conditions. The α-amanitin-sensitive activity exhibits a similar evolution (+100% at 2 h) when measured under low ionic strength conditions. However, the same activity, measured under high ionic strength conditions, is constant during the first 6 h after hormone treatment.

Since enzyme activity measured under high ionic strength conditions mainly reflects the number of enzyme molecules engaged in transcription, these results suggest that estradiol leads to an early increase in the number of α-amanitin-resistant molecules engaged in the process of transcription, while the number of α-amanitin-sensitive molecules remains constant. The increase in α-amanitin-sensitive activity obtained under low ionic strength conditions can be interpreted either by an increase in template activity, or by an activation of the molecules already engaged in the process of transcription, or both.

After estradiol administration, RNA polymerase activities have been measured in isolated nuclei of the uterus of prepupal rats, and various patterns for their increase have been reported (1-9). These activities have been determined essentially (a) in low ionic strength medium in the presence of Mg²⁺ for nucleolar activity, and (b) in high ionic strength medium in the presence of Mn²⁺ for extranucleolar activity. However, the measurement of RNA polymerase activities in intact nuclei raises two main problems. First, the effects of the various salt and divalent cation conditions on the polymerase themselves and on chromatin structure are far from being understood. Second, the enzymes are present in the nuclei in two forms, a fraction tightly bound to chromatin, difficult to extract and probably engaged in a DNA-enzyme-RNA transcription complex, and another part readily extractable from the cell nucleus and presumably inactive in transcription (10, 11). The respective contribution of these two polymerase fractions in the in vitro assays with isolated nuclei has not yet been established.

In this work, α-amanitin (40 μg/ml) was used to distinguish between nucleolar polymerase activity A (or I), resistant to the inhibitor, from nucleoplasmic polymerase activity B (or II), sensitive to the inhibitor (12-14, and for reviews see Refs. 15 and 16). Assays for measuring both A and B activities in low and high ionic strength media are presented, as well as a tentative interpretation of the results obtained under these different conditions. The possible contribution of the easily extractable enzyme to the low ionic strength nuclear activity has been evaluated, since its loss during the isolation of nuclei cannot be controlled. With the techniques which have been set up, the RNA polymerase activities of immature rat uterine nuclei have been measured at various times after estradiol injection.

MATERIALS AND METHODS

Chemicals— Estradiol prepared at Roussel-Uclaf was 99% pure as verified by thin layer chromatography. Actinomycin D was from Sigma. α-Amanitin and labeled nucleoside triphosphates (grade A reagents) were from Calbiochem. Tritiated uridine 5'-triphosphate

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(10 Ci/mmol) was purchased from the Radiochemical Centre (Amersham, Bucks, England) and checked for purity by thin layer chromatography before use.

Manganese sulfate, magnesium chloride, sodium ammonium phosphate, ammonium sulfate, and double-distilled glycerol were supplied by Prolabo (France). Trichloroacetic acid was purchased from Merek, and thiglycollar (purissimum) from Fluka (Switzerland). EDTA was purchased from Calbiochem. Trizma (2-amino-2-hydroxymethyl-1,3-propanediol) base was supplied by Sigma.

Buffer and Solutions—Stock solutions of 2 M Tris/HCl, pH 7.8 (4°C), 0.1 M EDTA adjusted to pH 7.0, 1.25 M thiglycollar, and 0.1 M dithiothreitol were diluted to prepare MS(25) buffer (50 mM Tris/HCl, pH 7.0, 10 mM thiglycollar, 0.1 mM EDTA, 0.1 mM dithiothreitol, and 25% (v/v) glycerol). Dithiothreitol was added immediately before use. Saturated ammonium sulfate was prepared at 25°C and neutralized with concentrated ammonium hydroxide until the pH of a 1:20 dilution was 7.5 at 4°C.

