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RNA Synthesis by Isolated Yoshida Ascites Hepatoma A.H. 130 Cells Mitochondria *

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Mitochondria isolated from Yoshida ascites hepatoma A.H. 130 cells are able to incorporate ^{3}H-UTP into RNA. The reaction has been extensively characterized and appear to be supported by a typical DNA-dependent RNA polymerase, and no special differences with the system of normal rat liver mitochondria have been found.

It has been clearly established that the biogenesis of mitochondria is under the control of two separated genetic systems, the nuclear cell-sap system of the eukariotic cell and the system localized in the organells itself. The mitochondrial genetic system consists of mitochondrial DNA and of a complete genetic apparatus to express the mitochondrial genoma. Although some properties of this apparatus have been clearly defined, many others still remain obscure. The mitochondrial DNA-dependent RNA polymerase, which is the enzyme used to transcribe the mitochondrial genome, has only been studied in a limited number of organisms because of the difficulty in characterizing and isolating it. Only recently, purification of mitochondrial RNA polymerase from rat liver (1, 7), *Neurospora crassa* (4), *Xenopus* (15), and yeast (10, 12, 13) has been reported. The purified enzymes from all four organisms display the same properties, with the possible exception of one yeast enzyme (12).

The results reported in this paper show that isolated mitochondria from ascites hepatoma A.H. 130 cells are able to incorporate ^{3}H-UTP into RNA. Using this system we have characterized the reaction

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demonstrating that ascites mitochondria possess their own DNA-dependent RNA polymerase.

Materials and Methods

^{3}H-UTP lithium salt was purchased from Schwarz Bio Research. The unlabeled ribonucleotides, piruvate kinase and phosphoenol piruvate from C.F. Boehringer (Mannheim); PPO (2,5-Diphenyloxazole) and dimethyl POPOP (1,4-bis-2-(4-Methyl-5-Phenyloxazolyl-Benzene) from Packard; actinomycin D from Schuhardt (München); ethidium bromide from Sigma; α-amanitin from Boehringer (Ingelheim); rifampicyn was kindly given by Lepetit (Milano); all other reagents were of analytical grade.

Male Wistar rats (120 g) were used in all experiments, 6-7 days after tumour transplantation.

For the mitochondrial preparation the rats were killed by cervical luxation. The skin was disinfected with alcohol and dissected to expose the muscular abdominal wall. The ascitic fluid was removed by abdominal incision and then collected and measured in a graduated cylinder packed in ice. Haemorragic fluid was discarded. All further procedures were carried out in the cold room.

The ascitic fluid was centrifuged for 5 minutes at 900 xg. The packed cells were washed twice with solution S (0.25 M sucrose containing 1 mM disodium EDTA, *p*H 4-5), resuspended with triple packed cell volume of solution S plus 0.1 M citric acid (400/1) and homogenized in a Dounce Potter packed in ice.

After centrifugation at 900 xg for 20 minutes the top two thirds of the supernatant solution were decanted, neutralized to *p*H 7.4 with Tris buffer and stirred in ice. The pellet was resuspended in the solution S plus citric acid and treated as before. The neutralized supernatants were then combined and centrifuged at 5,000 xg for 20 minutes. The pellet was suspended in solution T (0.25 M sucrose, 1 mM disodium EDTA and 20 mM Tris-HCl *p*H 7.4) and washed, twice at 8,000 xg for 10 min. and once at 12,500 xg for 10 minutes. The washed sediments were collected in a small quantity of solution S to a final protein concentration of about 15 mg/ml.

All solutions and materials were previously sterilized. Bacterial contamination was controlled by the «surface viable count technique».

The number of viable bacteria in the preparation was always less than 3,000 bacteria/mg of mitochondrial protein (3). Characterization of mitochondrial fraction thus prepared has already been reported (5). Protein was determined by measuring ultraviolet absorption in diluted alcali according to MURPHY (6).

