Contemporaneous Isolation of Deoxyribonucleic Acid-Dependent Ribonucleic Acid Polymerase and Poly(A) Polymerase from Rat Liver Mitochondria

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1. Poly(A) polymerase and DNA-dependent RNA polymerase from rat liver mitochondria can be completely separated by using two different chromatographic procedures.
2. Poly(A) polymerase can only incorporate ATP into acid-insoluble material and strongly depends on the addition of an endogenous factor (probably containing a mixture of oligoribonucleotides), but it is not stimulated by DNA.
3. RNA polymerase is fully DNA-dependent and rifampicin-sensitive, but was not stimulated by the endogenous factor mentioned above.
4. The chromatographic behaviour of the two enzymes, together with the properties described, suggest that they represent two different protein molecules.

Poly(A) sequences associated with mitochondrial RNA have been found in some higher eukaryotes, but they seem to be absent from mitochondria of yeast (Hirsch & Penman, 1973; Ojala & Attardi, 1974; Groot *et al.*, 1974). Little is known about the events that occur in the synthesis and in the addition of these poly(A) stretches to mitochondrial RNA. The presence of a mitochondrial poly(A) polymerase activity has so far been reported only in rat liver (Jacob & Schindler, 1972; Saccone *et al.*, 1974) and in Morris hepatomas (Jacob *et al.*, 1974), from which this enzyme has been purified and extensively characterized by Rose *et al.* (1975). However, although these authors used purified rat liver enzyme, no information on its purification or properties has, to our knowledge, so far been reported. During the purification of DNA-dependent mitochondrial RNA polymerase from rat liver, obtained in our laboratory (Gallerani *et al.*, 1972), we observed that a poly(A) polymerase activity was co-purified with RNA polymerase up to the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step, and several properties of the poly(A) polymerase activity at this stage were reported (Saccone *et al.*, 1974). The relationship between the two activities, however, was not clear, and their co-purification could suggest that the same molecule may be able to catalyse the two reactions in suitable conditions. Such a possibility has been demonstrated in *Escherichia coli* cells by Ohasa & Tsugita (1972), who showed that in pH conditions rather different from those used for RNA synthesis, the α subunit of the DNA-dependent RNA polymerase is able to polymerize ATP in poly(A) molecules. On the other hand, Rose & Jacob (1974) showed that the aging of polymerase II extracted from rat liver

nuclei at 37°C resulted in loss of RNA-polymerizing activity, with a corresponding increase in poly(A)-synthesizing activity. The above authors suggested that some subunit of DNA-dependent RNA polymerase II was responsible for poly(A) synthesis. To clarify the role and nature of these mitochondrial enzymes, we tested to see if poly(A) polymerase could be isolated from rat liver mitochondria and completely separated from the DNA-dependent RNA polymerase, and also to determine if the latter enzyme could act as poly(A) polymerase. In the paper we describe the isolation and subsequent separation by different chromatographic techniques of the two enzymes from rat liver mitochondria. Some properties of these two mitochondrial enzymes are also reported. The results obtained suggest that in rat liver mitochondria the two enzymic activities are probably due to different protein molecules.

Materials and Methods

Materials

Male albino rats starved overnight were used in all experiments. Sodium deoxycholate and dithiothreitol were purchased from Sigma Chemical Co., St. Louis, MO, U.S.A.; DNAase* (EC 3.1.4.5) was from Worthington Biochemical Corp., Freehold, NJ, U.S.A.; *E. coli* DNA (strain K12) was from General Biochemicals, Chagrin Falls, OH, U.S.A.; labelled nucleotides were from Schwarz-Mann, Orangeburg, NY, U.S.A.; rifampicin was a kind gift of Lepetit, Milan, Italy.

* Abbreviations: DNAase, deoxyribonuclease; RNAase, ribonuclease.

Methods

Preparation of $(\text{NH}_4)_2\text{SO}_4$ -precipitated fraction. Mitochondria were isolated from rat liver under sterile conditions as described previously (Saccone *et al.*, 1967). Partial purification of two enzymic activities was achieved by the procedure summarized in Fig. 1. The mitochondrial lysate, obtained by the addition of 1.5% (w/v) sodium deoxycholate to a mitochondrial suspension containing 0.3M- $(\text{NH}_4)_2\text{SO}_4$, was centrifuged for 1 h at 105000g at 0°C. The supernatant was passed through a column (2.8cm×50cm) of Sephadex G-25 to remove $(\text{NH}_4)_2\text{SO}_4$ and sodium deoxycholate. This mitochondrial lysate was incubated in the presence of DNAase to detach the DNA-dependent RNA polymerase from its template. After this purification step, $(\text{NH}_4)_2\text{SO}_4$ was added to a final concentration of 0.24g/ml. The suspension was centrifuged at 80000g for 30 min at 0°C, and the supernatant was processed as described below.

