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Chromatin-Bound and Free Forms of Poly(Adenylic Acid) Polymerase in Rat Hepatic Nuclei

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Isolated rat hepatic nuclei were shown to contain poly(A) polymerase in two distinct physiologically active forms. One form was associated with the chromatin fraction and was dependent on endogenous RNA, presumably mRNA. The other activity was localized in the nuclear sap in a 'free' form and was stimulated almost 30–40-fold by exogenously added poly(A). Isolated nucleoli were devoid of significant poly(A)-synthesizing activity.

Recent studies in eukaryotes have demonstrated that mRNA molecules for a number of specific proteins (for a review see Brawerman, 1974) contain a poly(A) sequence consisting of 150–250 adenylate residues. To clarify the physiological role of poly(A), factor(s) controlling its synthesis need to be explored. Such studies can be best performed in systems investigated *in vitro*. Enzymes that synthesize poly(A) have been well characterized in the nuclei (Winters & Edmonds, 1973*a*) and mitochondria (Rose *et al.*, 1975). Contrary to purified enzymes, isolated organelles, particularly nuclei, are known to retain most of the characteristics found *in vivo*. Thus transcription in isolated nuclei occurs with a high degree of fidelity, as shown by the synthesis of adenovirus-specific RNA (Wallace & Kates, 1973), avian-myeloblastosis-virus-specific RNA (Jacquet *et al.*, 1974), Rous-sarcoma-virus-specific RNA (Rymo *et al.*, 1974) and rRNA (Reeder & Roeder, 1972). Therefore it seemed reasonable to examine the characteristics of the polyadenylation reaction in isolated nuclei. During this investigation we observed that nuclei isolated from rat liver contain a 'free' and a 'bound' form of poly(A) polymerase (EC 2.7.7.19) associated with the nuclear-sap and chromatin fractions respectively, and that the activity of the former enzyme could be stimulated by the addition of exogenous polyribonucleotides.

Materials and Methods

Nuclei were isolated from rat liver essentially as described by Busch *et al.* (1967), and were suspended in 0.34M-sucrose (1 ml/g of original tissue). Nucleoli were isolated by sonication of the nuclei as described previously (Jacob *et al.*, 1967).

The nuclear-sap or supernatant fraction was obtained as follows. Nuclei were gently lysed by homogenization in buffer containing 50mM-Tris/HCl (pH8.5) containing 1mM-$MgCl_2$ and 0.04M-KCl (0.5ml/g of tissue) and centrifuged at 20000*g* for 10min to prepare the supernatant fraction.

In some experiments nuclei were suspended in buffer containing 50mM-Tris/HCl (pH8.5), 1mM-$MgCl_2$, 0.1M-EDTA, 2mM-dithiothreitol and 50mM-KCl. The nuclear suspension was then sonicated gently to minimize breakage of nucleoli and centrifuged at 46000*g* for 30 min.

Chromatin was isolated from the nuclei essentially as described for kidney (Jänne *et al.*, 1975), and the final chromatin preparation from 12g of liver was suspended in 3ml of 0.4mM-Tris/HCl buffer (pH8) to yield a final DNA concentration of approx. 0.5–1 mg/ml.

A typical poly(A) polymerase assay system contained nuclei derived from 50mg of liver in a reaction volume of 350μl. The reaction mixture contained 50mM-Tris/HCl (pH7.9), 10mM-phosphoenolpyruvate, 1μg of pyruvate kinase, 1mM-$MnCl_2$, 40mM-KCl, 0.48mM-[2,8-^{3}H]ATP (2.4×10^4d.p.m./nmol; New England Nuclear Corp., Boston, MA, U.S.A.) and 5μg of actinomycin D. The reaction was conducted at 37°C for 1h, was terminated by addition of 10% trichloracetic acid and the mixture was processed as described previously (Jacob & Schindler, 1972) to determine the nmol of AMP incorporated into product. ATP was present in saturating quantities and the reaction was linear for at least 1h.

Results and Discussion

Preliminary experiments with isolated nuclei indicated that the low poly(A)-synthesizing capacity in the isolated nuclei could be stimulated significantly by the addition of exogenous polyribonucleotides. Several synthetic polynucleotides, such as poly(A), poly(U), poly(A)·poly(U) and poly[d(A-T)]·poly[d(A-T)], as well as native RNA molecules, such as

Table 1. *Stimulation of poly(A) polymerase activity by exogenous polynucleotides*

Synthetic nucleotides and RNA, all used in saturating quantities, were added at the concentrations indicated. Poly(A) polymerase activity was assayed as described in the text. The 100% activity corresponded to 160nmol of AMP incorporated/h per g of tissue.

Polynucleotide	Concentration (μg/ml)	Activity (%)
Poly(A)	300	100
Nuclear RNA or tRNA	571	14
Poly(U)	285	54
Poly(A)·poly(U)	285	51
Poly[d(A-T)]·poly[d(A-T)]	571	4
Mitochondrial RNA	571	10
None	0	3

tRNA, nuclear RNA and mitochondrial RNA, were tested for their stimulatory effect on poly(A) synthesis in isolated nuclei. Synthetic poly(A) (Miles Laboratories, Elkhart, IN, U.S.A.) gave the highest activity on a weight basis but not necessarily on a molar basis (Table 1). Increasing the concentrations of polynucleotides beyond the values given in Table 1 did not further increase the poly(A)-synthesizing activity. In subsequent experiments, poly(A) was used.

