CHICK EMBRYO POLY (rA:dT)-DEPENDENT DNA POLYMERASE

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1. Introduction

It has been shown in several laboratories that RNA oncogenic viruses contain an RNA-dependent DNA polymerase activity (reverse transcriptase) [1, 2] associated with DNA-dependent polymerase and DNA ligase activities [3, 4]. According to Temin's DNA provirus hypothesis [5] this system may be involved in the replication of the RNA tumor viruses through a DNA intermediate. Similar enzymatic activities are found also in transformed mammalian cells [6, 7] and even in normal cells [7, 8].

RNA-dependent DNA polymerases from virions and transformed cells can use as template double-stranded homopolymers of the type (rA:rU) or (rA:dT) [9]. On the other hand, the enzyme from normal cells uses preferentially (rA:dT) polymer [7, 8]. This fact and other more recent results suggest that the enzymes prepared from these two kinds of materials are different.

The hypothetical role of these enzymes in gene amplification during cell differentiation and the possibility that the virion enzyme might be of cellular origin led us to investigate whether reverse transcriptase activity is also present in embryonic tissues.

A poly (rA:dT)-dependent DNA polymerase has been characterized and purified about 1000 times from the heart of chick embryos. This enzyme specifically copies the poly A strand of the (rA:dT) hybrid and very weakly poly A alone. The enzyme is distinct from at least two DNA-dependent DNA polymerases also present in tissue extracts.

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2. Materials and methods

2.1. *Materials*

Polythymidylic acid was purchased from Biopolymers Laboratory; all other polynucleotides were from Miles Laboratory Inc. ^{3}H-TTP (18.7 Ci/mmole) was purchased from New England Nuclear, ^{3}H-dATP (5.6 Ci/mmole) from Schwartz Bioresearch Inc. and ^{3}H-dGTP (4 Ci/mmole) from the Radiochemical Centre, Amersham. Nonidet P 40 was a gift from the Shell Company. Denatured DNA was prepared by heating calf thymus DNA at 100° for 10 min followed by immediate cooling to 0°. The polymers (rA:dT), (rA:rU) and (rI:rC) were prepared according to Spiegelman [9]. QβRNA was kindly supplied by Dr. D. Kolakofsky.

2.2. *Methods*

The hearts of 200 sixteen-day old embryos were washed in saline and homogenized in a Potter in the presence of 0.05 M Tris-HCl (pH 7.5), 5 mM $MgCl_2$ and 5 mM β-mercaptoethanol. The homogenate was sonicated for 60 sec at 4° using the MSE sonicator and then centrifuged 20 min at 3000 *g*. The precipitate was incubated 15 min at 37° in the presence of 0.4 percent Nonidet P 40. After centrifugation the supernatant was combined to that obtained from the 3000 *g* centrifugation and the mixture was centrifuged at 105,000 *g* for one hour. The material in the supernatant, precipitating between 33% and 70% ammonium sulfate, was dialyzed and chromatographed on a DEAE cellulose column equilibrated with 0.05 M Tris-HCl pH 7.9, 0.1 mM EDTA, 1 mM β-mercaptoethanol and 20% glycerol (v/v) (buffer A)

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Table 1
Purification of (rA:dT)-dependent DNA polymerase.

Step	Protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield percent	Ratio: (rA:dT)-dependent / (DNA)-dependent
Crude extract:	70,000	6,650	0.95	100	0.18
Ammonium sulfate fraction: 33–70%	1,400	1,540	1.1	23	0.13
DEAE cellulose fractions: 0.15 M KCl	45	1,305	29	19	0.11
Phosphocellulose fractions: 0.55 M KCl	0.4	378	947	5	2.20

using stepwise elution with 0.15-0.25 M KCl. The fractions eluting at 0.15 M KCl were collected, precipitated with 70% ammonium sulfate and rechromatographed on a phosphocellulose column prepared as indicated by Burgess [10]. The column was washed with buffer A containing 0.25 M KCl and then a gradient of 0.25–0.8 M KCl was applied (total volume 80 ml). The fractions containing the bulk of the activity and eluting approximately at 0.55 M KCl were pooled, concentrated 5 times by vacuum dialysis and stored at 4° in the presence of 20% glycerol. Protein concentration was determined by the method of Lowry et al. [11].

For the determination of enzymatic activities all reaction mixtures contained in 0.1 ml: 5 μmoles Tris-HCl (pH 7.9), 0.02 μmole dithiothreitol, 1 μCi either of ^{3}H-TTP, ^{3}H-dATP or ^{3}H-dGTP and enzyme as indicated. Reactions with synthetic polymers as templates contained 0.05 μmole $MnCl_2$, 7.5 μmoles KCl, 0.5 μg polymer unless otherwise stated; those with DNA as template contained 0.5 μmole $MgCl_2$, 2 μg denatured DNA and 0.05 μmole each of unlabelled dATP, dGTP and dCTP if ^{3}H-TTP for example was used. The same amounts of the unlabelled triphosphates were added when RNA served as template; in this case all other conditions were identical to those used for synthetic polymers. The reaction was stopped after addition of 50 μg of yeast RNA and one volume of 0.1 M sodium-pyrophosphate by the addition of two volumes of 20% trichloroacetic acid (TCA). After 10 min at 0° the TCA precipitate was filtered over a Millipore filter and its radioactivity was determined.

