Enzymatic synthesis of DNA complementary to mitochondrial mRNA via reverse transcription

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ABSTRACT

The poly(A)-containing mitochondrial mRNAs of rat liver were tested for their ability to serve as templates for the DNA synthesis by means of reverse transcription in the presence of the oligo(dT) primer and the RNA-directed DNA-polymerase from avian myeloblastosis virus. The mt-mRNA does not support the DNA synthesis in the standard conditions sufficient for effective reverse transcription of rabbit globin mRNA and of poly(A) in the presence of oligo(dT) primers. After a mild alkaline treatment of the mRNA and subsequent polyadenylation of the 3'-termini of the generated fragments with ATP:RNA adenylyltransferase from E.coli the poly(A)(+) polyribonucleotides are able to serve as templates for reverse transcription in the presence of oligo(dT) and the reverse transcriptase. A conclusion is made that a "structural stop" exists in mitochondrial mRNA non-translatable regions adjacent to the poly(A) terminal sequence. The "structural stop" is suggested to be caused by post-transcriptional modification of mRNA (methylation, etc.) or by a particularly stable secondary structure in this region of the mRNA molecules.

INTRODUCTION

The RNAs of various origin have been shown to function as templates for reverse transcription (cf. the reviews ¹⁻³) and therefore, there is no restriction for synthesis of complementary DNAs if appropriate primers are available. It is known ^{4,5} that mitochondrial mRNAs like mRNA isolated from cytoplasm of animal cells contain poly(A) sequences. These observations provide a basis for attempts to use mt-mRNA as templates for reverse transcription in the presence of oligo(dT) primer. The reasons for synthesizing cDNA on the mt-mRNA is obvious since cDNA is invaluable tool for the investigation of many aspects of molecular genetics of mitochondria, i.e. genomic structure, gene mapping, regulation of transcription, etc. This approach

as far as we are concerned has not yet been described in the literature.

The purpose of this study is to synthesize cDNA along the mitochondrial mRNA via reverse transcription.

MATERIALS AND METHODS

Deoxyribonucleoside triphosphates and the HEPES buffer were purchased from Sigma; poly(A)·oligo(dT), oligo(dT)₁₀ and oligo(dT)-cellulose (type 7) were from PL Biochemicals, radioactive triphosphates from Radiochemical Center, Amersham; reagents for electrophoresis from Merck and Serva, Protosol from NEN, the Sephadex G-50 (fine) and poly(U)-Sephadex from Pharmacia Fine Chemicals. Other reagents were of analytical grade.

A highly purified reverse transcriptase from avian myeloblastosis virus was purified according to method ⁶ as described ⁷. The nuclease S₁ was isolated by method ⁸, ATP:RNA adenylyltransferase from E.coli as described ⁹.

The mitochondrial mRNAs of rat liver were obtained as described earlier ¹⁰ from intramitochondrial polyribosomes with a subsequent isolation of the poly(A) containing fraction on the poly(U)-Sephadex. From the total mt-mRNA a fraction was isolated soluble in 1.5 M NaCl. Approximately 70% of this fraction represented a homogeneous material with a sedimentation coefficient of 7.5S. This fraction was designated as 7.5S mRNA. Both mRNA fractions contained the poly(A) sequences ⁵.

The mt-mRNA fragmentation was achieved with incubation in 0.1 M Na₂CO₃/NaHCO₃ (pH 11.0) for 10 min at 37°C. The reaction was stopped by adding 0.2 volumes of the cold 1 M HEPES (pH 7.0), after cooling the pH was lowered to 7.6 by HCl; the fragmented mRNA was desalted with gel filtration on the Sephadex G-50 (fine) column.

Polyadenylation of the fragments obtained was performed at 37°C for 1 hour in the incubation mixture composed of 25 mM Tris-HCl (pH 7.9), 0.25 M NaCl, 0.01 M MgCl₂, 0.002 M MnCl₂, 1·10⁻⁴ M dithiothreitol, 2·10⁻⁴ M [³H]ATP (specific activity 100 cpm/pmol), 0.025 mg/ml RNA, 0.5 mg/ml of bovine serum albumin, 5% glycerol and 5 units/ml of ATP:RNA adenylyltransferase (EC 2.7.7.19) from E.coli ⁹.