The poly(A)-synthesizing activity either in the presence or in the absence of poly(A) was insensitive to actinomycin D (generally included in the assay), indicating that the reaction was not dependent on DNA. Moreover, the reaction was carried out in the absence of the other three nucleoside triphosphates that were obligatory to RNA synthesis. Inclusion of pancreatic ribonuclease and bacteriophage T_1 ribonuclease at the end of the reaction did not render the product acid-soluble, thus demonstrating that the product is most probably poly(A). The possibility that the stimulation with exogenous polynucleotides is due to a decrease in the activity of poly(A)-degrading nucleases (Sporn *et al.*, 1969) was ruled out by the following experiment. [^{3}H]Poly(A) (1.2×10^4 d.p.m./μg) was added to the nuclei at concentrations ranging from 7 to 300μg/ml, and the trichloroacetic acid-insoluble radioactivity was examined before and after incubation for 60min in the standard reaction conditions. No detectable decrease in the radioactivity was observed after incubation of the nuclei even when poly(A) was present at 300μg/ml, suggesting the absence of any nuclease activity under the assay conditions. Only when the incubation was carried out in the presence of Mg^{2+} and phosphate (to shift the reaction equilibrium to the hydrolysis of poly(A) formed *in vitro*) did the degradation of poly(A) occur.

The average size of the product obtained *in vitro* was determined by subjecting the reaction product (obtained after substituting [^{14}C]ATP for [^{3}H]ATP) to hydrolysis with 0.3M-KOH followed by chromatography on polyethyleneimine-cellulose paper (Randerath & Randerath, 1967). The average lengths of the product synthesized in the absence and in the presence of poly(A), as estimated by the ratio of radioactivity in AMP to that in adenosine, were 163 and 13 respectively.

Table 2. *Poly(A) polymerase in nuclear subfractions*

Nuclei were subfractionated as described in the text, and poly(A) polymerase was assayed in each fraction as described in the text. A typical assay system contained nuclei derived from 50mg of tissue or supernatant derived from 66mg of tissue or chromatin derived from 900mg of tissue. Activities for chromatin and nucleoli were normalized for 30–40% recovery of these fractions from isolated nuclei. In experiments with nucleases, nuclei were preincubated with 10μg of ribonuclease A and 100 units of T_1 ribonuclease for 15min before enzyme assay.

Enzyme source	Poly(A) polymerase activity (nmol of AMP incorporated/h per g of tissue) Endogenous	+Poly(A)
Nuclei	5.47	216
Nuclei+ribonuclease A+ T_1 ribonuclease	0.13	222
Chromatin	1	1.2
Nuclear sap	11.72	477
Nucleoli	0.01	0.02

The intranuclear localization of poly(A) polymerase activities was determined by fractionation of the nuclei into chromatin, nucleolar and supernatant (nuclear-sap) fractions as described in the Materials and Methods section. Poly(A) polymerase activity of these nuclear fractions was determined in the presence and in the absence of poly(A) (Table 2). Nucleoli were virtually devoid of any detectable poly(A)-synthesizing activity. Almost all the activity detected in the presence of exogenous poly(A) was localized in the nuclear-sap or supernatant fraction. The chromatin contained the bulk of the activity observed in the absence of poly(A). To demonstrate that the chromatin-associated poly(A) polymerase was dependent on endogenous RNA, nuclei were preincubated with pancreatic ribonuclease and bacteriophage T_1 ribonuclease to hydrolyse non-poly(A)-containing RNA (Table 2). The degradation of nuclear RNA resulted in complete loss of the poly(A)-synthesizing activity in the absence of added poly(A), indicating the absolute requirement of this enzyme for endogenous RNA, presumably mRNA associated with the chromatin

fraction. The lack of activity in isolated nucleoli coupled with polyadenylation of pre-existing RNA in the chromatin fraction indicate that the chromatin-bound poly(A) polymerase might be involved in the polyadenylation of the mRNA. The enzyme in the nuclear sap was further characterized by glycerol-density-gradient centrifugation with the use of suitable markers as described previously (Rose *et al.*, 1975). The peak activity of poly(A) polymerase was at about 4S (results not shown), indicating that this enzyme is not associated with the 30S ribonucleoprotein particles or other large-molecular-weight moieties, but is present mostly as a 'free' enzyme in the nuclear sap.

The present studies demonstrate that poly(A) polymerase from rat liver nuclei exists in 'bound' and 'free' forms with different intranuclear localizations. The chromatin-associated poly(A) polyermase may be involved in initiating the polyadenylation of mRNA synthesized on the chromatin. The average chain length of 163 adenylate residues added to the endogenous RNA is about the size of poly(A) added to mRNA *in vivo* (see Brawerman, 1974). The shorter size of the product synthesized by the 'free' enzyme may be a reflection of the high concentrations of polynucleotides used in the assay, since these are known to decrease the product size (Winters & Edmonds, 1973*b*). The poly(A) polymerase activity present in the nuclear supernatant fraction might be involved in the extension of the poly(A) tract already added post-transcriptionally to the chromatin-associated mRNA.

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