ALTERED CHARACTERISTICS OF MAMMALIAN RNA POLYMERASE FOLLOWING SOLUBILIZATION FROM NUCLEI

Samson T. Jacob, Elizabeth M. Sajdel and
Hamish N. Munro
Department of Nutrition & Food Science
Massachusetts Institute of Technology
Cambridge, Massachusetts

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Unlike the bacterial enzyme, mammalian RNA polymerase is usually assayed as an "aggregate enzyme" preparation consisting of the polymerase tightly bound to its deoxyribonucleoprotein template. In consequence changes in enzyme activity cannot be distinguished from alterations in available template in the aggregate. To overcome this difficulty, attempts have been made to release polymerase free of DNA from chicken embryo (Furth and Loh, 1963), bovine lymphosarcoma (Furth and Ho, 1965), rat testes (Ballard and Williams-Ashman, 1966) and from isolated rat liver nuclei (Ramuz et al., 1965; Cunningham and Steiner, 1967; Seifert and Sekeris, 1967) followed by measurement of the activity of the isolated enzyme using purified DNA as template. None of these authors obtained total release of the enzyme and in most cases it is evident that only a small proportion of the activity was solubilized. Since polymerase activity occurs both in Mg^{2+}- and Mn^{2+}-dependent forms in whole nuclei (Widnell and Tata, 1964) and in nucleoli (Jacob et al., 1968), extraction of only a fraction of the total activity does not allow meaningful conclusions to be drawn about the quantities of each type of activity present in intact nuclei. In the present communication, we report a procedure for release of more than 80% of the total polymerase activity from rat liver nuclei.

Materials and Methods

ATP, UTP, GTP and CTP, Cleland's reagent (dithiothreitol) and spermine were purchased from Calbiochem. Schwarz Bioresearch Inc.

provided UTP-C^{14} (specific activity 110 mc/mmole). DNase (electrophoretically pure) and RNase were obtained from Sigma. Gifts of actinomycin D from Merck, Sharpe and Dohme and of rifamycin from Professor Tecci of the University of Rome are gratefully acknowledged.

Nuclei were isolated from the livers of male albino rats (Charles River Breeding Labs.) by the method of Busch et al., (1967) in which the tissue is first homogenized in 2.2 M sucrose containing 10 mM Mg acetate and 0.25 mM spermine. The nuclei isolated from 15 gm. of liver were washed once with 15 ml of 0.88 M sucrose containing 1 mM Mg acetate and then lysed by gentle homogenization with 10 ml of 0.05 M Tris-HCl buffer, pH 9.1 containing 0.025 M KCl, 0.004 M Mg acetate and 0.005 M dithiothreitol. The lysed nuclei were incubated for 45 min. at 35° with gentle shaking, 4.0 ml glycerol was then added and the mixture shaken vigorously at room temperature. The preparation was finally centrifuged at 110,000 x g for 40 min at 10° in the SW 50 rotor of the Spinco L2 centrifuge. The enzyme was recovered free of DNA in the supernatant fluid while the DNA sedimented in the nuclear residue; normally, the first 1 cm of the fluid above this residue was discarded. The solublized enzyme retained 80% of its activity when stored at -40° for a month.

DNA was prepared from liver nuclei according to Marmur's (1961) procedure. In some experiments DNA was denatured by heating it for 3 min at 100° in the buffer used for the preparation of the enzyme, followed by rapid cooling in ice. DNA was measured either by Giles and Myers (1965) procedure or by UV absorption at 260 mμ. Assay for RNA polymerase was carried out in duplicate by a modification of the method of Jacob et al. (1968). Protein was determined by the procedure of Lowry et al. (1951).

