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IRON STIMULATED RNA-DEPENDENT DNA POLYMERASE ACTIVITY FROM GOLDFISH EGGS

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Abstract - A partially purified protein fraction from fish eggs presents a DNA polymerase activity dependent on globin messenger RNA, oligo dT, four deoxyribonucleoside-5'-triphosphates, magnesium, and iron. The reaction is inhibited by pre-incubation with ribonuclease A and not in the presence of actinomycin D. The product is resistant to ribonuclease A and KOH, but digested by DNA-A. The product of the globin mRNA templated reaction hybridized to a globin DNA probe and not to another probe.

Key words : Rabbit globin mRNA, reverse transcriptase, cDNA, mRNA transcription, Southern blot technique, complementary sequence.

STIMULATION PAR LE FER DE L'ACTIVITE DE L'ADN POLYMERASE ARN DEPENDANTE ISOLEE DES OEUFS DE POISSON ROUGE

Résumé- Une fraction enzymatique partiellement purifée des oeufs de poisson se comporte comme une ADN polymérase dépendante de l'ARN messager de la globine, oligo dT, quatre désoxyribonucléoside-5'-triphosphates, magnésium et fer. La réaction est inhibée par préincubation avec la ribonucléase A; elle n'est pas inhibée par l'actinomycine D. Le produit est résistant à la ribonucléase A et au KOH; il est digéré par l'ADN-A. Le produit synthétisé sur la matrice de l'ARN-m de la globine s'hybride avec l'ADN-sonde de la globine, mais non avec d'autres sondes.

Mots clés : ARNm de la globine du lapin, transcriptase inverse, ADNc, transcription de l'ARNm, Southern blot technique, séquences complémentaires.

INTRODUCTION

RNA dependent DNA polymerase (reverse transcriptase, abbreviated as RT) was first discovered in retroviruses (Baltimore, 1970; Temin and Mizutani, 1970). The RNA of these viruses is transcribed into DNA. The viral enzyme is capable of using purified rabbit globin mRNA for in vitro synthesis of complementary DNA (cDNA) (Ross et al., 1972). The role of RT in the virus life cycle and cell transformation has been extensively discussed (Baltimore, 1985; Temin, 1976; Warmus and Swanstrom, 1982). It was suggested that RT in Drosophila melanogaster might be responsible for the transposition of movable genetic elements (Shiba and Saigo, 1983). Yeast transposon (TY) elements were shown to transpose via an intermediate RNA. The pattern of sequence inheritance in progeny TY insertions follows the predictions of

a model of retroviral reverse transcriptase (Boeke et al., 1985). The reader is refered to a review on reverse transcription in the eukaryotic genome (Temin, 1985) in which retrotranscripts and retrotransposons have been thoroughly discussed. The reverse transcriptase activity would have to be present in germe cells or to be brought in by infection.The presence of RT activity was reported in fish eggs (Niu, 1981). Now we report the partial purification of the enzyme and its requirements for in vitro transcription of rabbit globin mRNA into complementary DNA.

MATERIALS AND METHODS

Chemicals :

Deoxynucleoside-5'-triphosphates (dNTP), oligo dT_{12-18} were purchased from P.L. Biochemicals Inc. (USA); ^{3}H-labelled TTP (specific activity 30 ci/mM) and ^{3}H-dCTP (20 ci/mM) from Amersham (England); pancreatic RNA-A and DNA-A 1 from Worthington (USA); actinomycin D from Calbiochemicals (USA); DEAE-cellulose (De-52) and Whatman glass filters GF/C and GF/A from W.R. Balsorn (England); Tris (hydroxymethyl) aminomethane and dithiothreitol (DTT) from Sigma (USA); Sephadex G-150 from Pharmacia (Sweden). Rabbit globin mRNA was purchased from Bethesda Research Laboratory (USA) for comparison with our own preparations. All solutions in the following experiments were freshly made using deionized sterile distilled water.

Isolation and purification of rabbit globin mRNA :

The procedure for reticulocyte collection, mRNA isolation, and purification has been described elsewhere (Warmus and Swanstrom, 1982).