One unit of enzyme activity was defined as the number of pmoles of TMP recovered in the TCA-precipitable material per mg of protein after an incubation of 1 hr at 37°.

3. Results

3.1. *Purification of poly (rA:dT)-dependent DNA polymerase*

Specific activities of the poly (rA:dT)-dependent DNA polymerase found in different fractions during the purification are summarized in table 1. The enzyme has been purified 1000 fold with a total yield of 5 percent. Indications have been obtained that in the heart tissue extracts there are also at least two DNA-dependent DNA polymerase activities which can be separated on DEAE cellulose column by being eluted at 0.15 M KCl and at 0.25 M KCl respectively. They can furthermore be distinguished by their differential behavior in the presence of potassium ions (unpublished results).

Most of the DNA-dependent activities were separated from the poly (rA:dT)-dependent activity after DEAE cellulose and phosphocellulose chromatography. The purified (rA:dT)-dependent fractions recovered after the phosphocellulose step still contained DNA-dependent activity. However, filtration on Sephadex G-100 of the DEAE cellulose fraction eluted at 0.15 M KCl leads to complete separation of these two activities (fig. 1).

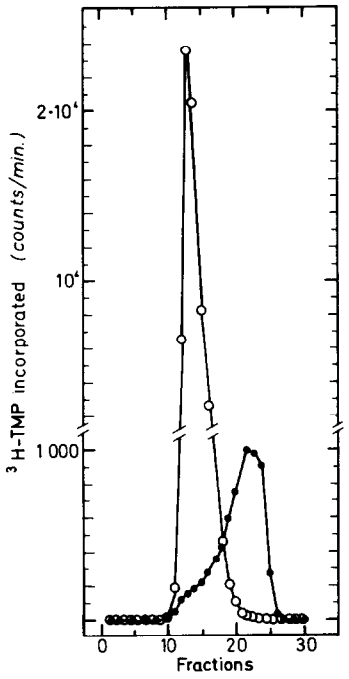

Fig. 1. Chromatography of chick embryo DNA polymerases on Sephadex G-100. A 2-ml sample of the peak eluting at 0.15 M KCl from DEAE cellulose chromatography was layered over a Sephadex G-100 column (2 × 62 cm) previously equilibrated with buffer A containing 0.2 M KCl. Fractions (2.4 ml) were collected and 25 μl aliquots were assayed for DNA and poly (rA:dT)-dependent DNA polymerase activity as described in Methods. •——• poly (rA:dT)-dependent polymerase; ○——○ DNA-dependent polymerase.

3.2. *Molecular weight*

The molecular weight of the poly (rA:dT)-dependent DNA polymerase was determined using the Sephadex gel filtration technique and various proteins as standards. The value of approximately 50,000 daltons was obtained for the polymerase (fig. 2).

3.3. *Template specificity*

The results reported in table 2 show that in the conditions used, only the poly (rA:dT) hybrid is effective in promoting polymerisation and that the enzyme copies exclusively the the rA strand. A weak activity is observed in the presence of poly A and none in the presence of several other synthetic polymers and physiological RNAs tested. The results also show that Mn^{2+} but not Mg^{2+} is effective with (rA:dT).

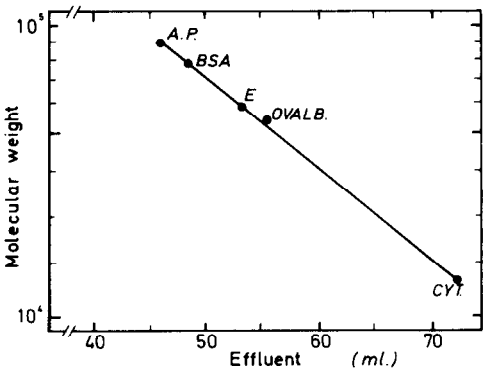

Fig. 2. Molecular weight determination of chick embryo poly (rA:dT)-dependent DNA polymerase by gel filtration. All experiments were carried out using the same column (2 × 62 cm) packed with Sephadex G-100 (medium) and equilibrated with buffer A plus 0.2 M KCl. The void volume was 32 ml. The column was calibrated with blue dextran (MW 2×10^6), alkaline phosphatase (MW 80,000), bovine serum albumin (MW 67,500), ovalbumin (MW 45,000) and horse cytochrome *c* (MW 13,400). All samples had been dialyzed against buffer A containing 0.2 M KCl before being applied to the column; 2.4 ml fractions were collected. Poly (rA:dT)-dependent DNA polymerase activity was assayed as described in fig. 1.

Both Mn^{2+} and Mg^{2+} are ineffective with all other polymers tested (not shown in the table).

