SYNTHESIS OF POLY(A) CONTAINING RNA IN ISOLATED MITOCHONDRIA FROM RAT LIVER.

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SUMMARY

Rat liver mitochondria are able to incorporate labelled nucleotides in RNA species which are retained by Millipore filters or oligo(dT)-cellulose column. The radioactivity retained was completely RNAse sensitive when [^{3}H] UTP was used as radioactive precursor whereas by using [^{3}H] ATP this fraction was only 30% RNAse sensitive. These results demonstrate that rat liver mitochondria are able to synthesize poly(A) containing RNA species and furthermore that the polyadenylation reaction occurs within the organelle.

Data regarding the presence of poly(A) containing RNA in mitochondria are scanty and often controversial. The presence of these RNA species in mitochondria was first demonstrated in HeLa cells and their mitochondrial origin was established by hybridization experiments (1,2). However, although these species were extracted from mitochondrial polysomes, no direct evidence of a possible messenger function was given. In yeast cells Cooper and Avers (3) reported the presence from mitochondrial polysomes of poly(A) containing RNA species possessing messenger activity but, in a subsequent paper Groot et al. (4) criticized these data since they were not able to detect poly(A) containing RNA species in Saccharomyces cerevisiae and attributed the previous results to contamination of the mitochondrial preparations with intact protoplasts. The same authors suggested that, in contrast to animal mitochondria, the yeast organelles like bacterial cells do not contain poly(A)-RNA and that in animal mitochondria the presence of such RNA species is probably due to the adaption of a prokaryotic-type system to the demands of higher eukaryotes. The presence of a class of RNA containing poly(A) sequences was also reported by Avadhani et al. (5) in Erlich Ascites mitochondria. However, their origin was first attributed to nuclei (6) but subsequently (7) the same authors found in the mitochondrial polyribosomes of the same organism poly(A) containing RNA that

hybridized with mtDNA. These authors, however, have not been able to demonstrate the synthesis of poly(A) containing RNA in isolated organelles (7). In this paper, from studies of RNA synthesis in isolated organelles, we present experimental evidence which indicates that rat liver mitochondria are able to synthesize in vitro poly(A)-containing RNA species and also that the synthesis of poly(A) is a mitochondrial process probably catalyzed by a specific mitochondrial poly(A) polymerase enzyme.

MATERIALS AND METHODS

The mitochondria, isolated in sterile conditions from rat liver as previously described (8), were partially swollen by incubation at 30° for 15 min in order to increase their permeability to ribonucleoside triphosphates. The organelles were then incubated in the medium previously described (8) containing either $[^3H]$-ATP or $[^3H]$-UTP as the labeled precursor (Bioschwarz Sp.act. 1 Ci/mmole) at a protein concentration of 8 mg/ml in a final volume of 5.0 ml. After incubation for 10 min at 30°C the reaction was stopped by adding 5 ml of 2 mM ATP (or 2 mM UTP) in a buffer containing 100 mM NaCl, 1 mM EDTA, 10 mM Tris-HCl pH 7.5. The mitochondria were then washed twice with the same buffer, collected by centrifugation at 12,500 g for 10 min, and used for RNA extraction which was performed according Hirsch and Penman (9). After extraction, the RNA was precipitated with two volumes of ethanol and stored at -20°C overnight. The pellet dissolved in the appropiate buffer was used for subsequent analysis.

RNA was analyzed by polyacrylamide gel electrophoresis as previously described (10). After the run the gel was frozen in dry ice and cut with the Yeda macrotome. The slices (about 1.4 mm each) were digested with H_2O_2 30% overnight to 60° and put into 10 ml of Bray's solution for counting. Hybridization experiments were carried out according to Aaij et al. (11). The Millipore filter binding of extracted RNA was measured according to Lee et al. (12), while the fractionation on oligo(dT)-cellulose columns (Collaborative Research) was performed as described by Hirsch and Penman (9).

Protein was assayed by the Waddel method (13). All glassware was acid-cleaned and sterilized. Wherever possible, sterilized reagents and sterile techniques were used.