The reaction was stopped with phenol-chloroform deproteinization, and the polyadenylated fractions were separated on the oligo(dT)-cellulose column as described ¹². For the cDNA synthesis a fraction was taken able to bind with the oligo(dT) column.

The cDNA synthesis was undertaken in the incubation mixture containing 0.05 M Tris-HCl (pH 8.3), 40 mM KCl, 6 mM MgCl₂, 2 mM dithiothreitol, 0.1 mM of each deoxyribonucleoside triphosphates including [³H]dCTP (14 Ci/mM), 10 µg/ml of (dT)₁₀, 0.1 mg/ml of actinomycin D, 20 µg/ml of mt-mRNA and 25 unit/ml of reverse transcriptase. After incubation for 1 hr at 37°C the mixture was deproteinized with phenol and 2.5 volumes of ethanol was added to the aqueous phase in the presence of the 0.2 M sodium acetate (pH 5.0). After centrifugation the precipitate was dissolved in 0.3 ml of 0.3 N KOH and incubated for 5 hr at 37°C, after neutralization the solution was applied to the Sephadex G-10 column equilibrated with the buffer containing 0.1 M NaCl, 0.01 M Tris-HCl (pH 7.2), 1 mM EDTA and 0.1% sodium dodecylsulphate. The cDNA-containing fractions were combined and precipitated with 2.5 volumes of ethanol in the presence of 0.4 M LiCl and tRNA-carrier (50 µg/ml).

The disc electrophoresis was run in 4% polyacrylamide gel in 98% formamide as described ¹³. However bisacrylamide cross-link was substituted for the ethylenediacrylate one. After electrophoresis the gels were scanned with the "Scan-400" (Joyce Loebel) densitometer at 265 nm and then cut into 2 mm slices. In the case of RNA, the gel cuts were minced in 0.5 ml of protosol, incubated for 1 hr at 60°, cooled and 5 ml of toluene scintillator was added. Radioactivity was measured with Intertechnique SL-30. In the case of DNA 0.1 ml of 1.5 M NaOH was added to each slice incubated for 40 min at 70°C, neutralized with HCl, combined with trichloroacetic acid (TCA) up to final concentration of 10% in the presence of the RNA-carrier (0.1 mg/ml). The samples were transferred on the nitrocellulose filters (AUF5, Chemapol), washed with 5% TCA and counted as described.

Hybridization in solution was run in the mixture containing 0.3 M NaCl, 0.03 M sodium citrate, 0.1% sodium dodecylsul-

fate, 40% formamide, 0.05 $\mu\text{g/ml}$ of DNA product and 5 $\mu\text{g/ml}$ of mt-mRNA. The mixture was gradually cooled within 16 hr from 60° down to 28°C, 2° off every hour. After hybridization the mixture was diluted with buffer used for S_1 nuclease hydrolysis.

The hydrolysis with S_1 nuclease was carried out in the mixture containing 0.1 M NaCl, 0.03 M sodium acetate (pH 4.6), 1 mM ZnSO_4 , 5% glycerol, 20 $\mu\text{g/ml}$ of denatured DNA, 4 units/ml of S_1 nuclease purified as described⁸. The reaction was stopped with addition of TCA to the final concentration of 10% in the presence of RNA-carrier (50 $\mu\text{g/ml}$). The samples then were transferred on the AUFS filters, washed with 5% TCA and assayed as described above.

RESULTS AND DISCUSSION

The mitochondrial poly(A)⁺-mRNAs do not support the DNA synthesis at standard conditions favourable for effective reverse transcription of rabbit globin mRNA and of poly(A) in the presence of oligo(dT) primer (Table 1). This is not due to the presence of poly(U), co-eluted from the affinity column, preventing the formation of the poly(A)-oligo(dT) initiation duplex

TABLE 1

ABSENCE OF REVERSE TRANSCRIPTION OF MITOCHONDRIAL mRNAs
IN THE PRESENCE OF REVERSE TRANSCRIPTASE, (dT)₁₀
AND ACTINOMYCIN D

Template	Incorporation of [³ H]precursors into the acid insoluble fraction, pmol/ μg RNA			
	dCMP	dGMP	TMP	TMP in the absence of other triphosphates
mt-mRNA				
7.5S fraction	2.0	0.8	38.0	56.0
total	0.6	not determined		
rabbit globin mRNA	70	70	300	1200
globin mRNA + 7.5S fraction	69*	71*	not determined	

* with respect to globin mRNA.

because globin mRNA after elution from the same batch of poly(U)-Sephadex column exactly in the same way is fully active as the template for reverse transcriptase. Moreover, we observed an absence of reverse transcription of heteropolymeric part of the mt-mRNAs eluted from oligo(dT)-cellulose as well.