Egg and oocyte crude extract :

3000 carp (Cyprino carpio L.) or goldfish eggs (Carassius auretus L.) at early cleavage, embryos with yolk sac, or oocytes were suspended in 10 ml of Tris buffer (10 mM Tris-HCl, pH 7.5, 60 mM KCl, and 1% Triton X-100). They were broken in a glass homogenizer and centrifuged at 10,000 RPM for 10 min. in a cold room (4°C). The supernatant, called crude extract, was dialyzed against Tris buffer (10 mM Tris-HCl, pH 7.5, 60 mM KCl, 0.1 mM Mg^{++} acetate, and 10 mM DTT (Buffer A). After dialysis, the protein content was estimated by the Biuret reaction (Gornal et al., 1949). Ammonium sulphate was added (w/v, 17 %) to precipitate ribosomal materials. The precipitate was pelleted by centrifugation at 10,000 RPM for 5 min. and discarded. The protein in the supernatant was precipitated by ammonium sulphate (70%), collected by centrifugation, and dialyzed against Tris buffer (10 mM Tris-HCl, pH 7.5, 60 mM KCl and 0.1 mM Mg^{++} acetate) (Buffer B). A batch of the crude extract was used for isolation of total RNA by the classical phenol and chloroform method (Niu et al., 1981).

Column chromatography :

1. A DEAE cellulose column (42 x 1.7 cm) was equilibrated with Tris buffer B. The ammonium sulphate precipitated proteins were applied onto the DEAE column. A linear elution gradient with 100 ml Tris buffer (10 mM Tris-HCl, pH 7.5) and 100 ml Tris buffer containing 0.7 M KCl (pH 7.5) was used. Fractions of 2.5 ml were collected. The enzymatic activity of every other fraction was tested. The most active enzyme was found in the last 20 fractions. All active fractions were pooled, precipitated by ammonium sulfate (70%), and dialyzed.

2. A Sephadex G-150 column (42 x 1.7 cm) was equilibrated with Tris buffer (B). The active enzyme from DEAE column was used for fractionation on the Sephadex column. Each eluted fraction was tested for RT activity using rabbit globin mRNA as a template. All fractions with enzymatic activity were pooled and precipitated by ammonium sulphate. The precipitate was collected by centrifugation and dialyzed against the same buffer overnight at 4°C.

Polyacrylamide gel electrophoresis :

The above partially purified proteins were electrophoresed on a polyacrylamide gel (Southern,

1975).

Enzyme assay :

The reaction mixture (150 µl) contained 25 µM Tris-HCl, pH 7.7, 2 µM $MgCl_2$, 0.6 nM each dATP, dCTP, dGTP, and 0.6 nM ^{3}H-TTP (100,000 CPM), 0.2-0.5 µg rabbit globin mRNA, 2 µg Fe^{+++} (freshly prepared solution, pH 6.5), 0.1 µg oligo dT_{12-18}, and 5-50 µg enzyme (protein). After incubation at 37°C for 10 min., the reaction was stopped by adding an equal volume of cold trichloroacetic acid (10% TCA) containing 0.02 M pyrophosphate . The precipitate was washed on Whatman glass filters GF/A or GF/C with 5% TCA plus 0.02 M pyrophosphate, and 95% ethanol and dried. The radioactivity was measured in 5 ml of scintillation fluid with a Beckman 9000 scintillation counter. The results were expressed as CPM in TCA precipitable material.

Identification of the reaction product (cDNA) :

Quadruplicate samples of the reaction product were pooled on ice. The sample was adjusted with KOH to a concentration of 0.3 M, incubated at 37°C overnight or 60°C for an hour, and then neutralized. The cDNA was extracted twice with a mixture of phenol and chloroform (1 : 1) and once with chloroform (Niu et al., 1981). Potassium acetate was added (2%) to the supernatant, it was stirred and then 2 1/2 volumes of cold 95% alcohol was added. The precipitate was collected by centrifugation and electrophoresed on agarose (1%). Only one of the bands, about 450 nt, was distinct. It was eluted, re-electrophoresed, and transfered to a nitrocellulose membrane. A full lenght globin B_1 cDNA plasmid ($pRcB_1$) was a gift from Prof. Thomas Maniatis of Harvard University. This plasmid was nick translated (10^8 CPM/µg) for use as a probe. Molecular hybridation was done according to standard procedure (Southern, 1975). The same ^{32}p-$pRcB_1$ was used for the hybridization with a rat albumin cDNA clone, a gift from Dr. Thomas Sargent of the National Institute, Bethesda, MD.

RESULTS

Early results suggested the presence of RT activity from crude extracts of fish eggs. In this report, the crude extracts of carp and goldfish oocytes and eggs were partially purified by ammonium sulphate precipitation, DEAE cellulose column, and Sephadex column chromatography. Polyacrylamide gel electrophoresis was used to analyse these partially purified proteins. It can be seen in Fig.1 that the number of bands decreased in accordance with steps of purification.

