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ISOLATION AND CHARACTERIZATION OF DNA-DEPENDENT RNA POLYMERASES FROM RABBIT MAMMARY GLAND*

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RNA polymerases AI, AII, BI, BII, CI and CII were found in the mammary gland from lactating rabbits. The enzymes obtained from total cell homogenates were partially purified and separated by DEAE-Sephadex chromatography. Their chromatographic properties, α-amantin-sensitivity, template specificity, ionic strength and divalent cation requirements are described.

Several DNA-dependent RNA polymerases occur in eukaryotic cells (Roeder & Rutter, 1969). The available data indicate that nucleolar RNA polymerase A and nucleoplasmic RNA polymerase B may be involved in the transcription of rRNA precursor and heterogeneous nuclear RNA, respectively (Blatti et al., 1970; Zylber & Penman, 1971; Reeder & Roeder, 1972), whereas RNA polymerase C is responsible for the synthesis of the 5S RNA component of ribosomes and of tRNA precursors (Weinman & Roeder, 1974; Weil & Blatti, 1976). In several cases multiple forms of RNA polymerases of all types have been observed (Chambon, 1974).

This report provides evidence for the existence of six chromatographically distinct DNA-dependent RNA polymerases in lactating rabbit mammary gland and describes their chromatographic and catalytic properties with calf thymus DNA as a template. The enzymes were classified on the basis of their sensitivity to the toxin α-amantin and designated AI, AII, BI, BII, CI and CII according to the nomenclature proposed by Chambon and coworkers (Kedinger et al., 1970; 1971).

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MATERIALS AND METHODS

Tris (puriss.) was purchased from Koch-Light (Colnbrooks, Bucks., England); double distilled glycerol from Merck (Darmstadt, F.R.G.); calf thymus DNA from Worthington (Freehold, U.S.A.); double-stranded poly[d(A-T)] and α-amanitin from Calbiochem (San Diego, U.S.A.); crystalline bovine serum albumin from B.D.H. (Poole, England); actinomycin D and ammonium sulphate pyrogen-free from Serva (Heidelberg, F.R.G.); DEAE-Sephadex A-25 was obtained from Pharmacia (Uppsala, Sweden) and processed before use according to Schwartz et al. (1974). [5-³H]Uridine-5'-triphosphate (11 Ci/mmol) was from the Radiochemical Centre (Amersham, U.K.); after removal of ethanol, it was diluted with unlabelled UTP to final concentration of 2100 Ci/mol. Phenylmethylsulphonyl fluoride (PhMeSO₂F) was obtained from Serva and dissolved in absolute ethanol.

Buffers employed were: buffer A (0.05 m-Tris/HCl, pH 7.9 at 23°C, 25% (v/v) glycerol, 5 mM-MgCl₂, 0.1 mM-EDTA:Na₂, 0.5 mM-dithiothreitol and 0.1 mM-PhMeSO₂F; buffer B, which was of the same composition as buffer A but contained in addition 0.05 m-(NH₄)₂SO₄. To both buffers dithiothreitol and PhMeSO₂F were added immediately before use.

Calf thymus DNA (2 mg/ml) was dissolved at 4°C in 10 mM-Tris/HCl, pH 7.5, containing 10 mM-NaCl. Denatured calf thymus DNA was prepared by heating DNA (200 µg/ml) in 1 mM-Tris/HCl, pH 7.5, containing 1 mM-NaCl at 100°C for 10 min. After rapid cooling in an ice-water bath, it was immediately used. Concentration of (NH₄)₂SO₄ was determined by measuring the conductivity of the tenfold diluted solution, using a standard curve.

Protein was assayed after precipitation with cold 10% trichloroacetic acid by the method of Lowry et al. (1951), using crystalline bovine serum albumin as a standard.

RNA polymerases were isolated from the mammary gland of Great Ppolieno-White rabbits (primiparous) on the 5-7 day of lactation. To analyse a total cellular population of RNA polymerases, they were solubilized from tissue homogenate containing cytoplasmic and nuclear fractions.