One would suggest the mRNA preparation contains an inhibitor inactivating the enzyme or interfering with its action. It is not the case however since as shown in Table 1 the complementary copying of the heteropolymeric portion of the globin mRNA molecule is not impaired by the presence of the mt-mRNA. The incorporation of [^3H]TMP with mt-mRNA as template (Table 1) confirms directly the presence of poly(A) sequence in the template probably at the 3' end of mt-mRNA and shows also that the enzyme is active in the presence of the given preparation of mt-mRNA. Therefore, it remains to assume that between the poly(A) part of the mt-mRNA and its heteropolymeric part there is a "structural stop" for reverse transcription which has to be by-passed in order to obtain cDNA.

If the mt-mRNA besides 3'poly(A) terminus contains other homopolymeric sequences, one may try to use primers complementary to these regions in order to make reverse transcripts lying to the left of the oligohomopolymeric regions of the mRNA molecule. However neither (dG)₁₀ nor (dC)₁₀ primes any noticeable synthesis along mt-mRNA (Table 2). This result prompted an indirect evidence that the mt-mRNA presumably lacked oligocytidilic and oligoguanilic sequences long enough and accessible for complex formation with the complementary oligonucleotides.

Another pathway for obtaining cDNA was described ¹¹ where the poly(A)(-)RNA was subjected to fragmentation, the poly(A) was enzymatically synthesized at the 3'-ends of the fragments followed by reverse transcription initiated by oligo(dT). Following this approach we subjected mt-mRNA to a mild alkaline treatment to achieve fragmentation into sufficiently large polynucleotides and then to polyadenylate by means of ATP:RNA adenyltransferase, as it had been done for ribosomal RNA fragments ¹¹.

Fig. 1A shows that fractionation of the total mt-mRNA brings about a wide range of molecular weight distribution -

TABLE 2

REVERSE TRANSCRIPTION OF VARIOUS mRNAs DEPENDING ON
THE NATURE OF THE PRIMERS

Template	$[^3\text{H}]\text{dCMP}$ incorporation, $\mu\text{mol}/\mu\text{g RNA}$ (dG) ₁₀	(dC) ₁₀
mt-mRNA		
7.5S fraction	0.3	1.2
total	0	0
globin mRNA	7.2	2.6

(dG)₁₀ and (dC)₁₀ - 0.1 A₂₆₀/ml each. Incubation for 1 hr at 37°.

Fig.1. Electrophoresis in a 4% polyacrylamide gel in 98% formamide of fragmented total mt-mRNA before (A) and after (B) polyadenylation.

from ten to hundreds of thousand of dalton. After adenylation $[^3\text{H}]\text{AMP}$ is found in all fractions, but mainly in 4-7S region (Fig. 1B). The presence of radioactivity in all fractions demonstrates that the polyadenylation is independent on the polynucleotide length. The preparation obtained was passed through a column with oligo(dT)-cellulose to establish what part of the RNA fragments contained sufficiently long poly(A) 3'-termini after enzymatic polyadenylation. The column appeared to retain from 1/3 or 1/2 of RNA applied; 90-93% of all $[^3\text{H}]\text{AMP}$ radioactivity incorporated into RNA was retained too. Hence, the major

rity of the molecules passing through the column had not been adenylated at all or contained very short oligo(A) stretches. The average length of the poly(A) stretches in the poly(A)(+) fragments obtained was calculated to correspond to ~15 nucleotides.

