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PRIMER DIRECTED POLY(A) SYNTHESIS IN RAT LIVER MITOCHONDRIA

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SUMMARY

Rat liver mitochondria contain an endogenous factor highly specific in stimulating the homologous poly(A) polymerase. By using an *in vivo* labelling with [^{32}P] orthophosphate it is possible to prepare a labelled factor and to demonstrate that it is stably incorporated in an acid insoluble molecule. This suggests that the factor probably acts as a primer in the polymerization of ATP molecules, being involved in the recognition between the mitochondrial poly(A) polymerase and the homologous RNA molecules which have to be polyadenylated.

The presence of a specific poly(A) polymerase in rat liver mitochondria has been demonstrated by Jacob (1) and in our laboratory (2). In particular Jacob showed that this enzyme is dependent on an "endogenous factor" partially dializable and of ribonucleotide nature (1). Subsequently we confirmed the specific requirement of this factor for rat liver mitochondrial poly(A) polymerase and devised a method which allows a partial purification of poly(A) polymerase and its separation from the homologous DNA dependent RNA polymerase (3).

The dependence of this enzyme on a specific endogenous molecule differentiates mitochondrial poly(A) polymerase from other poly(A) polymerases extracted from different sources (see ref. 4 for a review). Nuclear and cytoplasmic poly(A) polymerases so far isolated show a non specific primer requirement and are able to add poly(A) tracts to RNAs of different nature. Nothing is known about the mechanism of action of the mitochondrial endogenous factor; however, its extensive purification (manuscript in preparation) and its high specificity in stimulating the synthesis of poly(A) (5) have suggested to us that it could contain the signal (probably a specific

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nucleotide sequence originally present in an RNA molecule) recognized by the enzyme for the addition of AMP residues. This implies that the factor may act as a primer and remain bound to the reaction product.

In order to demonstrate the incorporation of the so-called endogenous factor in a poly(A) containing molecule synthesized in vitro by the partially purified mitochondrial enzyme, we have prepared a labelled factor by injecting the animals with [^{32}P] orthophosphate. In this paper we demonstrate that during polymerization of ATP molecules the endogenous factor is converted into an acid insoluble product which behaves as a molecule containing poly(A) sequences.

MATERIALS AND METHODS

Oligo-(dT) Cellulose was from Collaborative Research Inc. Waltham, Massachussetts, U.S.A., Bio-Gel P2 was from Bio Rad Laboratories, Richmond California, U.S.A., [^{32}P]-orthophosphate (Sp. Act. 14 Ci/mg P. and [^{3}H]-ATP (Sp. Act. 29 Ci/mmol) were from Amersham. Insta gel was from Packard Instrument Company Inc. Downers Grove Illinois, U.S.A.

Male albino rats weighing about 80 gr. received each 1 mCi of [^{32}P] orthophosphate in Tris sterile and were killed 12 h after injection.

The mitochondria were prepared by a partial modification of a standard procedure (6). Centrifugation at 600 xg was repeated twice and the last centrifugation at 12,000 xg was substituted with a second at 8,000 xg.

The mitochondrial endogenous factor and the homologous poly(A) polymerase were prepared according to standard procedures (7,5).

The same enzyme preparation was used throughout the experiments described in this paper. The incorporation in acid insoluble material of labelled compounds was measured as already reported (3). The specific activity of the enzyme used in the experiments described in this paper was 4,100 Units/mg of protein. One unit corresponded to a pmol of [^{3}H]-ATP incorporated in acid insoluble material in standard conditions (3).

The protein concentration of partially purified poly(A) polymerase was determined by the Waddel method (8).

RESULTS AND DISCUSSION

The specificity of rat liver mitochondrial poly(A) polymerase for an endogenous factor (1,3,5) has suggested that it could contain a specific nucleotide sequence or a specific nucleotide originally present at the 3' OH termination of an RNA molecule conferring on it the signal which is recognized by the homologous enzyme in order to start the addition of AMP residues.