3.4. *Effect of enzyme concentration and time dependence*

It can be seen in fig. 3 that the polymerisation of TMP in the presence of (rA:dT) is dependent on enzyme concentration and that the rate of polymerisation remains constant for at least two hours when low enzyme concentrations are used.

3.5. *Effect of poly (rA:dT) and of poly A concentration*

From the data presented in fig. 4 it follows that already at a concentration of 0.025 μg of (rA:dT) per 0.1 ml of incubation mixture the polymerisation is about 50 percent of that observed in the presence of 1 μg of polymer. This shows that the enzyme has a very high affinity for (rA:dT). Poly A alone is also used by the enzyme but with much lower efficiency.

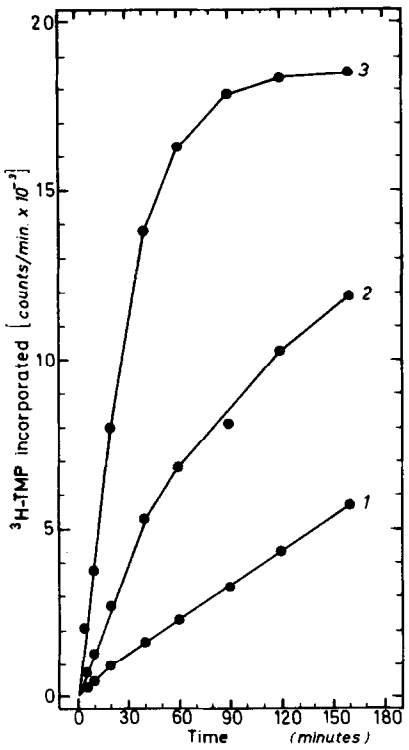

Fig. 3. Poly (rA:dT)-dependent DNA polymerase activity; enzyme concentration and time dependence. The incubations were performed for times indicated, and the conditions described under Methods using the following amounts of phosphocellulose fraction: 0.5 μg (curve 1), 1 μg (curve 2) and 2 μg (curve 3).

4. Discussion

The results reported in the present paper demonstrate the existence in chick embryo heart of an enzyme which polymerizes TMP in the presence of poly (rA:dT). Similar activity has also been observed in chick embryo liver and will be reported later.

The enzyme has been separated from at least two DNA-dependent DNA polymerase activities also present in tissue extracts. This suggests that the DNA- and (rA:dT)-dependent DNA polymerase activities correspond to different enzymes.

The characteristics of the (rA:dT)-dependent DNA polymerase are distinct from those of the enzymes isolated from RNA viruses. The enzyme described here does not respond to the presence of poly (rA:rU)

Table 2
Template specificity.

	Template (0.5 μg)	cpm Incorporated		
		dTMP	dAMP	dGMP
1	No template added	35		
2	Poly (rA:dT)	5,600	40	
3	Poly (rA:dT) $-Mn^{2+} + Mg^{2+}$	150		
4	Poly T	38		
5	Poly A	180		
6	Poly U		40	
7	Poly AG	48		
8	Poly (rA:rU)	75		
9	Poly (rI:rC)			80
10	*E. coli* 16 S RNA	80		
11	Rat liver RNA	82		
12	Qβ RNA	85		
13	Chick embryo RNA	70		

In each experiment 0.5 μg of the phosphocellulose enzyme was used. The conditions are as described in Methods. Rat liver and chick embryo RNAs were extensively treated with pancreatic DNase which was then removed by phenol extraction. These RNAs were used after ethanol precipitation and dialysis. Except in 3 each incubation mixture contained 0.5 μmole $MnCl_2$.

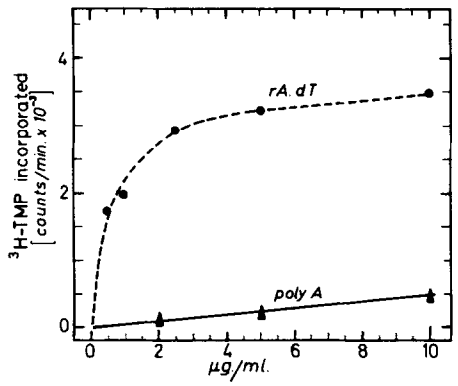

Fig. 4. Effect of poly (rA:dT) or poly A concentration. The standard reaction mixtures were as indicated in Methods, except that the concentrations of polymers were as given in the figure. Each assay tube contained 0.5 μg of phosphocellulose fraction.

whereas viral enzymes are active with this polymer. Moreover, its molecular weight is different from that of enzymes isolated from viruses [8].

The specificity of chick embryo enzyme for (rA:dT) polymer could indicate that in physiological conditions it is able to transcribe the RNA strand from (RNA:DNA) double-stranded hybrids. Some preliminary indications which are in favor of this hypothesis are being obtained in our laboratory. However, we do not know at the present time if the enzyme that has been isolated is a true "reverse transcriptase" or is a part of another enzyme which possibly uses only DNA in physiological conditions. Further experiments now in progress should give an answer to this question.

Acknowledgements

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