The Sephadex purified fraction was incubated under optimal conditions for the RT activity assay using rabbit globin mRNA as template. The formation of ^{3}H labelled acid insoluble product required 4 dNTP and Mg^{++} (Table 1).

It was sensitive to pancreatic RNA-A and was not inhibited by actinomycin D. In the absence of Fe^{+++} (40 nM), the cDNA synthesized was only 1/5 of that with the complete reaction mixture.

In order to monitor the process of enzyme purification, RT activity from each step of purification was assayed with and without the primer (oligo dT_{12-18}) as well as with the primer plus Fe^{+++}. The results are given in Table 2.

Three points are clear, namely (i) using rabbit globin mRNA as template, crude extract exhibited low levels of enzyme activity as judged by the amount of ^{3}H-labelled product. Sequential purification (see column 1) increased RT specific activity approximately 80 times, (ii) as the steps of purification advanced, the need for the primer and for Fe^{+++} became apparent (see bottom row of Table 2), and (iii) optimal concentration of Fe^{+++} was 40 nM

(2 µg) per assay.

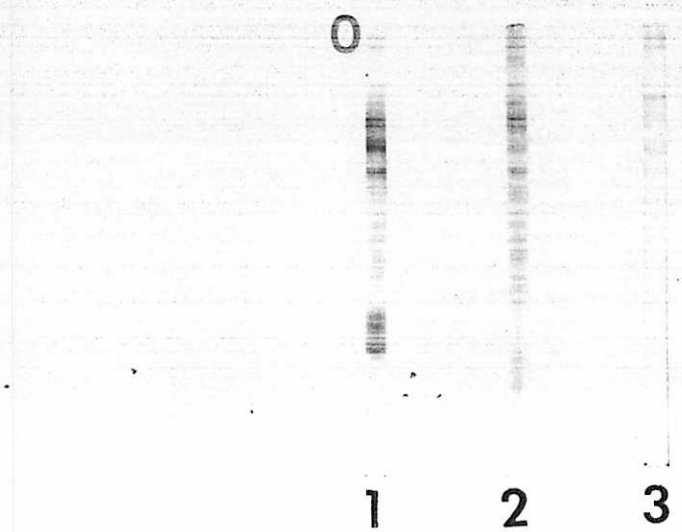

Figure 1

Polyacrylamide gel electrophoresis of the proteins from $(NH_4)SO_4$ precipitates (1: 30 µg), DEAE column (2: 25 µg), and Sephadex-150 (3: 20 µg). Note the reduction in number of the bands as the steps of purification advanced.

Table 1

Requierements for RT catalyzed c DNA synthesis

Reaction system	C.P.M. mean values	S.D.	% inhibition
Complete	4263 ±	239	0
- dCTP	1097 ±	94	74
- dATP	1325 ±	85	69
- dGTP	931 ±	136	78
- $MgCl_2$	2256 ±	115	47
- Fe^{3+}	749 ±	100	82
+ RNA-A (heated at 100°C 10 min., 10 µg)	1022 ±	89	75
+ Preincubeted mRNA whith RNA-A (same as above)	62 ±	8	100
+ Actinomycin D (10 µg)	4202 ±	174	0

Incubation conditions (see Materials and Methods) : 0.1 µg oligo (dT), 0.2 µg rabbit globin mRNA, and 10 µg Sephadex enzyme per assay. Similar results were obtained when ^{3}H-dCTP was used instead of ^{3}H-TTP. Above data were average of results of experiments performed at four different dates.

In figure 2, it can be seen that goldfish DNA is a poor substrate for the enzyme. In order to show that this goldfish DNA is functional, its activity as a template for bacterial DNA dependent DNA polymerase was tested. When goldfish DNA (0.6 µg) was used as template, bacterial DNA polymerase (Beljanski and Beljanski, 1974) catalyzed the synthesis of cDNA with 5500 CPM, while the Sephadex purified RT catalyzed cDNA with 833 (15% of the former).

Identification of the reaction product (DNA) :

Aliquots of the ^{3}H-labelled product were tested separately with pancreatic RNA-A, KOH and pancreatic DNA-A, 1 (Table 3).

Table 3

Chemical properties of the rabbit globin mRNA transcript from the in vitro synthesizing system.