Results and Discussion

Polymerase activity in the intact nuclei and in the nuclear residue was assayed separately in the presence of Mg^{2} and of Mn^{2+} with $(NH_4)_2SO_4$. The whole nuclei incorporated 450 μμ moles UTP-C^{14}/mg DNA in the presence of Mg^{2+} and 945 μμ moles/mg DNA in the

presence of Mn^{2+} and $(NH_4)_2SO_4$. The nuclear residue left after extraction showed activities of 35 and 170 μμ moles/mg DNA respectively. This suggests that more than 80% of the original

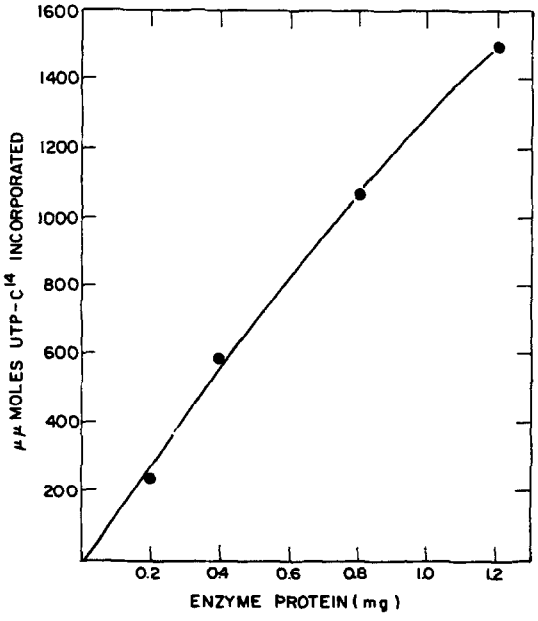

Figure 1: Relationship between enzyme concentration and activity. The enzyme was assayed at concentrations ranging from 0.2 mg - 0.8 mg of protein. DNA concentration was 45 μg. The incubation medium for the enzyme assay contained in 0.75 ml (μ moles): Tris-HCl, (pH 8.0), 40; $MgCl_2$, 2.0; $MnCl_2$, 1.5; NaF, 2.0; Cysteine, 7.0; spermine, 2.0; ATP, 0.35; CTP and GTP, 0.3 each; UTP, 0.07; UTP-C^{14}, 0.0002. Each sample also contained 0.015 ml saturated $(NH_4)_2SO_4$, pH 8.0.

Incubation was carried out for 20 min at 37°. The reaction was stopped by the addition of cold UTP (500 mμ moles) and about 2 mg of liver cell sap protein, followed by 3 ml of 10% TCA containing 0.1 M $Na_4P_2O_7$. The acid-insoluble material was transferred to Millipore filters. The filters were washed three times with 3 ml of 5% TCA containing 0.1 M $Na_4P_2O_7$, once washed with 5 ml of ice-cold glass-distilled water and then dried. The radioactivity was determined in a Nuclear Chicago gas-flow counter with a Micromil^R window. A unit of enzyme was defined as that amount of enzyme which catalyzes, with liver DNA as primer the incorporation of 1 μμ mole of labeled UTP into an acid-insoluble product in 20 min at 37°. Specific activity was expressed as units per mg of protein.

activity assayed in the presence of each ion had been extracted from the nuclei. In agrement with this, the extract contained large amounts of polymerase activity which had to be assayed with added DNA as template, since the DNA extracted along with the enzyme was less than 1% of the original DNA of the nuclei. It is consequently not possible to compare the activity of the original whole nuclear enzyme with that recovered as soluble enzyme, because the template for one is the deoxyribonucleoprotein of the aggregate enzyme, but is isolated DNA for the other.

Addition of DNA is an absolute requirement for activity of the soluble enzyme. At a fixed DNA concentration (45 μg), the soluble polymerase activity is proportional to enzyme concentration (Fig. 1). The enzyme activity of 0.4 mg enzyme protein is also proportional to the amount of DNA and is almost saturated at about 15 μg DNA (Fig. 2). In contrast to the soluble enzyme prepared from rat testes (Ballard and Williams-Ashman, 1966) and lymphosarcoma (Furth and Ho, 1965), denatured DNA is more effective than native DNA as the template for our liver RNA