Animals—Immature female Sprague-Dawley rats (9 to 21 days old, 60 g) were purchased from Charles River. They received intraperitoneally 1 µg of estradiol dissolved in 0.1 ml of saline (0.9% NaCl solution) with 10% (v/v) ethanol. Controls received the same volume of solvent.

Preparation of Nuclei—The animals were killed by exsanguination at different times after estradiol injections. Their uteri were immediately removed, weighed, and homogenized in 0 volumes of 0.2 M sucrose, 1 mM MgCl₂ (Medium A) at 2°C, with 10 strokes in a glass-glass conical homogenizer. The homogenate was diluted 2-fold with Medium A and centrifuged at 4°C for 10 min at 900 × g in a Claris centrifuge. The crude nuclear pellet was washed twice with Medium A and once with MS(25) and resuspended in a MS(25) medium to give approximately 100 µg of DNA/ml. Nuclei were stored in liquid nitrogen until use. DNA was estimated by the diphenylamine procedure (17) with calf thymus DNA as standard.

Washing of Nuclei—Nuclei were thawed and the suspension in MS(25) was made in 0.15 M ammonium sulfate (100 µg of DNA/0.1 ml). After centrifugation at 4°C for 30 min at 30,000 × g in a MSE ultracentrifuge, the recovery of DNA in the pellet used for polymerase assay was about 80%.

RNA Polymerase Assay—Unless otherwise specified, the incubation medium (0.25 ml) for low ionic strength assays contained 50 mM Tris/HCl, pH 8.4, 4 mM MgSO₄, or MgCl₂, 2 mM ATP, CTP, and GTP, 0.2 mM ['H]UTP (100 to 300 mCi/mmol), and about 100 µg of DNA. In the high ionic strength assay, the same components in the same volume (0.125 ml) contained only 50 µg of DNA. When indicated, 40 µCi of 5'-a-amanitin or 80 µg/ml of actinomycin D were added to the incubation medium.

The enzymatic reaction was run at 25°C over a period of 6 min. Enzymatic activity was measured at various times by chilling each tube on ice and immediately adding 1 ml of a cold solution of bovine serum albumin (1 mg/ml) as carrier and 2 ml of cold 10% trichloroacetic acid in 0.02 M sodium pyrophosphate. After 30 min the precipitate was isolated by centrifugation, washed once with cold 5% trichloroacetic acid, and dissolved in 1 M sodium/ammonium phosphate and finally reprecipitated with 10% trichloroacetic acid. This step was repeated twice and the final pellet was dissolved in 0.5 ml of Soluene 350 (Packard) and counted in 10 ml of Omnifluor (New England Nuclear Chemicals), 4 g/liter of toluene. The tritium efficiency was 35 to 40% in an Intertechnique liquid scintillation counter. A blank was estimated from a parallel incubation at 0°C and subtracted; its value is constant over the 6-min period of the assay.

Ribonuclease Activity—UMP incorporation was stopped by addition of actinomycin D (20 µg/0.25 ml), and the incubation was run for an additional 6 min at 25°C. Ribonuclease activity was estimated from the decrease of radioactivity in the recovered RNA.

RESULTS

Enzymatic Assay

Under low ionic strength conditions, incorporation of ['H]UMP depends on the presence of the 4 nucleoside triphosphates and is completely suppressed by actinomycin D (Table 1). Similar results were obtained in high ionic strength medium (data not shown). The Km for UTP was measured under low and high ionic strength conditions (Fig. 1). Values of 0.03 to 0.05 mM were obtained, being of the same order of magnitude as the Km's of soluble RNA polymerases obtained from rat uterus and measured on an exogenous template (18).

In order to have an excess of substrate, the ['H]UTP concentration was fixed to 0.2 to 0.3 mM, and the concentration of the other nucleoside triphosphates was 2 mM. Under these conditions, ['H]UMP incorporation was linear during the first 6 min of the reaction, irrespective of the ionic strength and divalent cation conditions (Fig. 2). After 6 min, the apparent rate of the RNA synthesis decreased in some experiments and was no longer linear. Therefore, in order to obtain Vmax, an incubation time of 6 min was adopted. Ribonuclease activity was determined in each nuclear preparation, as indicated under "Materials and Methods." At 25°C, RNA degradation never exceeded 10%, a value within the limits of the overall experimental error.