The addition to poly(A)(+) mt-mRNA fragments of reverse transcriptase, oligo(dT) and other components necessary for reverse transcription led to [^3H]dCMP incorporation into the acid insoluble fraction (Table 3). Neither before, nor after passing through the oligo(dT)-cellulose column incorporation was observed in the control samples containing the fractionated but not polyadenylated template (Table 3). This implied that fragmenta-

TABLE 3
REVERSE TRANSCRIPTION OF
MITOCHONDRIAL mRNAs AFTER VARIOUS
TREATMENTS

Template	Incorporation of [^3H]dCMP into the acid insoluble fraction, pmol/ μg RNA
mt-mRNA	
7.5S fraction	< 1
total	< 1
Fragmented mt-mRNA	
total	< 1
7.5S fraction	< 1
7.5S fraction after fractionation on the oligo(dT)- cellulose column	
retained	< 1
passed	< 1
Fragmented and poly- adenylated mt-mRNA retained on the oligo(dT)-cellulose	
total	30
7.5S fraction	28

All tubes contained (dT) $_{10}$ (10 $\mu\text{g}/\text{ml}$)
 and were incubated for 30 min at
 37°C.

tion had not resulted in generating of endogeneous primers which, in principle, might be possible after fragmentation due to the formation of the terminal hairpins having free 3'-OH groups. The polyadenylated mRNA fragments seems to be not so good templates for reverse transcription as for instance rabbit globin mRNAs. This might be caused by a number of factors like for example, different content of the cytidine residues in the globin cDNA as compared to the mt-mRNA, a non-similar poly(A) length of these mRNAs which might decrease a fraction of the efficiently primed mRNAs, a presence of more rigid secondary structure, etc. The electrophoresis in the polyacrylamide gel under denaturing conditions (Fig. 2) shows that the products formed correspond roughly by their length to a set of polyadenylated mt-mRNA fragments. The reverse transcription is presumably not affected by the length of the fragments and the cDNA molecules are full-representative copies of the fragments being templates. A more precise conclusion on this point can be made after quantitative molecular hybridization data being under way.

The RNA-cDNA hybrids formed after reannealing of cDNA with excess of mt-mRNA are resistant to S_1 nuclease hydrolysis (Fig. 3). This result supports the complementary nature of synthesis. The cDNA preparation themselves are hydrolyzed almost completely with S_1 nuclease and consequently, the cDNA obtained in the presence of actinomycin D appears to be single stranded and devoids of a rigid secondary structure.

Hybridization of the cDNA with various RNAs is shown in Table 4. In accord with the data of Fig. 3 the cDNA-7.5S poly(A)⁺-mt-mRNA hybrids are 92% resistant to S_1 nuclease hydrolysis. Almost no hybridization was observed with cytoplasmic RNAs and 21S mt-rRNA. The relatively high level of hybridization of cDNA with high molecular weight poly(A)⁺-mt-mRNA and 14S mt-rRNA is due to cross contaminations of the given RNA preparations. The base-sequence complexity calculated from the $Cr_0 t_{1/2}$ value corresponds to 350 nucleotides in a very good agreement with the molecular weight of the 7.5S mt-mRNA template (to be published elsewhere).

Thus the fragmentation and polyadenylation of the mt-mRNA fragments helps to avoid the transcription block located some-

Fig. 2. Electrophoresis in a 4% polyacrylamide gel in 98% formamide of DNAs complementary to the fragmented and polyadenylated mt-mRNA: total mt-mRNA (A) and 7.5S fraction (B).

Fig. 3. S_1 nuclease resistance of the cDNA-mRNA hybrids. cDNA₁ is synthesized in the presence of actinomycin D on the total mt-mRNA, cDNA₂ - on the 7.5S fraction after fragmentation and polyadenylation. Controls: cDNA₁ (A) and cDNA₂ (B) treated with S_1 nuclease before (x-x-x) and after (Δ-Δ-Δ) denaturation. Experiments: cDNA₁ (A) and cDNA₂ (B) annealed with respective templates and digested with S_1 nuclease (o-o-o).

where between the heteropolymeric and the poly(A) parts of the mt-mRNA. Simultaneously, these results prove the existence of such a block since the reverse transcription of the fragments having different length runs quite smoothly.