Treatment	Acid precipitable CPM	% degradation
1. Untreated	2460	0
2. DNA-A	362	85
3. RNA-A	2406	0
4. Treated with KOH	2380	0

The volume of the reaction mixture was five times the size of that described in standard assay (see Materials and Methods). Aliquots (100 µl) were taken and ^{3}H-labelled product precipitated with 5% TCA solution containing 0.02 M pyrophosphate, then filtered and washed on millipore filters. Treatment with 20 µg of DNA-A (RNA-A free) : 1 ml Tris-HCl buffer 0.02 mM, pH 7.5 + Mg^{++}, 1hr. at 36°C. Treatment with 20 µg of RNA-A (preheated for 1hr. at 100°C) for 1hr. at 36°C. KOH : 0.1 N for 24 hrs at 36°C. The filters were washed with 5% TCA solution containing 0.02 M pyrophosphate, dried and radioactivity measured.

The results clearly indicate that the H-labelled acid-insoluble material produced was DNA transcribed with a rabbit globin mRNA template.

The cDNA was electrophorised and hybridized with ^{32}P-pRcB$_1$ (10^8 CPM/µg) and rat ^{32}P-albumin cDNA clone. The development of a band representing the hybridization between the cDNA and rabbit B_1 DNA, but no band appeared between cDNA and the rat albumin gene probe (Fig.3).

Table 2

Comparative activity of sequencially purified enzymes from carp eggs with and without the primer and ferric iron.

Experiments	P moles ^{3}H-TTP incoporated per 100 µg protein (10 minutes)				
Reaction systems / Enzymes recovered	No oligo	+ oligo dT_{12-18}	+ dT_{12-18} $+Fe^{+++}$ (1µg)	+ dT_{12-18} $+Fe^{+++}$ (2µg)	+ dT_{12-18} $+Fe^{+++}$ (5µg)
Ammonium sulfate fraction	3.0	4.0	9.0	18.0	25.0
DEAE column fraction	30.0	80.0	200	340.0	384.0
Sephadex column fraction	266.0	1660.0	3520.0	5700.0	6620.0

Incubation conditions (see Materials and Methods). Enzyme (µg) per assay : Ammonium sulfate fraction, 50 µg ; DEAE fraction , 8.3 µg ; Sephadex fraction, 1.3 µg.

In the presence of Sephadex purified enzyme the amount of ^{3}H-labelled product increased with the amount of rabbit globin mRNA (Fig.2) as well as with time (data not shown).

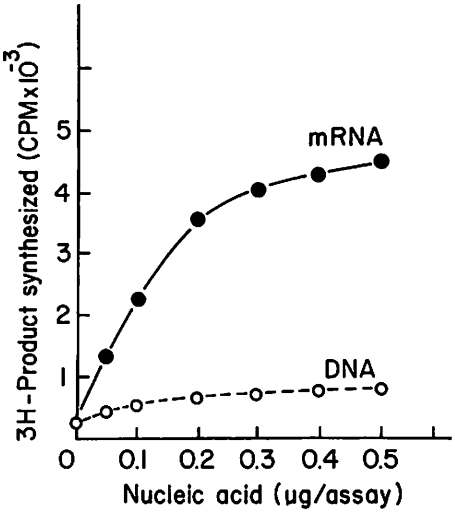

Figure 2

Concentration effect of rabbit globin mRNA and fish DNA on the incorporation of ^{3}H-TTP into acid insoluble materials (for incubation conditions, see Materials and Methods). Note the significant difference between the mRNA and fish DNA in templating the synthesis of cDNA.