As already reported for the RNA polymerases of rat liver nuclei (19-21), the presence of Mg²⁺ or Mn²⁺ at various concentrations influences the enzymatic activities of immature rat uterine nuclei. Fig. 3 shows that at low ionic strength, ['H]UMP incorporation is maximal for both Mg²⁺ and Mn²⁺ at a 4 mM concentration. However, the nucleotide incorporation with Mn²⁺ is twice that with Mg²⁺. It has already been reported (19-22) that nuclear RNA polymerase activities are higher in high ionic strength than in low ionic strength. With the prepuberal rat uterine nuclei, maximal activity is ob-
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Fig. 2. Incorporation of [3H]UMP by nuclei varying the ionic strength and divalent cation concentrations. Nuclear fractions from immature rat uteri were incubated at 25°C for various time periods as described under "Materials and Methods." Left panel, nuclear fractions (100 μg of DNA) were incubated without ammonium sulfate (AS); in the presence of 4 mM Mn2+ (●), of 4 mM Mn2+ and 40 μg/ml of α-amanitin (○○○), and of 4 mM Mg2+ (××××). Right panel, nuclear fractions (50 μg of DNA) were incubated in the presence of 0.25 M ammonium sulfate; with 4 mM Mn2+ (○○○), with 4 mM Mn2+ and 40 μg/ml of α-amanitin (○○○). No incorporation indicated for ammonium sulfate concentration ranging from 0.2 to 0.5 M (Fig. 4).

Effects of α-Amanitin

At Low Ionic Strength – In the presence of Mg2+, α-amanitin has practically no effect on [3H]UMP incorporation whatever the Mg2+ concentration (Fig. 3). Consequently, RNA synthesis may be attributed exclusively to RNA polymerase A activity. In the presence of Mn2+, the α-amanitin-resistant RNA polymerase activity is approximately constant for Mn2+ concentration ranging from 2 to 6 mM and is of the same order as that measured with 4 mM Mg2+ (Table II). The α-amanitin-sensitive activity is maximum when Mn2+ concentration is 4 mM and approximately equal to A activity (Table II). Therefore, using α-amanitin (40 μg/ml), it is possible to evaluate both polymerases under the same conditions, i.e. low ionic strength (without ammonium sulfate) in the presence of 4 mM Mn2+.

At High Ionic Strength (0.25 M Ammonium Sulfate) – In the presence of Mn2+ (4 mM), RNA polymerase activity is approximately 10 times greater than when measured in low ionic strength (Table II), and α-amanitin (40 μg/ml) inhibits this activity up to 90%. This result confirms other data (19–22) which have suggested that nuclear polymerase B activity is considerably increased in high ionic strength medium. Under these conditions, the α-amanitin-resistant activity is identical with that obtained under low ionic strength conditions in the presence of either Mg2+ or Mn2+.

Thus, in the presence of α-amanitin, RNA polymerase A activity in nuclei can be measured under three experimental conditions which give similar results: in low ionic strength medium in the presence of Mg2+, in low ionic strength medium in the presence of Mn2+ (4 mM), and in high ionic strength medium with Mn2+ (4 mM). RNA polymerase B activity can be measured in the presence of Mn2+ (4 mM) in low ionic strength medium and in the presence of ammonium sulfate (0.25 M). This is done by subtracting the A activity from the total nuclear activity.