TABLE 4

HYBRIDIZATION OF cDNA SYNTHESIZED ALONG 7.5S FRACTION
OF mt-mRNA TEMPLATE WITH VARIOUS RNAs

RNA	Resistance of [³ H]cDNA to S ₁ nuclease
7.5S poly(A) ⁺ -mt-mRNA	92
Low molecular weight poly(A) ⁻ -mt-mRNA	12
High molecular weight poly(A) ⁺ -mt-mRNA	18
21S mt-rRNA	6.1
14S mt-rRNA	12.3
Poly(A) ⁺ -mRNA from cytoplasmic ribosomes	7.8
28S rRNA from cytoplasm	5.7

The conditions of hybridization as described ¹⁷. Each tube contained 5,000 cpm of [³H]cDNA.

The nature of this block is unknown. It may be located somewhere in a non-translatable region between the terminal codon and the poly(A) part of the mt-mRNA. The existence of non-translatable sequences in this region has been firmly established for other mRNAs ^{14,15}. Two assumptions can be made with respect to the nature of the transcription block - either this area may contain modified base or bases unable to form canonical hydrogen bonds with any of the four deoxyribonucleoside triphosphates or there should be a rigid secondary structure which arrests the movement of the reverse transcriptase along the template. The second supposition seems to be less probable as the enzyme is known to carry on synthesis along the structured templates including those having a rigid structure ¹⁶. With respect towards the post-transcriptionally modified nucleosides, this supposition seems to be probable due to overall distribution of modified bases among the ribosomal, viral and transfer RNAs.

Successful synthesis of the cDNA containing information of mt-mRNA offers new possibilities for the study of mitochondrial genome, i.e. the gene number, mapping, expression and processing.

The data on the cDNA synthesis along the mt-mRNA were not found in available literature. This may be due to the presence of the "structural block" in the mt-mRNA described here.

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REFERENCES

- 1 Green, M. and Gerard, G.F. (1974) Progr. Nucl. Acids Res. and Mol. Biol. 14, 188-334.
- 2 Frolova, L.Yu. (1976) Molecular Biology (series) v. 7, p.132-193, VINITI, Moscow.
- 3 Verma, I. (1977) Biochim. Biophys. Acta 473, 1-38.
- 4 Attardi, G., Albing, M., Amalrie, F., Gestand, R., Griffith, I., Lynch, D., Menkel, Ch., Murphy, W. and Ojala, D. (1976) in: Genetics and Biogenesis of Mitochondria and Chloroplasts (Bucher, Th. et al., eds.), p. 573-585, North-Holland, Biomed. Press, Amsterdam.
- 5 Gaitskhoki, V.S., Kisselev, O.I. and Klimov, N.A. (1973) FEBS Letters 37, 260-263.
- 6 Kacian, D.L., Watson, K.F., Burny, A. and Spiegelman, S. (1971) Biochim. Biophys. Acta 246, 365-383.
- 7 Kisselev, L.L., Frolova, L.Yu., Gasaryan, K.G., Tarantul, V.Z. and Engelhardt, V.A. (1973) Doklady Akademii Nauk SSSR 213, 1203-1206.
- 8 Vogt, V. (1973) Eur. J. Biochem. 33, 192-200.
- 9 Sippel, A.E. (1973) Eur. J. Biochem. 37, 31-40.
- 10 Kisselev, O.I., Gaitskhoki, V.S. and Neifach, S.A. (1977) Molec. Cell Biochem. 14, 91-96.
- 11 Hell, A., Young, B. and Birnie, G. (1976) Biochim. Biophys. Acta 442, 37-49.
- 12 Aviv, H. and Leder, P. (1972) Proc. Nat. Acad. Sci. USA 62, 1408-1412.
- 13 Maniatis, T., Jeffrey A. and van de Sande, H. (1975) Biochemistry 14, 3787-3794.
- 14 Milstein, C., Brownlee, G.G., Cartwright, E.M., Javris, J.M. and Proudfoot, N.J. (1974) Nature 252, 354-359.
- 15 Proudfoot, N.J. and Brownlee, G.G. (1974) Nature 252, 359-362.
- 16 Frolova, L.Yu., Metelyev, V.G., Ratmanova, K.I., Smirnov, V.D., Shabarova, Z.A., Prokofyev, M.A., Berzin, V.M., Jansone, I.V., Gren, E.J. and Kisselev, L.L. (1977) Nucl. Acids Res. 4, 2145-2159.
- 17 Gummerson, K.S., Williamson, R. (1974) Nature 247, 265-267.