The standard incubation medium for RNA polymerase activity contained 50 mM Tris-HCl *p*H 7.4, 50 mM KCl, 2.5 mM $MgCl_2$, 2 mM PEP (phosphoenolpiruvate), 2.5 μg PK (piruvate kinase), 50 μM ATP, 50 μM CTP, 50 μM GTP, 50 μM UTP, 5 μM ^{3}H-UTP (10 μC) in a final volumen of 0.1 ml.

Variations were made in the compositions of the standard incubation mixture in order to determine the optimal conditions for each component. Incubation was carried out at 35° for 10 minutes in a Dubnoff thermoregulated shaker. Reaction was started by addition of mitochondrial protein and stopped with 3 ml of ice-cold 5 % trichloroacetic acid containing saturated pyrophosphate (10:1). The acid insoluble material was collected on Whatman GF/C 25 mm ∅ glass fiber paper filters by suction and washed 5 times with 5 ml each of ice-cold 5 % trichloroacetic acid. The filters were dried and dipped in 10 ml of a scintillation mixture containing 50 mg PPO (2.5-Diphenyloxazole) and 3 mg Dimetyl POPOP (1.4-bis-2-[4-Methyl-5-Phenyloxazolyl]-

Benzene) in 10 ml Toluene. Radioactivity was measured in a Packard Tri-Carb Scintillation Counter.

Results

In order to study RNA synthesis in isolated mitochondria in the absence of any permeability barrier for nucleotides and for some high molecular weight inhibitors, the organelles were swollen by phosphate as described for rat liver (9). In these conditions isolated mitochondria were able to incorporate ^{3}H-UTP into acid insoluble material almost linearly for about 10 minutes reaching a plateau value after 20 minutes of incubation (fig. 1). At 10 minutes 20 pmoles ^{3}H-UMP were incorporated for mg protein. This specific activity seems to be slighty higher than that reported for normal rat liver mitochondria (9). The radioactive acid insoluble material was characterized as RNA because of its sensitivity to alkaline or acid hydrolysis and to RNAase treatment. RNA synthesis by Yoshida ascites hepatoma cell mitochondria measured in these conditions was independent of exogenous added DNA as described for other mitochondrial systems, but dependent on the presence of the four nucleoside triphosphates in the incubation mixture (results not shown).

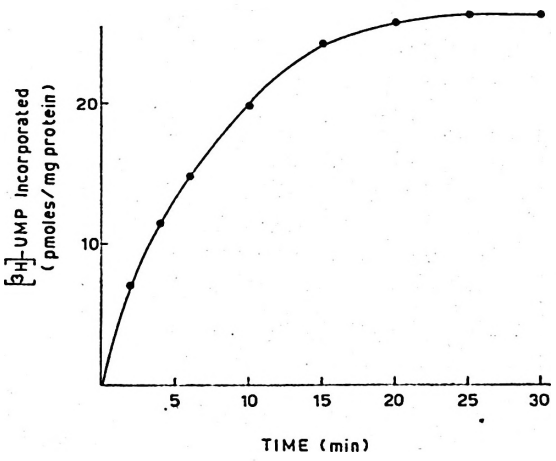

Fig. 1. *Time course of ^{3}H-UTP incorporation into RNA in the presence of swollen mitochondria.*
Mitochondria were isolated under semisterile conditions. The reaction mixture contained: 5 μmoles tris-HCl *p*H 7.4, 5 μmoles KCl, 0.25 μmoles $MgCl_2$, 0.2 μmoles, PEP, 0.25 μg piruvate kinase, 5 nmoles ATP, 5 nmoles CTP, 5 nmoles GTP, 5 nmoles UTP, 0.4 nmoles ^{3}H-UTP (10 μCi) and 0.2 of mitochondrial protein in a final volume of 0.1 ml. Experiments were performed at *p*H 7.4 and 35°. Other experimental details in Material and Methods.

Figure 2 shows that the incorporation of radioactive nucleotides into RNA by swollen mitochondria is a linear function of the amount of protein present in the incubation mixture up to about 3 mg protein per ml.

The optimal temperature of the reaction was studied by incubating the organelles for 10 minutes at different temperatures. The results of figure 3 show that the extent of the reaction at 10 minutes increases with the temperature, reaching the optimal value at 35° C.