Previous studies (10, 11) have shown that nuclear RNA polymerases are either readily extracted or tightly bound to...
the chromatin. During the isolation of nuclei, there is an uncontrolled loss of the readily extractable fraction. If the free molecules can initiate RNA synthesis during the in vitro assay at low ionic strength, the results will depend on these unknown losses. Consequently, a comparison of the RNA polymerase activity of intact nuclei, as they are used routinely, with a nuclear preparation previously washed with 0.15 M ammonium sulfate was undertaken. The 0.15 M ammonium sulfate washing has been shown to remove all free, easily extractable enzyme (18). Table III shows that [3H]UMP incorporation in washed and in unwashed nuclei is identical, whatever the ionic strength and cation conditions of the enzymatic assay. Moreover, an additional control has been obtained by adding RNA polymerase preparation, solubilized from prepuberal rat uterine nuclei (18), to unwashed nuclei. No effect was observed, as indicated in Table IV. From these results, it is concluded that under the present conditions, [3H]UMP incorporation due to chain initiation by free nuclear enzymes is negligible. A similar result was obtained with rat liver chromatin (23).

TABLE III
UMP incorporation in intact and 0.15 M ammonium sulfate-washed nuclei

Nuclei were prepared and washed with 0.15 M ammonium sulfate. Enzyme assays were performed under standard conditions (see under "Materials and Methods"). Ammonium sulfate, Mn\(^{2+}\), and Mg\(^{2+}\) were present in the incubation medium at the indicated concentrations. A activity was measured in the presence of α-amanitin (40 μg/ml).

<table>
<thead>
<tr>
<th>Ammonium sulfate</th>
<th>Divalent cation (4 mM)</th>
<th>α-Amanitin</th>
<th>A activity (pmol/min/mg of DNA)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>Mn(^{2+})</td>
<td>0</td>
<td>4.4</td>
</tr>
<tr>
<td>0</td>
<td>Mn(^{2+})</td>
<td>10</td>
<td>2.6</td>
</tr>
<tr>
<td>0</td>
<td>Mg(^{2+})</td>
<td>0</td>
<td>3.0</td>
</tr>
<tr>
<td>0.25</td>
<td>Mn(^{2+})</td>
<td>0</td>
<td>26</td>
</tr>
<tr>
<td>0.25</td>
<td>Mn(^{2+})</td>
<td>10</td>
<td>2.8</td>
</tr>
</tbody>
</table>

Fig. 5. Variation of nuclear RNA polymerase A and B activities in immature rat uterus after estradiol administration. Enzymatic activities of nuclei were measured at different time periods after a single estradiol injection. Upper panels, activities measured in the presence of 0.25 M ammonium sulfate (AS) and 4 mM Mn\(^{2+}\). Lower panels, in the absence of ammonium sulfate. RNA polymerase A activity, measured in the presence of 40 μg/ml of α-amanitin and 4 mM Mn\(^{2+}\) (○-○), or 4 mM Mg\(^{2+}\) (△-△), ○, RNA polymerase B activity. This activity corresponds to the difference in the activities measured in the absence or in the presence of α-amanitin (40 μg/ml). Each point (+S.D.) is the average of five independent experiments (seven animals for each point of each experiment).

TABLE IV
UMP incorporation in nuclei after addition of solubilized RNA polymerases from immature rat uteri

Nuclei (100 μg of DNA) from immature rat uterus were incubated for 6 min under standard conditions in the presence of 4 mM Mn\(^{2+}\) and ammonium sulfate at the indicated concentration, either in the presence or absence of α-amanitin (40 μg/ml). Solubilized RNA polymerases were obtained from immature rat uterus (18). UMP incorporation by this enzyme preparation under the same conditions without ammonium sulfate, using a calf thymus DNA template, was 3 and 1.2 pmol in the absence and presence of α-amanitin, respectively. For the control, solubilized enzymes were denatured at 60° for 30 min.