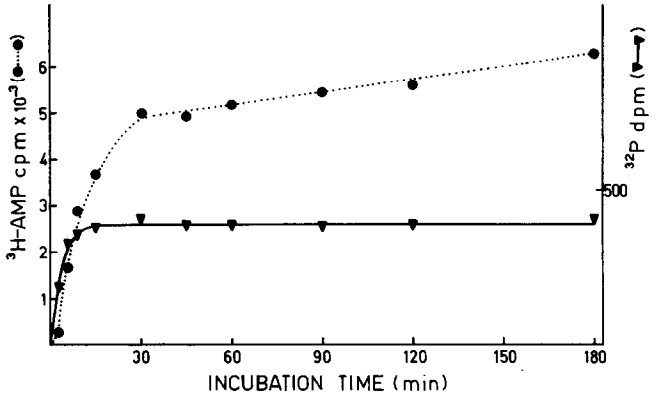

Fig. 1 - Time course of [^{32}P] labelled endogenous factor and [^{3}H]-ATP incorporation in acid insoluble material in presence of partially purified mitochondrial poly (A) polymerase.
The [^{32}P] labelled endogenous factor purified through Bio Gel P_2 column (see Fig. 2) (about 90,000 dpm) was mixed with 1.11 mg of poly(A) polymerase in an incubation mixture of standard composition (3) having a final volume of 1 ml. 80 µl aliquots were taken out each time (including the zero time) and chilled in 1 ml of cold 5% trichloroacetic acid. Zero time for [^{32}P] radioactivity was 80 dpm, for [^{3}H] radioactivity 450 c.p.m.

In order to demonstrate that the poly(A) polymerase adds the AMP residues to this specific molecule using it as a primer, it is necessary to show that it remains stably linked in the reaction product. For this purpose we prepared a [^{32}P] labelled endogenous factor by an *in vivo* labelling with [^{32}P]-orthophosphate. The experimental results reported in this paper clearly demonstrate that the product synthesized in the presence of [^{32}P] labelled endogenous factor, labelled or unlabelled precursor and partially purified poly(A) polymerase contain a poly(A) sequence covalently bound to a [^{32}P] labelled molecule. The results reported in Table I show that after incubation in the presence of cold ATP and poly(A) polymerase the endogenous factor is incorporated in an acid insoluble molecule containing the unlabelled sequence of poly(A). The same conclusion can be drawn by the analysis of data reported in Fig. 1 showing the time dependent incorporation of [^{32}P] and [^{3}H] labelling in acid insoluble material. The constant

TABLE I

[^{32}P]-LABELLED ENDOGENOUS FACTOR INCORPORATION IN ACID INSOLUBLE MATERIAL.

SYSTEM		% of control
a	Complete	100
b	Poly(A) polymerase omitted	22
c	Incubation mixture omitted	35
d	Poly(A) polymerase and incubation mixture omitted	25

An aliquot of 120 µg of partially purified mitochondrial poly (A) polymerase was incubated in standard conditions in the presence of 49,700 dpm of [^{32}P] labelled endogenous factor and in the presence of a suitable incubation mixture containing cold ATP (3). The reaction was stopped by adding 5% trichloroacetic acid and the acid insoluble material formed was determined by the millipore technique. About 0,8% (375 dpm) of the initial amount of [^{32}P] labelled endogenous factor was converted into acid insoluble material. All the assays were repeated in triplicate. Counting efficiency was about 95%. The background was 10 dpm. The experiment was carried out by simultaneously incubating three different controls (b,c,d) in the mentioned conditions.

level reached after 15' by the [^{32}P] acid insoluble radioactivity suggests that the [^{32}P] primer molecules taken up during this time in acid insoluble product remain stably incorporated in it.

As far as the [^{3}H] labelling is concerned, two different rates of incorporation may be noticed. One (up to 30') which probably corresponds to the initiation of poly(A) sequences and to the contemporaneous elongation of already initiated molecules and the other (30'-180') probably corresponding only to the elongation of product molecules initiated in the first thirty minutes.

The existence in the reaction product of two different labelled portions is clearly confirmed by the elution pattern obtained by desalting the [^{32}P] endogenous factor on Bio Gel

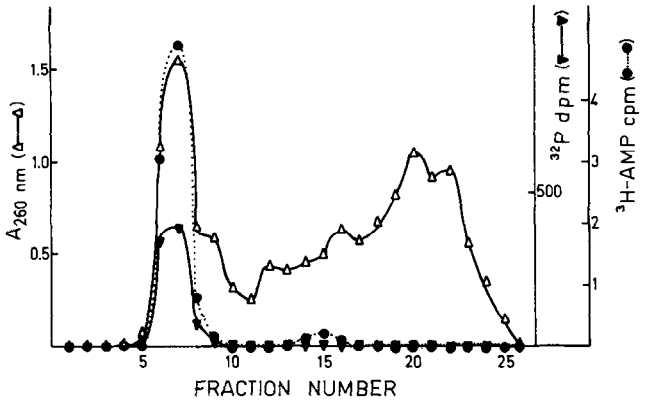

Fig. 2 - Bio-Gel P_2 chromatography of [^{32}P]-labelled endogenous factor.