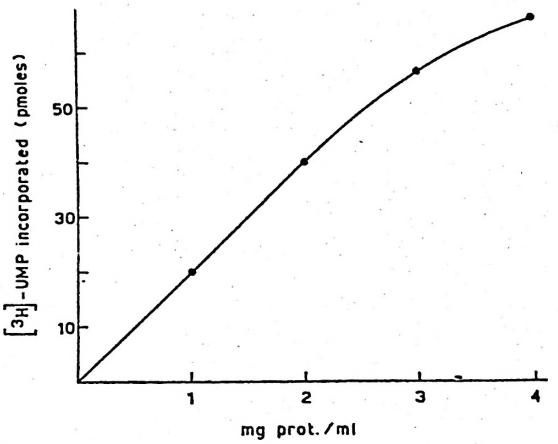

Fig. 2. *Dependence of ^{3}H-UTP incorporation on the amount of mitochondrial protein in the reaction mixture.*
Experimental conditions as in figure 1. Incubation was carried out a 35° for 10 minutes.

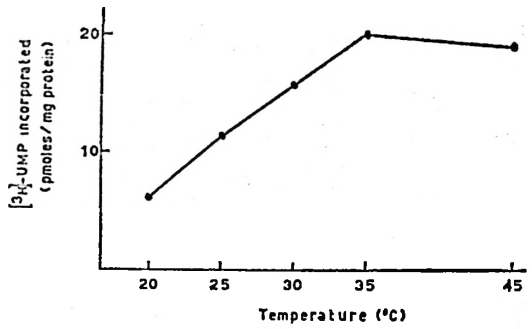

Fig. 3. *Temperature dependence of ³H-UTP incorporation into RNA.*
Incubation was carried out for 10 minutes at different temperatures. Other experimental condition as in figure 1.

KCl concentration between 10 and 75 mM does not seem to affect greatly the reaction (table I). Divalent cations otherwise are essential for RNA polymerase activity. The incorporation of ³H-UTP increases by increasing Mg^{2+} concentration up to 5 mM, optimal Mn^{2+} ion concentration is on the contrary 1 mM (fig. 4). In table II the effect of the addition of the two ions at various concentrations is reported. The effect of the two ions does not seem to be additive.

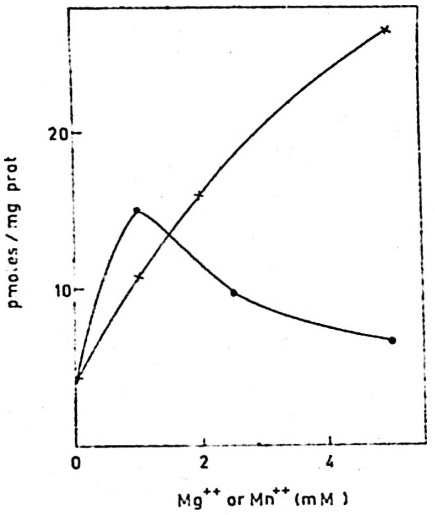

Fig. 4. *Divalent cations concentration on ³H-UMP incorporation into mitochondrial RNA.* Mg^2 or Mn^2, at the concentration reported in figure, were present in standard incubation mixture. Other experimental conditions as in figure 2.

Table I. *Effect of KCl concentration on ³H-UMP incorporation into swollen mitochondria of Yoshida ascites hepatoma A.H. 130 cells.*
All experimental conditions as in figure 1.