DEAE-Sephadex A-25 chromatography. $(\text{NH}_4)_2\text{SO}_4$ was removed from the preceding fraction on a column (1.8cm×40cm) of Sephadex G-25 equilibrated with 'buffer B' [25mM-Tris/HCl, pH7.4, 5mM-MgCl₂, 0.1mM-EDTA, glycerol (20%, v/v), 1mM-dithiothreitol, 0.25M-KCl]. The proteins eluted from this column were adsorbed on a column of DEAE-Sephadex A-25 equilibrated with the same buffer. After adsorption the column was washed with 15ml of this buffer B and then eluted with 20ml of 'buffer B₁' (buffer B with 0.5M-KCl) [see Fig. 1 ('Fraction 1/A'), and Fig. 2].

Phosphocellulose P-11 chromatography. The $(\text{NH}_4)_2\text{SO}_4$ fraction was passed through a column (1.8cm×40cm) of Sephadex G-25 equilibrated with 'buffer A' [50mM-potassium phosphate, pH6.8, 5mM-MgCl₂, 0.1M-EDTA sodium salt, 0.1M-dithiothreitol, 50mM-KCl, glycerol (20%, v/v)]. The proteins in buffer A were adsorbed on a column (1.1cm×15cm) of P-11 phosphocellulose and, after washing with 20ml of this buffer, the column was eluted with a linear gradient of 0.05–0.8M-KCl.

Measurement of poly(A) polymerase activity. The poly(A) polymerase activity was measured by incubating 30 µl samples of each fraction in 110 µl final volume of an incubation mixture containing: 30mM-Tris/HCl, pH8; 5.8mM-MgCl₂; 2.8mM-MnCl₂; 50mM-KCl; 9mM-phosphoenolpyruvate; pyruvate kinase (62 µg/ml); 0.06mM-ATP; [³H]ATP (sp. radioactivity 1mCi/µmol); 1.9E₂₆₀ units of stimulating factor/ml prepared by the method of Jacob *et al.* (1974). After 30min incubation at 30°C the reaction was stopped by the addition of 100pmol of non-radioactive ATP and 110 µl of 10% (w/v) trichloroacetic acid containing 0.04M- $\text{Na}_4\text{P}_2\text{O}_7$. The precipitate was then collected on Whatman GF/C filters, washed at 0–4°C with 5% trichloroacetic acid, and

counted for radioactivity in a Packard Tri-Carb liquid-scintillation counter by using a scintillation mixture containing 5g of PPO (2,5-diphenyloxazole) and 0.3g of dimethyl POPOP [1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene] per litre of toluene. A unit of enzyme is the amount of protein that incorporates 1 pmol of [³H]ATP into acid-insoluble material in the mentioned conditions.

Measurement of RNA polymerase activity. The RNA polymerase activity was measured by incubating 30 µl samples of each fraction in 100 µl final volume of an incubation mixture containing: 55mM-Tris/HCl, pH7.4; 3mM-MgCl₂; 1mM-MnCl₂; bovine serum albumin (250 µg/ml); ATP, UTP, CTP and GTP at 0.5mM each; [³H]UTP (0.2Ci/mmol); *E. coli* K12 DNA (100 µg/ml). The incubation time was 10min at 30°C and the reaction was stopped by the addition of 1ml of cold 5% trichloroacetic acid containing 0.04M- $\text{Na}_4\text{P}_2\text{O}_7$. The precipitate was further processed as reported for the poly(A) polymerase activity.

Measurement of protein concentration. Protein concentration was measured by the method of Waddell (1956), with bovine serum albumin as standard.

Results

Chromatographic separation of poly(A) polymerase and RNA polymerase

The isolation of the two enzymes can be obtained by using the procedure described in Fig. 1. After the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step, a column of DEAE-Sephadex A-25 or of phosphocellulose P-11 can be used. The poly(A) polymerase activity was measured as described in the Materials and Methods section. After the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step, the addition of the endogenous primer first described by Jacob *et al.* (1974) and called here 'stimulating factor', was essential for the detection of activity. The nature of this factor is still unknown. It is neither a protein nor RNA because it is heat-stable and is unaffected by ribonuclease treatment. Other properties, such as sensitivity to KOH treatment, its absorbance spectrum and chromatographic analysis, seem to suggest, in agreement with the results of Jacob *et al.* (1974), that it represents a mixture of polyribonucleotides of relatively low molecular weight. The elution profile of the DEAE-Sephadex column is shown in Fig. 2. The first peak eluted at about 0.35M-KCl contained the poly(A) polymerase activity, since it was able to incorporate only [³H]ATP and was stimulated 80% by the addition of the stimulating factor. The peak eluted at 0.5M-KCl contained, on the other hand, the mitochondrial RNA polymerase, which was fully DNA-dependent and rifampicin-sensitive. The specific activity of poly(A) polymerase after DEAE-Sephadex elution was 22000pmol/30min per mg of protein, but, when analysed by sodium dodecyl sulphate/