An aliquot of about $5x10^6$ dpm of solubilized [^{32}P]-labelled endogenous factor (7) was resuspended using 1.5 ml of buffer A/10 (potassium phosphate 5 mM pH 6.8, $MgCl_2$ 0.5 mM, EDTA 0.02 mM, KCl 5 mM) and applied to a (1.2x36 cm) Bio-Gel P_2 column equilibrated with the same buffer. Each fraction was assayed for the [^{32}P]-labelled endogenous factor content incubating 50 µl aliquots in the presence of the incubation mixture already reported (3) and 40 µg of partially purified mitochondrial poly(A) polymerase. Each fraction had a volume of 2.6 ml. Flow rate was 18 ml/h. The fractions (6-8) were pooled, lyophilized and used in the experiments described in Fig. 3.

P_2 column (Fig. 2) and by the characterization of the reaction product on Oligo-(dT) cellulose (Fig. 3). The correspondence of the acid insoluble radioactivity profile, due to [^{3}H] labelling, with that of [^{32}P] is in agreement with our previous conclusions.

The experiments by affinity chromatography on the reaction product (see Fig. 3, panels A-B) confirm the action mechanism of the endogenous factor and show that about 20% of the reaction product is made by molecules long enough to be retained at 4°C by the column being eluted only by LSB buffer. The nature of the [^{3}H] labelled part of the product is also confirmed by its chemical and enzymatic characterization. In fact it is sensitive to snake venom phosphodiesterase, insensitive to pancreatic ribonuclease and hydrolyzed by concentrated alkali (9).

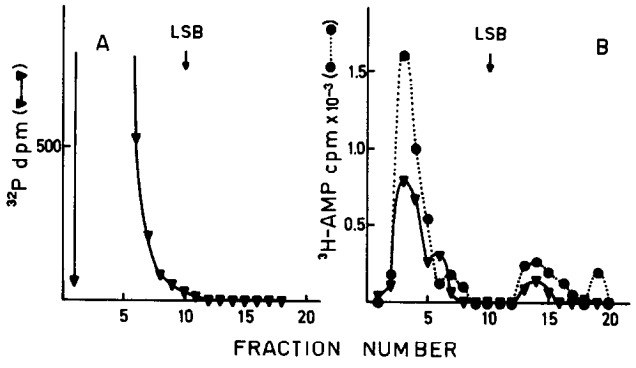

Fig. 3 - Oligo-(dT) cellulose chromatography of the reaction product made in vitro by the mitochondrial poly(A) polymerase in presence of [^{32}P]-labelled endogenous factor and [^{3}H]-ATP.

About 30,000 dpm of [^{32}P] labelled endogenous factor fractionated through a Bio-Gel P_2 column (see Fig. 2) were absorbed on a 0.5x2.5 cm Oligo-(dT) cellulose column and left at 4°C for about 30'. The column was eluted using HSB buffer (NaCl 0.5 M, Tris 10 mM pH 7.4) (fractions 1-9) and LSB buffer (Tris 10 mM pH 7.4) (fractions 10-18). Each fraction had a volume of 0.5 ml. Radioactivity was counted by mixing each fraction in 10 ml of INSTA GEL (Fig. 3/A). The same amount of [^{32}P] labelled endogenous factor used in the previous experiment was incubated in standard conditions in the presence of 370 µg of mitochondrial poly(A) polymerase. After 30' incubation unlabelled ATP was added to a final concentration of 12 mM and NaCl to a final concentration of 0.5 M. After further 30' the column was eluted by HSB and LSB (see above). To each fraction 0.5 ml of 10% trichloroacetic acid was added and acid insoluble radioactivity determined as reported elsewhere (3) (Fig. 3/B).

The existence of two different classes of acid insoluble molecules (Fig. 3/B, fractions 2-6 and 13-17) can be explained by assuming that those eluted at high salt concentration (fractions 2-6) contain poly(A) sequences which are too short to be retained by the column in our experimental conditions.

The structure and the origin of the endogenous primer remain unknown. Its partial purification (manuscript in preparation) led us to the conclusion that it can be solubilized in form of a trinucleotide and preliminary results suggest that it can be generated during the solubilization procedure mainly from poly(A) containing mitochondrial RNA molecules. Studies on the molecular structure and its chemical composition

are now in progress in our laboratory in order to verify its physiological role and its interaction with the homologous poly(A) polymerase.

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