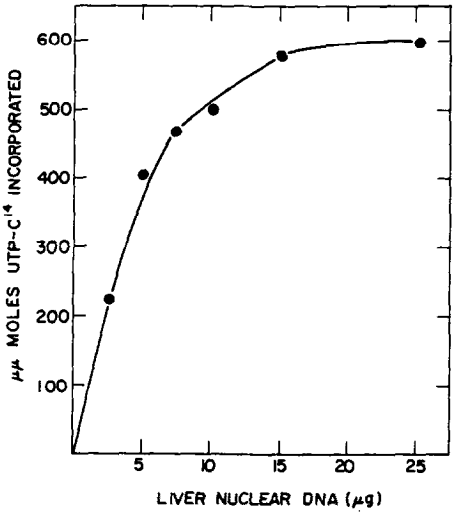

Figure 2: Relationship between template concentration and polymerase activity. Concentrations of DNA ranging from 2.5 - 25 μg were used. The enzyme assay was carried out as described in the legend to Fig. 1.

TABLE I

Type of primer	$\mu\mu$ moles of UTP-C^{14} incorporated/mg protein
Native DNA	1350
Native DNA - $(NH_4)_2SO_4$	415
Denatured DNA	6550
Denatured DNA - $(NH_4)_2SO_4$	1030

Table 1: Effect of heat denaturation of DNA on the RNA polymerase activity. Denaturation of DNA was carried out as described in the text. The enzyme assay was performed according to the conditions described in the legend to Fig. 1.

polymerase (Table 1). This difference may be due to the use of calf thymus DNA as the primer in the other investigations. Bremer and Bruner (1968) have recently reported that purified E. coli RNA polymerase can synthesize more RNA molecules on denatured T_4 DNA templates than on the native template. It is possible that strand separation due to denaturation makes available to the polymerase a larger number of attachment sites.

The characteristics of the soluble enzyme are summarized in Table 2. The DNA-dependence of the enzyme is further evidenced by a considerable loss of activity after preincubation of the DNA with DNase or with actinomycin D. Rifamycin, which is known to inhibit bacterial RNA polymerase (Hartmann et al., 1967), does not have a significant effect on the liver polymerase. Omission of either GTP or CTP diminishes enzyme activity. The enzyme appears to depend only on Mn^{2+} and $(NH_4)_2SO_4$. Omission of Mg^{2+} alone does not reduce incorporation significantly whereas removal of Mn^{2+} and $(NH_4)_2SO_4$ reduces its activity to levels comparable to omission of the enzyme, thus indicating complete loss of the Mg^{2+}-dependent activity. Since essentially all Mg^{2+}-

TABLE 2

Additions	μμ moles of UTP-C^{14} incorporated/mg protein
Complete	1245
Complete + DNase (50 μg)	230
Complete + RNase (50 μg)	535
Complete + Actinomycin D (12.5 μg)	77
Complete + Rifamycin (200 μg)	924
Omit DNA	240
Omit Enzyme	170
Omit GTP	290
Omit CTP	400
Omit Mg^{2+}	1195
Omit Mn^{2+}	200
Omit Mg^{2+} and $(NH_4)_2SO_4$	415
Omit Mn^{2+} and $(NH_4)_2SO_4$	145

Table 2: Requirements for RNA polymerase activity with liver DNA as primer. The polymerase assay is described in the legend to Fig. 1. In experiments using inhibitors, the enzyme was preincubated for the same period in the same volume of buffer as used for inhibitors.

dependent activity present in the original liver nuclei was removed by the extraction procedure, it must either have been destroyed or been converted to Mn^{2+}-dependent activity with native DNA as the primer (Table 2). The latter explanation seems more likely, and implies that Mg^{2+} is an active cofactor only in the presence of deoxyribonucleoprotein.

It has been thought that $(NH_4)_2SO_4$ enhances Mn^{2+}-dependent polymerase activity in whole nuclei by causing removal of

inhibitory proteins from the DNA template (Marushige and Bonner, 1966). However, in our studies $(NH_4)_2SO_4$ increased the activity of the extracted polymerase even when the template used was isolated DNA (Table 1). Alternatively, $(NH_4)_2SO_4$ could have induced a conformational change in either the template or the enzyme. Since the salt enhanced the polymerase activity with denatured DNA as the primer (Table 1), it is unlikely that $(NH_4)_2SO_4$ has induced any significant structural alterations in the template. It is thus possible that the enzyme itself is affected by the salt.

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