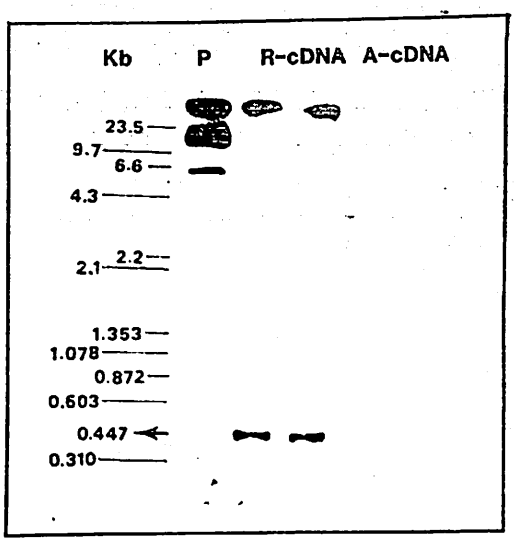

Figure 3

Autoradiogram of molecular hybridization (Southern, 1975) between the probe, ^{32}P-pRcB$_1$ (1 : 1.5 x 10^7 CPM in 10 ml medium) and the cDNA (0.6 µg) from (i) two *in vitro* globin mRNA reverse transcripts and (ii) rat albumine cDNA clone (for negative control). P, globin B$_1$ gene plasmid (pRcB$_1$) (for positive control); R-cDNA (the left lane is from Bethesda Research Laboratory's globin mRNA reverse transcript); A-cDNA is cloned rat albumin cDNA, a gift from Dr Thomas D.Sargent of the National Cancer Institute, Bethesda, MD. The bands at 447 base pairs of the experimental (i) represent complementary sequence between *in vitro* globin mRNA reverse transcript (globin cDNA) and the cloned globin gene of ^{32}P-pRcB$_1$. The numbers under Kb are where the marker bands are located.

DISCUSSION

Since the discovery of RT in retroviruses (Baltimore, 1970; Temin and Mizutani, 1970), the interest in reverse transcription has increased. Some groups have used extracts or partially purified enzymes for studies of the activity (Bauer and Temin, 1979; Kang and Temin, 1972; Mayer *et al.*, 1974; Mondal and Hofschneider, 1983; Weiss *et al.*, 1983, 1984), while others have isolated the enzyme and characterized the RT that utilized small RNA but not mRNA for DNA synthesis (Beljanski and Beljanski, 1974; Dutta *et al.*, 1977, 1980).

Recently, the transposition of movable genetic elements in *Drosophila melanogaster* (Shiba and Saigo, 1983) and *Saccharomyces* (Cameron *et al.*, 1979) appeared to involve RNA intermediates (Boeke *et al.*, 1985). In processed pseudogenes, there are segments which were copies of small cellular RNAs (Beljanski and Le Goff, 1986; Jelinek and Schmid, 1982). In contrast, the experiments reported here were done on RT of goldfish and carp oocytes and developing eggs using rabbit globin mRNA as a template. It should be noted that the amount of the 4 dNTP used in our experiments was less than that usually employed in cDNA synthesis. This was because the experiments using our partially purified egg enzyme had always given higher yield than those with higher concentration of the four dNTPs.

In vitro transcription of rabbit globin mRNA into cDNA was performed in a buffered reaction mixture containing four dNTP (one of them isotopically labelled), Mg^{++}, oligo dT_{12-18}, Fe^{+++}, globin mRNA, and Sephadex purified enzyme. The amount of ^{3}H-labelled product increased with increased concentration of globin mRNA. Conversely, if the time or mRNA was

held constant, the amount of reaction product (cDNA) varied in accordance with the amount of the enzyme. The sensitivity of the reaction to preincubation with RNA-A and the resistance of the reaction to actinomycin D are properties of reverse transcription. The product produced using a rabbit globin mRNA template was resistant to RNA-A and KOH, but degraded by DNA-A 1. These are characteristics of DNA. Hybridization of the in vitro synthesized cDNA (of 447 bp) from rabbit globin mRNA with ^{32}p-globin B_1 cDNA plasmid indicated that these two DNAs possessed complementary DNA sequences.

A further observation reported in this paper was that Fe^{+++} stimulated the synthesis of cDNA. Iron is present in eggs in general (Needham, 1935). We found that, during early embryogenesis, it stimulated the RT in fish eggs for the transcription of stored mRNA into cDNA. The effect of iron, although strongly manifested in partially purified enzyme, was slight in the crude enzyme extract. The demonstration of the iron-stimulated RT in fish eggs suggested the possibility that this RT might be capable of catalyzing mRNA for cDNA synthesis.

There are many types of stored mRNA in eggs, for exemple, histone (Gross and Arceci, 1981) tubulin (Raff et al., 1972), haemoglobin (Niu et al., 1981; Yu and Niu, 1981), albumin (Yu and Niu, 1981), etc.

Each of them may encode the synthesis of a specific cDNA. During early embryogenesis, each would be inserted into the genone, thus responsible for the development of cells or tissues of an organism. This train of thought leads to the hypothesis that different types of cDNA in cytoplasm are active agents for differential gene activation in embryogenesis. Our preliminary results are consitent with this hypothesis (Niu, 1981, 1985; Niu et al., 1981).

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