All operations were carried out at 0-4°C. After rinsing of the mammary gland with buffer A to remove the residual milk, 8 g of the mammary tissue were minced, and then homogenized in 24 ml of the same buffer for 2×15 s in a Unipan homogenizer, this being followed by 12 strokes in a motor-driven Teflon-glass homogenizer. After filtration of the crude extract through mira cloth, 4.0 mM-ammonium sulphate (adjusted to pH 7.9 with NH₄OH) was added to obtain the solution of 0.32 m-(NH₄)₂SO₄. The viscous mixture maintained below 0°C in an NaCl-ice bath was sonicated at high ionic strength (Roeder & Rutter, 1969) in a Techpan ultrasonic disintegrator (type UD-11) at power setting N, for six 10-s periods. The sonicate was centrifuged for 50 min at 220 000 g.

The ionic strength of the solution was reduced from 0.32 m through 0.1 m to 0.05 m-(NH₄)₂SO₄; at both concentrations, chromatin was removed by ultracentrifugation (220 000 g for 50 min), as described by Schwartz et al. (1974).
The final supernatant was aspirated and applied onto a 16 × 2.5 cm DEAE-Sephadex column (2 mg protein/ml bed volume). The column was washed with a 2.5 bed volume of buffer B, until A$_{280nm}$ of the effluent was nearly zero. The enzymes were eluted with a linear gradient of 0.05 - 0.5 M(NH$_4$)$_2$SO$_4$ in buffer A (three bed volumes). Fractions of 130 drops (approx. 7.2 ml) were collected, and aliquots assayed for conductivity, protein content and RNA polymerase activity.

Because of the low protein concentration in the eluates, the enzymes were stabilized by the addition of bovine serum albumin (1.2 mg/ml). The fractions showing the maxima of enzyme activity were stored in liquid nitrogen.

RNA polymerase activity was determined by measuring the amount of [³H]UMP incorporated into acid-precipitable RNA. The incorporation was linear with respect to time and enzyme concentration. Unless otherwise specified, the reaction mixture contained in a final volume of 75 μl: 70 mM-Tris/HCl, pH 7.9, 8.3% (v/v) glycerol, 2 mM-MnCl$_2$, 1.6 mM-MgCl$_2$, 0.03 mM-EDTA, 0.03 mM-dithiorthreitol, 0.03 mM-PH$_4$SO$_4$, 0.6 mM each ATP, GTP and CTP, 0.02 mM-[³H]UTP, 7.5 μg calf thymus DNA, 30 μg bovine serum albumin and appropriate amounts of the enzymatic fractions containing (NH$_4$)$_2$SO$_4$ to obtain desired salt concentrations.

Samples were incubated for 20 min at 30°C and the reaction tubes were transferred to an ice-bath to stop the reaction. The aliquots (60 μl) were withdrawn and absorbed on the Whatman GF 83 square filters (2 cm × 2 cm), which were washed with 7% trichloroacetic acid containing 25 mM-sodium pyrophosphate and were processed batchwise according to Friedman (1968).

Radioactivity was estimated by liquid scintillation in toluene using a Nuclear-Chicago Isocap 300 counter. The results were corrected using controls incubated without DNA.

RESULTS AND DISCUSSION

Figure 1 illustrates DEAE-Sephadex chromatography of the DNA-dependent RNA polymerases solubilized from rabbit mammary gland. As a rule, six activity maxima were obtained. The enzyme activity was assayed in the absence or presence of α-amanitin in order to distinguish between the three classes of RNA polymerases: class A, insensitive to α-amanitin; class B, inhibited at low concentration (10$^{-9}$ - 10$^{-8}$M), and class C, inhibited at high concentration (10$^{-5}$ - 10$^{-4}$M) of α-amanitin (Chambon, 1974).

It is evident that the A$_{280nm}$ curve did not correspond to the enzyme activity profile obtained with the salt gradient.