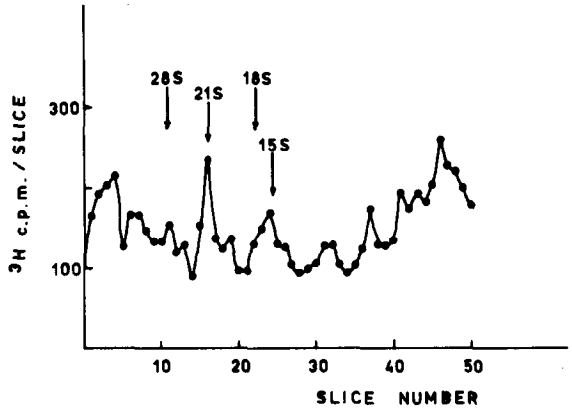

Fig. 1. Polyacrylamide gel electrophoresis of the RNA incorporated *in vitro* using [^{3}H]-ATP as labeled precursors. The analysis was performed as reported in Materials and Methods.

RESULTS

In order to investigate if isolated mitochondria are able to synthesize poly(A) containing RNA species and, furthermore, if the poly(A) sequence could be labeled under the same conditions, we have followed mitochondrial RNA synthesis *in vitro* using either [^{3}H]-UTP or [^{3}H]-ATP as labeled precursors. The RNA was extracted after 15 min incubation and treated as described in Materials and Methods. Hybridization experiments carried out as control demonstrated that the newly-synthesized mitochondrial RNAs only hybridize with mitochondrial DNA but not with E. coli or nuclear DNA in agreement with our previous results (11). The above mentioned properties, together with the criteria used in our previous paper (8,15), clearly demonstrate that the incorporation of labeled nucleotides into mitochondrial RNA occurring *in vitro* is wholly of mitochondrial origin and is not due to the activity of other fractions contaminating the mitochondrial preparation. The labeled RNA using [^{3}H]-ATP as labeled precursor, when analyzed by polyacrylamide gel electrophoresis (Fig. 1), shows two peaks in the region 21-14 S, probably representing the mitochondrial ribosomal RNA species and a polydisperse radioactivity between 12 S and 4 S. The presence of poly(A) sequences in *in vitro* synthesized mitochondrial RNA was measured using two

TABLE I

BINDING OF *IN VITRO* MITOCHONDRIAL RNA TO MILLIPORE FILTERS.

Labeled nucleotide	Input cpm	Addition	Binding cpm	%
[^{3}H]ATP	4000	none	480	12
	4000	RNase	322	8
[^{3}H]UTP	3800	none	266	7
	3800	RNase	40	1

The synthesized RNA (the incorporation of nucleotides into RNA was 0.3 pmoles[^{3}H]-ATP/μg isolated RNA/15 min and 0.13 pmoles [^{3}H]-UTP/μg isolated RNA/15 min) was submitted to millipore filters binding as reported by Lee et al. (12). The cpm bound to the millipore filters have been corrected for nonspecific binding (not more than 3%) measured using [^{3}H]-E. coli ribosomal RNA The binding of [^{3}H]-ATP and [^{3}H]-UTP carried out as a control was negligible.
The RNAse treatment was performed as described in Fig. 2. After incubation the reaction was stopped by adding ten volumes of the buffer (0.5 M KCl, 1 mM $MgCl_2$, 10 mM TRIS pH 7.5) used for millipore binding and chilled in ice for 10 min. before carrying out the binding.

different techniques, the Millipore filter binding technique (12) and fractionation on oligo(dT)-cellulose columns (13). The results of the Millipore filter technique are given in Table I. When [^{3}H]-UTP was used as the labeled precursor about 7% of incorporated radioactivity is retained by the filter. The percentage of incorporated radioactivity bound increased up to 12% when [^{3}H]-ATP was used as precursor. Since the content of the four nucleotides is almost the same in rat liver mitochondrial RNA (8), this suggests that also the poly(A) sequences are labeled *in vitro*.