Fig. 1. Procedures for separating poly(A) polymerase and DNA-dependent RNA polymerase extracted from rat liver mitochondria

Technical details and buffer compositions are described in the Materials and Methods section, and in Gallerani *et al.* (1972).

polyacrylamide-gel electrophoresis, this peak of material revealed several bands of mol.wt. ranging from 20000 to 100000, whereas the RNA polymerase peak showed the usual 62000-mol.wt. band as described previously (Gallerani & Saccone, 1974). Fig. 3 shows the pattern obtained by eluting a phosphocellulose column charged with the same protein fraction as in Fig. 2, and by using a linear KCl gradient (0.05–0.8M). In this case too, the enzymes are completely separated. Nevertheless, the specific activity of poly(A) polymerase eluted by the phosphocellulose column was about 950 pmol/30min per mg of protein, much lower than when the DEAE-Sephadex column was used, owing to the greater portion of proteins retained by the phosphocellulose column.

Characterization of poly(A) polymerase and RNA polymerase

Table 1 shows several properties of the poly(A) polymerase activity obtained by using the above

chromatographic procedures. The enzyme purified by the two different columns displayed the same properties, e.g. almost complete dependence on the addition of the endogenous factor, no inhibition by rifampicin, no specificity for ribonucleoside triphosphates other than ATP. The activity was unaffected by the addition of RNAase or DNAase and was also not stimulated by the addition of DNA, poly(A) or RNA of different sources both polyadenylated or not. This latter property differentiates the mitochondrial poly(A) polymerase from other similar enzymes of extramitochondrial origin (Giron & Huppert, 1972; Niessing & Sekeris, 1972). The product synthesized after 30 or 60 min incubation by mitochondrial poly(A) polymerase displayed a sedimentation coefficient higher than 4S when analysed by gel electrophoresis in the presence of 12% acrylamide by the method of Loening (1967). The size of the product was unaffected by treatment with ribonucleases A and T₁ and with Pronase.

The mitochondrial RNA polymerase was 100%

Fig. 2. Elution pattern of DEAE-Sephadex A-25 column

The column (1 cm $\times$ 12 cm) was eluted at a flow rate of 0.15 ml/min. Fractions (3 ml) were collected from fractions 1–12; from fraction 13 to the end, each tube contained 1.5 ml. RNA polymerase activity was measured from tubes 10 to 23 in the absence and in the presence of template and rifampicin (50 μ g/ml). No significant incorporation into acid-insoluble material was detected in either case. $\blacktriangle$, E_{280} ; $\triangle$, [KCl]; $\circ$, [3 H]AMP incorporation (d.p.m.) in the presence of stimulating factor; $\bullet$, [3 H]UMP incorporation (d.p.m.) in the presence of DNA.

Fig. 3. Elution pattern of phosphocellulose P-11 column

The flow rate was 0.25 ml/min. The linear gradient was made up by using 20 ml of buffer A and 20 ml of the same buffer containing 0.8 M-KCl. Tubes 1–20 contained 3 ml; tubes 21–37, 2 ml. The symbols are as described in the legend of Fig. 2. RNA polymerase activity, by using fractions 17–37, was also measured in the presence of rifampicin (50 μ g/ml) and in the absence of template. In both cases no significant incorporation into acid-insoluble material was detected.

DNA-dependent and sensitive to rifampicin and displayed all the properties previously reported by us or others for the purified mitochondrial DNA-dependent RNA polymerases (Küntzel & Schäfer, 1971; Wu & Dawid, 1972; Gallerani & Saccone, 1974; Scragg, 1974). The properties of RNA polymerase

prepared by phosphocellulose column (Table 2) further indicate that the activity was unaffected by the addition of the endogenous primer and that the enzyme was unable to polymerize [3 H]ATP either in the presence or the absence of the stimulating factor. Aging of the enzyme or any other treatment, such as

Table 1. *Properties of poly(A) polymerase from rat liver mitochondria partially purified by two different chromatographic techniques*

The results reported in the Table are obtained by using the collected fractions 13–15 of the DEAE-Sephadex column and fractions 24–25 of the phosphocellulose column, and by incubating 30 μ l samples under the conditions described in the Materials and Methods section. The enzyme activities are expressed as pmol of [3 H]AMP incorporated into acid-insoluble material/30 min per mg of protein. By substituting [3 H]ATP with labelled CTP, GTP, or UTP at the same concentrations and specific radioactivity, no incorporation into acid-insoluble material was detected. Both polyadenylated and non-polyadenylated RNA were extracted and separated from rat liver cytoplasm by the method of Hirsch & Penman (1973).