The first two enzymatic maxima separated on DEAE-Sephadex, AI and AI, were eluted from the column at about 0.05 m and 0.155 M(NH$_4$)$_2$SO$_4$, respectively. They were not inhibited by α-amanitin at either concentration used.

The enzymes eluted correspond probably to the rat mammary gland RNA polymerase AI and AI, forms, isolated from nuclei by Guzik & Anderson (1976). Similar chromatographic heterogeneity of the class A enzyme was found in several other tissues (Gissinger & Chambon, 1975; Fuhrman & Gill, 1976; Duceman & Jacob, 1977; Nowock et al., 1978).
Fig. 1. DEAE-Sephadex chromatography of RNA polymerases from rabbit mammary gland on the 7th day of lactation. Elution was performed with an 0.05 m - 0.5 m-(NH₄)₂SO₄ gradient in buffer A. Aliquots (25 μl) of each column fraction were assayed for RNA polymerase activity with native calf thymus DNA as a template in the absence (-----) or presence of α-amanitin (1 μg/ml), (-----). The active fractions were reassayed in the presence of α-amanitin (150 μg/ml) (●). (-----) (NH₄)₂SO₄ gradient.

Two forms of RNA polymerase B, BI and BII, were eluted at about 0.20 m and 0.26 m-(NH₄)₂SO₄, respectively. They were sensitive to low α-amanitin concentration (1 μg/ml).

It is noteworthy that so far two or even three forms of enzyme B were obtained on chromatography of the enzymes from mouse plasmocytomas cells (Schwartz & Roeder, 1975), hen oviduct (Krebs & Chambon, 1976), pig kidney (Duceman & Jacob, 1977) and insect wing epidermis (Nowock et al., 1978).

It was previously suggested that RNA polymerase BII could be an artifact due to proteolysis of subunit SBI during purification of RNA polymerase BI (Weaver et al., 1971). In our experiments PhMeSO₄F, a potent protease inhibitor (Pringle, 1970) was present during enzyme extraction and chromatographic fractionation. The addition of the inhibitor did not change the BI/BII ratio during purification of the enzymes from K B cells (Sugden & Keller, 1973), calf thymus (Kedinger et al., 1974) and hen oviduct (Krebs & Chambon, 1976).

The activities CI and CII (Fig. 1) eluted as the last fractions from DEAE-Sephadex at about 0.30 m and 0.40 m-(NH₄)₂SO₄ concentration, respectively, exhibited a moderate sensitivity toward α-amanitin. Both enzymes were resistant to low and sensitive to high concentrations of α-amanitin. In most of the higher organisms this property evidenced the presence of RNA polymerase C even when this enzyme
Fig. 2. Effect of divalent cations on the activity of solubilized mammary gland RNA polymerases from lactating rabbits. DEAE-Sephadex A-25 column fractions were analysed with native calf thymus DNA as a template (7.5 μg/ml); see Methods. Concentration of: ●, Mn²⁺ (at a constant Mg²⁺ concentration of 1.6 mM); and ○, Mg²⁺ (Mn²⁺ omitted). RNA polymerase activity was expressed as percentage of the maximum activity observed in the system. Under these conditions the maximum activities of the enzymes were (d.p.m.): AI, 1512; AII, 2008; BI, 1090; BII, 670; CI, 3427; CII, 630.

Fig. 3. Activity of rabbit mammary gland RNA polymerases as a function of (NH₄)₂SO₄ concentration. DEAE-Sephadex A-25 column fractions with enzymic peak activity were assayed with native calf thymus DNA as described in Methods, in the presence of increasing concentrations of (NH₄)₂SO₄ and 2 mM-MnCl₂ (except for enzyme CII, 1 mM-MnCl₂). RNA polymerase activity was expressed as percentage of the maximum activity observed in the system; ●, forms I; ○, forms II.

was not clearly resolved chromatographically from other RNA polymerases (Amalric et al., 1972; Seifart et al., 1972).