<table>
<thead>
<tr>
<th>Ammonium sulfate</th>
<th>α-Amanitin</th>
<th>UMP incorporated (pmol)</th>
</tr>
</thead>
<tbody>
<tr>
<td>M</td>
<td>0</td>
<td>2.4</td>
</tr>
<tr>
<td>0</td>
<td>10</td>
<td>1.4</td>
</tr>
<tr>
<td>0.25</td>
<td>0</td>
<td>15.6</td>
</tr>
</tbody>
</table>

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**Table V**

RNA polymerase A and B activities in co-incubation of nuclei from control and 6-h treated animals

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>A activity</th>
<th>B activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.84 ± 0.06</td>
<td>0.6 ± 0.12</td>
</tr>
<tr>
<td>6</td>
<td>2.58 ± 0.06</td>
<td>2.1 ± 0.24</td>
</tr>
<tr>
<td>Control + 6</td>
<td>3.12 ± 0.3</td>
<td>2.76 ± 0.3</td>
</tr>
</tbody>
</table>

**Effects of Estradiol**

RNA polymerase A and B activities of prepuberal rat uterine nuclei have been measured after injection of 1 μg of estradiol in five series of experiments (Fig. 5).

As seen in Fig. 5, α-amanitin-resistant activity, measured under low ionic strength conditions in the presence of either Mn⁺⁺ or Mg⁺⁺, increases very early (1 to 2 h) by more than 50%. This increase reaches 100% at 6 h and remains constant until 24 h after hormone injection. The same pattern is obtained when the enzyme activity is measured in the presence of ammonium sulfate.

α-Amanitin-sensitive activity, as measured in high ionic strength conditions, remains unchanged during the first 6 h after estradiol injection but is found to have increased by 70% at 24 h. The results are different when the activity is measured under low ionic strength conditions, the increase (~100%) is seen earlier (at 2 h) and remains constant until 6 h after hormone administration (Fig. 5).

In order to exclude that the observed increases could be attributed to the inhibition of a factor(s) involved in the degradation of newly synthesized RNA, assays of polymerase activities were performed with a mixture of nuclei from the uteri of 6-h treated animals and of control animals. The resulting incorporation is strictly the sum of the UMP incorporations measured separately in nuclei of estradiol-injected and control animals, irrespective of the assay conditions used (Table V).

**DISCUSSION**

RNA polymerase activities in rat uterus nuclei have been most frequently assayed by measuring RNA synthesis at 37° after a rather long time of incubation and in the presence of a limiting nucleotide concentration. Under these conditions, RNAse activity is significant and nucleotide incorporation has reached a plateau (1–9). In the present work, RNA polymerase activity was measured by UMP incorporation at a low temperature (25°) to diminish RNAse activity (which becomes negligible) over short time intervals (from 0 to 6 min) in the presence of an excess of nucleotide (about 10 times the apparent Kₐ).

Previous studies (10, 11) have shown that nuclear RNA polymerases are either readily extractible or tightly bound to the chromatin. Washing of the nuclei with 0.15 M ammonium sulfate (18) eliminates any readily extractible RNA polymerases while keeping those enzyme molecules that are tightly bound to the chromatin. Since increasing the ionic strength prevents initiation of RNA synthesis and results in the release from the DNA template of RNA polymerase molecules which are not engaged in a ternary transcription complex (18), it is very likely that the enzyme activity which remains tightly bound to the chromatin corresponds to the enzyme fraction which is actively engaged in transcription in the form of enzyme-DNA-RNA ternary complexes. The RNA polymerase activities of the 0.15 M ammonium sulfate-washed nuclei are the same as those measured in intact nuclei, whatever the divalent cation and the ionic strength conditions used in the enzymatic assay. Moreover, UMP incorporation into RNA in "intact" nuclei is not modified by addition of a preparation of RNA polymerase molecules solubilized from immature rat uteri. Therefore, it can be assumed that, under our in vitro assays, new initiations (if any occur) do not affect significantly the nucleotide incorporation and that RNA synthetised in vitro is only attributable to the RNA polymerase molecules which have initiated RNA synthesis in vivo.