KCl mM	³H-UMP Incorporation (pmoles/mg prot.)
10	22.4
25	22.6
50	21.4
75	19.5

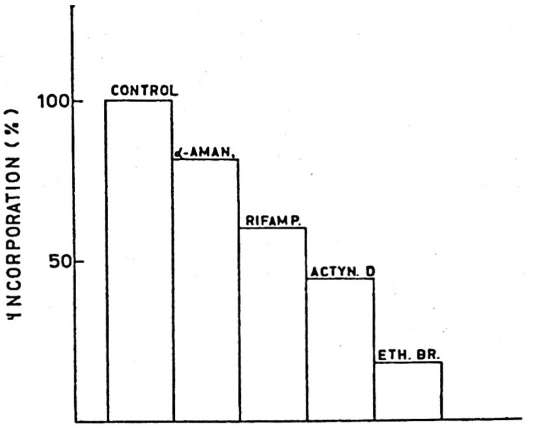

Fig. 5. *Effect of some inhibitors of RNA synthesis on ³H-UTP Incorporation reaction.* Final concentrations of inhibitors were: 40 μg/ml of α-amanitin, 100 μg/ml of rifampicin, 50 μ/ml of actinomycin D and 10 μg/ml of ethidium bromide. Inhibitors were added to the standard incubation mixture at zero time. The incubation was carried out at 35° C for 10 minutes. The activity of the control was: 20 pmoles of ³H-UMP incorporated/mg prot./10 minutes incubation.

Figure 5 shows the sensitivity of the mitochondrial RNA polymerase to different inhibitors. The enzyme is almost insensitive to α-amanitin and only partially

Table II. *Effect of divalent cations concentration on ^{3}H-UMP incorporation into swollen mitochondria of Yoshida ascites hepatoma A.H. 130 cells.*
All experimental conditions as in figure 2.

Mg^2 mM	Mn^2 mM	^{3}H-UMP incorporation (pmoles/mg prot.)
1	1	20.33
1	5	5.63
2.5	1	19.40
2.5	5	5.92
5	1	13.97
5	5	6.40

sensitive to rifampicin. On the other hand, both actinomycin D but principally ethidium bromide strongly affected the reaction.

Discussion

The reported results show that isolated Yoshida A.H. 130 tumour cell mitochondria, are able to incorporate *in vitro* ^{3}H-UTP into RNA, as has been previously reported for normal rat liver mitochondria (9). This enzyme, like the rat liver one, seems to be DNA dependent because of the strong inhibition by some inhibitors like actinomycin D which interacts with DNA. Moreover, we can assume that in our experiments RNA synthesis was directed by endogenous mitochondrial DNA, because of the ineffectiveness of exogenously added DNA on mitochrondial RNA synthesis and because of the strong inhibition of UTP incorporation produced by ethidium bromide, a well known inhibitor of circular double stranded DNA-dependent RNA synthesis (11).

The incorporation of ^{3}H-UTP into mitochondrial RNA is ascribed to a mitochondrial enzyme, and not to contamination by nuclear enzymes, since the reaction is partly sensitive to rifampicin and quite resistant to α-amanitin. With regard to the sensitivity of mitochondrial RNA polymerase to rifampicin some discrepancies have been reported in literature. RNA synthesis in isolated mitochondria from yeast (14) and *Neurospora* (12) do not seem to be affected by rifampicin, however enzymes purified from the same organisms are rifampicin sensitive (4, 10). According to KÜNTZEL *et al.* (4) it would be possible that the native enzyme contains a co-factor which protects it against rifampicin and which could be lost during purification. Another explanation may be that in the whole organelles the enzyme-template complex under conditions used in measuring the reaction is not accessible to the drug, therefore the sensitivity to rifampicin must be measured with the purified enzyme (1, 8).

The properties of the enzyme here reported seem to indicate that it behaves like other mitochondrial RNA polymerase already studied.

The ionic strength dependence, the optimal divalent cations concentration and the sensitivity to inhibitors are not different from those reported for rat liver and other mitochondrial enzymes.

From the results here reported we conclude that Yoshida ascites hepatoma A.H. 130 cells possess a mitochondrial RNA polymerase which can be easily distinguished from nuclear polymerases and whose function is probably similar to that of the same enzyme in normal rat liver cells. The study of this activity and of the mechanism of mitochondrial transcription in tumor cells could be useful to the study of nuclear cytoplasmic interrelationship taking place in cancer cells.

Resumen

Las mitocondrias aisladas de células de hepatoma ascítico de Yoshida A.H. 130 son capaces de incorporar ^{3}H-UTP en RNA. La reacción ha sido ampliamente caracterizada y se realiza al parecer por una típica RNA polimerasa DNA-dependiente.

Este sistema no presenta diferencias notables con respecto al sistema de mitocondrias de hígado normal de rata.

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