Polymorphism of RNA polymerase C, detected by DEAE-Sephadex chromatography, was reported in various tissues of widely differing organisms: in calf thymus, mouse liver and spleen tissues (Schwartz et al., 1974), HeLa cells (Weil & Blatti, 1976), human peripheral lymphocytes (Jaehning et al., 1975), pig kidney (Duceman & Jacob, 1977) and rat uterus (Weil et al., 1977).

The enzymes studied were found to be DNA-dependent RNA polymerases, since their activity entirely depended on the addition of exogenous DNA and ATP,
GTP, CTP and UTP. Moreover, no [3H]UMP was incorporated into the acid-insoluble material in the presence of actinomycin D (20 μg/ml) (data not shown).

The divalent cation requirements of RNA polymerases from rabbit mammary gland are given in Fig. 2. The results were expressed in terms of relative enzyme activity in order to compare preparations of different specific activities. In the presence of calf thymus DNA (7.5 μg/ml), all the enzymes were stimulated both by Mn²⁺ and Mg²⁺. The maximum activity of the forms AI, AII, BII, BII and CI was found at 2 mM concentration of Mn²⁺, and that of CII, at 1 mM-Mn²⁺. The effect of Mg²⁺ on AI was most pronounced at 9.6 mM. The activity of the enzymes AII, BII and CII was maximally stimulated by 5.5 mM-Mg²⁺, and that of enzymes BI and CI, by 7.3 mM-Mg²⁺. At the concentration of 1.6 mM, Mg²⁺ failed to stimulate RNA synthesis by AI, AII, BII, CI and CII forms of RNA polymerase.

Enzymes AI and BI were almost equally stimulated by Mn²⁺ and Mg²⁺, while for all other forms Mn²⁺ was a more efficient activator than Mg²⁺. However, this property is not characteristic for all eukaryotic polymerases of class A (Roeder, 1974). Similar sensitivity to Mn²⁺ and Mg²⁺ was reported for the enzymes from rat liver nuclei (Roeder & Rutter, 1969), Xenopus laevis oocytes (Beebee & Butterworth, 1974) and developing wings of the oak silkworm (Nowock et al., 1978).

The effect of Mn²⁺ and Mg²⁺ on the enzymes BII, CI and CII (Fig. 2) was the same as described for RNA polymerases of class B and C isolated from mammalian cells (Roeder & Rutter, 1969; Sugden & Keller, 1973; Schwartz et al., 1974; Weil & Blatti, 1975, 1976).

When maximally effective concentrations of both Mn²⁺ and Mg²⁺ were present in the assay mixture, the activity of all the enzymes was lower than in the presence of maximally effective concentration of Mn²⁺ (data not shown).

The enzyme activities were markedly influenced by ionic strength (Fig. 3) as it is commonly observed with other systems. Enzyme AI exhibited the optimum activity at low ionic strength (below 17 mM-(NH₄)₂SO₄) and the enzymes AII, BI, BII, CI and CII at 80, 65, 110, 100 and 130 mM-(NH₄)₂SO₄ concentration, respectively. RNA polymerases CI and CII did not display the two maxima occasionally observed on transcription of native DNA templates (Roeder, 1974; Schwartz et al., 1974; Weil & Blatti, 1976).

Consistently with our results, monophasic curves for polymerase C were obtained with the enzymes of calf thymus (Weil & Blatti, 1975), although in this mammalian system only one form of enzyme C was demonstrated.

The mammary gland RNA polymerases differed markedly in their ability to transcribe DNAs (Table 1). Enzyme AI transcribed native calf thymus DNA almost as efficiently as the denatured form, whereas enzyme CII exhibited a marked preference for native DNA. For the remaining enzymes, the denatured conformation was preferred.