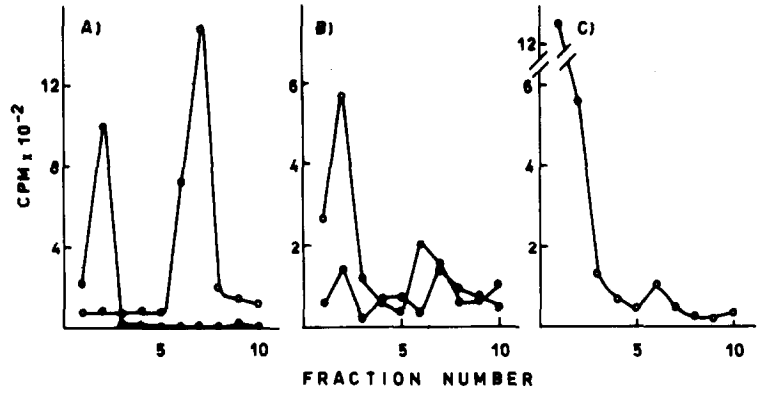

Fig. 2. oligo(dT)-cellulose chromatography of RNA.
A) o——o [^{3}H] poly(A); ●——● [^{3}H] E. coli ribosomal RNA
B) o——o [^{3}H] ATP labeled RNA before RNAse treatment
●——● [^{3}H] ATP labeled RNA after RNAse treatment
C) o——o [^{3}H] UTP labeled RNA before RNAse treatment

The separation of poly(A) containing RNA species was carried out according Hirsch and Penman (9), using 100 mM NaCl, 0.5% SDS, 10 mM Tris pH 7.4 as binding buffer, and 0.05% SDS, 10 mM Tris-HCl pH 7.4 as elution buffer. The RNA, dissolved in the binding buffer was applied to a 5 mm column in a Pasteur pipette and after washing with 5 ml of binding buffer the poly(A) containing RNA was eluted with elution buffer. The fractions collected (1 ml) were then precipitated with 5% trichloroacetic acid after the addition of 100 ug bovine serum albumin and the precipitate collected on GF/C Whatman filters and counted. The percentage of RNA retained was defined as the counts eluting in fractions 5-10. The RNAse treatment was performed by incubating the RNA dissolved in 0.3 M NaCl, 10 mM Tris-HCl pH 7.4 at 37°C for 30 min with ribonuclease A and T_1 at a final concentration of 20 µg/ml and 20 U/ml respectively. After the incubation the reaction was stopped by adding to the reaction mixture NaCl and SDS until the composition of binding buffer was reached.

This is confirmed by data obtained after RNase treatment: in fact the percentage of bound [^{3}H]-UTP labeled RNA drops to 1% after RNase treatment, whereas 8% of radioactivity is still bound to the filter when RNA labeled with [^{3}H]-ATP is used.

The results obtained with the millipore filter technique were confirmed by using the oligo(dT)-cellulose column. Fig. 2

shows the elution profile of the *in vitro* labeled RNA through this column. Using this technique we have found that with [^{3}H]-ATP as labeled precursor, about 15% of incorporated radioactivity is eluted at low salt concentration, while using [^{3}H]-UTP the radioactivity bound to the column was 7%. The RNase treatment of the RNA labeled with [^{3}H]-ATP reduced by 30% the radioactivity eluted with low strength buffer. As a control in any experiment [^{3}H]-labeled poly(A) and [^{3}H]-labeled E. coli ribosomal RNA were applied to two oligo(dT)-cellulose columns. The two RNA species were respectively eluted at low and at high salt concentration.

DISCUSSION

The results reported in this paper clearly demonstrate for the first time that isolated mitochondria from rat liver are able to synthesize poly(A) containing RNA species. Considering the results obtained with the RNA labeled with [^{3}H]-UTP using the two different techniques, we calculate that about 6% of the total *in vitro* synthesized RNA contains sequences of poly(A). The experiments performed using [^{3}H]-ATP as the labeled precursor roughly confirm these data because the percentage of RNA bound before and after RNase treatment is about 12-15% and 8-10% respectively, clearly showing that in the latter case also the sequences of poly(A) which are RNase resistant are synthesized *in vitro*. These data are particularly relevant to the problem of the physiological role of the poly(A) polymerase activity found in rat liver mitochondria. The presence of such mitochondrial enzyme has been so far described only in rat liver (16-17) and Morris hepatoma (18). Until now, however, no data about its significance and physiological role within the mitochondria are available. The experiments reported here suggest that this enzyme may be involved in the mitochondrial transcription process and that adenylation of mitochondrial RNA is occurring intramitochondrially. Our data are in conflict with those of Avadhani et al. who claim that synthesis of poly(A) containing RNA does not occur *in vitro* in isolated organelles (6,7) although a different source of mitochondria has been used in our case. The function of these poly(A) containing RNA species synthesized *in vitro* by isolated mitochondria is now under investigation in our laboratory.

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