	Specific activity (pmol/30 min per mg of protein)	
	DEAE-Sephadex A-25 chromatography	Phosphocellulose P-11 chromatography
Complete system	22100	950
–Stimulating factor	4510	29
–Stimulating factor + <i>E. coli</i> DNA (110 μ g/ml)	2000	25
–Stimulating factor +poly(A) (250 μ g/ml)	1800	38
–Stimulating factor +cytoplasmic RNA (330 μ g/ml)	3500	47
–Stimulating factor +poly(A) RNA (330 μ g/ml)	4000	43
+Rifampicin (90 μ g/ml)	22150	980

Table 2. *Properties of rat liver mitochondrial RNA polymerase prepared by phosphocellulose chromatography*

The results are obtained by using the collected fractions 31–32 of the phosphocellulose P-11 column, and by incubating 30 μ l samples under the conditions described in the Materials and Methods section for the RNA polymerase activity; 100% activity corresponded to 2700 pmol of [3 H]UMP incorporated/10 min per mg of protein. When [3 H]ATP was used as precursor, DNA and the other three ribonucleoside triphosphates were absent.

System	Enzyme activity (% of control)
Complete	100
–DNA	0
+Rifampicin 50 μ g/ml	0
+Stimulating factor (1.9 E_{260} units/ml)	98
[3 H]ATP alone (–stimulating factor)	2
[3 H]ATP alone (+stimulating factor)	3

incubation at a higher temperature or a different pH, also failed to convert the mitochondrial RNA polymerase into poly(A) polymerase.

Discussion

The results show that rat liver mitochondria contain a poly(A) polymerase activity that displays properties that differentiate it from other similar enzymes previously described in nuclear or cytoplasmic fractions (Niessing & Sekeris, 1972; Giron & Huppert, 1972). The fact that mitochondrial poly(A)

polymerase could arise from contamination of the mitochondrial preparation with other cellular fractions can be also excluded on the basis of our previous results demonstrating that incorporation of labelled ATP into acid-insoluble material by isolated mitochondria is almost completely inhibited by atractyloside. This indicates that for incorporation, the passage of ATP through mitochondrial membrane is required and, contemporaneously, that no other extramitochondrial activities are present in our mitochondrial preparation (Saccone *et al.*, 1967, 1969). Mitochondrial poly(A) polymerase and RNA polymerase are co-purified up to the $(\text{NH}_4)_2\text{SO}_4$ -fractionation step, but they can be separated by using two chromatographic columns with different properties. Poly(A) polymerase and RNA polymerase are eluted in both cases from the two columns at different salt concentration, and their separation is complete. The elution pattern shown in Fig. 2 is slightly different from that previously reported, since the DNA-dependent RNA polymerase is eluted here at 0.5 M-KCl, but, as we have already pointed out, the elution of the enzyme at a different salt concentration can be dependent on incomplete detachment of the enzyme from its template (Gallerani *et al.*, 1972). In the present experiments, however, RNA polymerase was 100% DNA-dependent, and this suggests that the treatment with DNAase may be a requisite for the purification of mitochondrial RNA polymerase and also for its separation from poly(A) polymerase. Mitochondrial RNA polymerase was eluted from the DEAE-Sephadex A-25 column in a virtually purified form, since only one band was detectable in sodium

dodecyl sulphate/polyacrylamide-gel electrophoresis as found by some of us (Gallerani *et al.*, 1972). On the other hand, the fractions containing poly(A) polymerase activity, although displaying a high specific activity, were not homogeneous on sodium dodecyl sulphate/polyacrylamide-gel electrophoresis, so that no information can at present be deduced on the size and structure of mitochondrial poly(A) polymerase of rat liver. Rose *et al.* (1975) reported a mol.wt. of 60000 for the poly(A) polymerase from Morris hepatoma, similar to the molecular weight of mitochondrial DNA-dependent RNA polymerases so far purified, which in various organisms are composed of a single polypeptide chain of mol.wt. ranging from 46000 to 64000 (Küntzel & Schäfer, 1971; Wu & Dawid, 1972; Gallerani & Saccone, 1974; Scragg, 1974). This property already excludes, for the mitochondrial enzyme, the situation in *E. coli* RNA polymerase or RNA polymerase II from rat liver, in which it has been suggested that one of the subunits may be active in synthesizing poly(A) (Ohasa & Tsugita, 1972; Rose & Jacob, 1974).

The sharp separation of the two activities obtained in the present experiments and the properties of the two enzymes suggest that the two activities may be due to different protein molecules. However, extensive characterization for chemical and physical properties of both purified enzymes will be very useful for understanding the relationship between the two enzymic activities, particularly for elucidating the chronology of events that occur during mitochondrial genome transcription, a process that is still hardly understood.

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