Furthermore, the enzymes CI and CII showed preferential utilization of the artificial template, poly[d(A-T)] (Table 2), like the enzymes C from other organisms (Schwartz et al., 1974; Weil & Blatti, 1975, 1976; Weil et al., 1977).
Table 1

Transcription of native (n) and heat-denatured (d) calf thymus DNA by rabbit mammary gland RNA polymerases

DEAE-Sephadex A-25 column fractions with enzymatic peak activity were assayed, as described in Methods, in the presence of 2 mM-MnCl₂ (enzyme CII at 1 mM-MnCl₂). The DNAs were added at the concentration of 7.5 µg/75 µl of assay medium. The RNA polymerase activity was different in various enzyme preparations, therefore the absolute values of incorporated [³H]UMP cannot be compared.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(NH₄)₂SO₄ (mM)</th>
<th>[³H]UMP incorporated (d.p.m.)</th>
<th>Activity ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>n-DNA</td>
<td>d-DNA</td>
</tr>
<tr>
<td>AI</td>
<td>17</td>
<td>997</td>
<td>862</td>
</tr>
<tr>
<td>AII</td>
<td>80</td>
<td>449</td>
<td>786</td>
</tr>
<tr>
<td>BI</td>
<td>66</td>
<td>892</td>
<td>3034</td>
</tr>
<tr>
<td>BII</td>
<td>112</td>
<td>411</td>
<td>532</td>
</tr>
<tr>
<td>CI</td>
<td>100</td>
<td>624</td>
<td>1175</td>
</tr>
<tr>
<td>CH</td>
<td>133</td>
<td>1071</td>
<td>798</td>
</tr>
</tbody>
</table>

Table 2

Template dependence of rabbit mammary gland RNA polymerase activity

DEAE-Sephadex A-25 column fractions with enzymatic peak activity were assayed as described in Methods, in the presence of 2 mM-MnCl₂ (enzyme CII at 1 mM-MnCl₂), at (NH₄)₂SO₄ concentrations indicated. Calf thymus native DNA and the polymer were added at the same concentration (7.5 µg/75 µl of assay medium).

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>(NH₄)₂SO₄ (mM)</th>
<th>[³H]UMP incorporated (d.p.m.)</th>
<th>Incorporation ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>poly[d(A-T)]</td>
<td>DNA</td>
</tr>
<tr>
<td>AI</td>
<td>20</td>
<td>271</td>
<td>704</td>
</tr>
<tr>
<td>AII</td>
<td>85</td>
<td>262</td>
<td>449</td>
</tr>
<tr>
<td>BI</td>
<td>70</td>
<td>490</td>
<td>827</td>
</tr>
<tr>
<td>BII</td>
<td>115</td>
<td>937</td>
<td>1066</td>
</tr>
<tr>
<td>CI</td>
<td>100</td>
<td>551</td>
<td>366</td>
</tr>
<tr>
<td>CH</td>
<td>130</td>
<td>1017</td>
<td>532</td>
</tr>
</tbody>
</table>

We have previously reported that rabbit mammary gland nuclei exhibit a higher ability for RNA synthesis in early lactation than in late pregnancy (Chomeyński et al., 1974, 1977). In addition, changes were observed in the contribution of RNA polymerases A and B to the total RNA synthesis during lactogenesis (Chomeyński et al., 1977). For understanding of the role of the multiple polymerase forms in mammary gland development, further studies are required on specific control of gene transcription.

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REFERENCES


IZOLACJA I CHARAKTERYSTYKA ZALEŻNYCH OD DNA POLIMERAZ RNA Z GRUCZOŁU MLECZNEGO KRÓLIKA

Streszczenie

Stwierdzono obecność sześciu form zależnych od DNA polimeraz RNA w gruczołach mlecznym królika w okresie laktacji. Enzmy izolowano z ekstraktu komórkowego i rozdzielano chrotnatograficznie na kolumnie z DEAE-Sephadex A-25. Na podstawie wrażliwości na działanie a-amanityny i elucji z kolumny klasyfikowano je jako polimerazy RNA A1, AII, B1, BII, C1, CII. Określono wymagania poprzednich enzymów w stosunku do matrycy, siły jonowej oraz stężenia jonów manganu i magnezu w badanym układzie.

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