RNA polymerase A and B activities of immature rat uterine nuclei were measured under different ionic strength conditions and with different concentrations of divalent cations. RNA polymerase A activity is the same whether measured at low ionic strength, in the presence of Mn⁺⁺ or Mg⁺⁺, or at high ionic strength (0.25 M ammonium sulfate) in the presence of Mn⁺⁺. On the contrary, under low ionic strength conditions, RNA polymerase B activity is undetectable in the presence of Mg⁺⁺, while in the presence of Mn⁺⁺ this activity is measurable and reaches a maximum at 4 mM Mn⁺⁺. At this concentration of Mn⁺⁺, RNA polymerase A and B activities are approximately equivalent. Under high ionic strength conditions, in the presence of 4 mM Mn⁺⁺, RNA polymerase B activity is increased 10 times. These results are different from those obtained with solubilized enzymes of immature rat uteri assayed on calf thymus DNA (18). In the latter case, both A and B activities can be measured under low ionic strength conditions, in the presence of Mn⁺⁺ or Mg⁺⁺. The ionic strength and divalent cation requirements are approximately the same for Enzymes A and B when they transcribe the same exogenous DNA. However, these requirements are very different for the transcription of their respective templates, nucleolar (Enzyme A) and extranucleolar chromatin (Enzyme B). A possible explanation is that the two chromatin templates have a different sensitivity to the condensing action of divalent cations and to the decondensing action of salts (24). A part of the increased nuclear activity of Enzyme B at high ionic strength could be due to the demasking of Enzyme B molecules, which had already initiated a RNA chain but whose elongation was inhibited by template condensation. For this reason, we suggest that Enzyme B activity measured at high ionic strength is a good reflection of the total number of Enzyme B molecules engaged in a RNA-DNA-enzyme transcription complex.

Polymerase A and B activities of immature rat uterine nuclei were measured after estradiol administration. RNA polymerase A activity evolves according to the same pattern: whether measured in low ionic strength medium in the presence of Mn⁺⁺ or Mg⁺⁺ or in high ionic strength medium in the presence of Mn⁺⁺, there is an early increase of 50% at 1 to 2 h, which attains 100% at 6 h. This increase is due either to an acceleration of elongation or to an increase in the number of transcription complexes, or both. As discussed above for Enzyme B, the fact that this increased activity is also obtained at high ionic strength supports the second interpretation. RNA polymerase B activity, measured under low ionic strength in the presence of Mn⁺⁺, increases by 70% as early as 2 h. On the contrary, in high ionic strength medium, there is no measurable change in RNA polymerase B activity during the first 6 h and the 70% increase is observed only after 24 h. These results are
suggest that, for the first 6 h, the number of RNA polymerase B molecules engaged in transcription complexes remains unchanged. Therefore, the early increase in RNA polymerase B activity observed in low ionic strength medium can be interpreted as the synthesis of longer RNA chains either by an increase in template capacity or by an activation of RNA polymerase B molecules already tightly bound to DNA, or both.

RNA polymerase activities have already been measured in isolated nuclei of rat uterus after estradiol administration (1-9). In general, RNA polymerase A activity is studied under low ionic strength conditions in the presence of Mn$^{2+}$ or Mg$^{2+}$ and RNA polymerase B activity in high ionic strength medium. The results we obtained under these conditions are in good correlation with those already reported. However, we were never able to reproduce the 200% increase in RNA polymerase B activity, which has been reported, 30 min after estradiol treatment (8, 9).

In a previous report, the activities of uterine nuclear RNA polymerases A and B tightly bound to DNA were measured after solubilization. The activities of both enzymes were assayed with an exogenous DNA template, and the number of enzyme B molecules was determined using binding with 1$^{14}$C]amamin (18). Such studies led to the conclusion that there was an early increase in the number of transcribing A enzyme molecules, while the number of transcribing B molecules was unchanged. Our present results fully support this interpretation.

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