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# DNA POLYMERASE ACTIVITIES IN BRAIN OF CHICK EMBRYO

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## 1. Introduction

The discovery of an RNA-instructed DNA polymerase in the virions of two oncogenic viruses [1, 2] was rapidly confirmed and extended to other RNA oncogenic particles as well as to normal [3] and tumor-infected cells and tissues [4, 5].

The study of DNA polymerase activities was extended to embryonic material [6], and Maia et al. [7] have succeeded in purifying a poly rA:dT-dependent DNA polymerase from chick embryonic heart.

It seemed interesting to know whether DNA polymerase activities from different subcellular fractions isolated from an organ of developing chicken embryo, show particular preferences for synthetic templates. Recently, it has been shown [8] that compensatory hypertrophic rat liver contains a DNA polymerase which copies the deoxyribo strands of poly rA:dT and poly rG:dC. We report here results concerning DNA polymerase activities from mitochondrial and nuclear fractions from brain of 11-day old chick embryos. We have shown that two DNA polymerase activities can be distinguished in mitochondrial and nuclear fractions (poly dC:dG-dependent and poly dG:rC-dependent, both of them polymerizing $^{3}$H-dGMP).

## 2. Material and methods

### 2.1. *Brain material*

Brains from 11-day old chick embryos (100–150 embryos) were removed into a beaker which was packed in ice and contained Tyrode's physiological medium (130 mM NaCl, 2 mM KCl, 1 mM $CaCl_2$, 1 mM $MgCl_2$, 0.4 mM $Na_2HPO_4$, 5 mM glucose, solution adjusted with $NaHCO_3$ at pH 7.2).

### 2.2. *Mitochondrial fraction*

Mitochondria were obtained as previously described [9]. They were suspended in buffer (50 mM Tris-HCl pH 7.8, 50 mM KCl, 1 mM EDTA, 1 mM dithiothreitol). Triton X100 was added to a final concentration of 0.4 per cent (v/v) [4]. The extract was incubated at 37° for 15 min to release mitochondrial-bound enzymes and centrifuged at 15,000 *g* for 15 min. After precipitation of the supernatant with 66 per cent (w/v) ammonium sulphate the mixture was centrifuged at 30,000 *g* for 20 min and the precipitate was suspended in a small volume of buffer, dialyzed against 400 volumes of this buffer and then stored at –20° in the presence of 50% (v/v) of glycerol, until used for the enzyme assays. Under such conditions of storage enzymes remained active for at least 4 months. Concentration of proteins was determined by the method of Lowry et al. [10].

### 2.3. *Nuclear fraction*

Nuclei were isolated according to Maggio et al. [11]. In order to solubilize nuclear enzymes nuclei were treated in exactly the same way as mitochondria.

### 2.4. *Templates and substrates*

Calf thymus DNA (Calbiochem) was "activated" by a partial hydrolysis with crystalline pancreatic DNAse (Worthington) as previously described [12].

Deoxycytidine 5′-triphosphate (5-$^{3}$H), deoxy-

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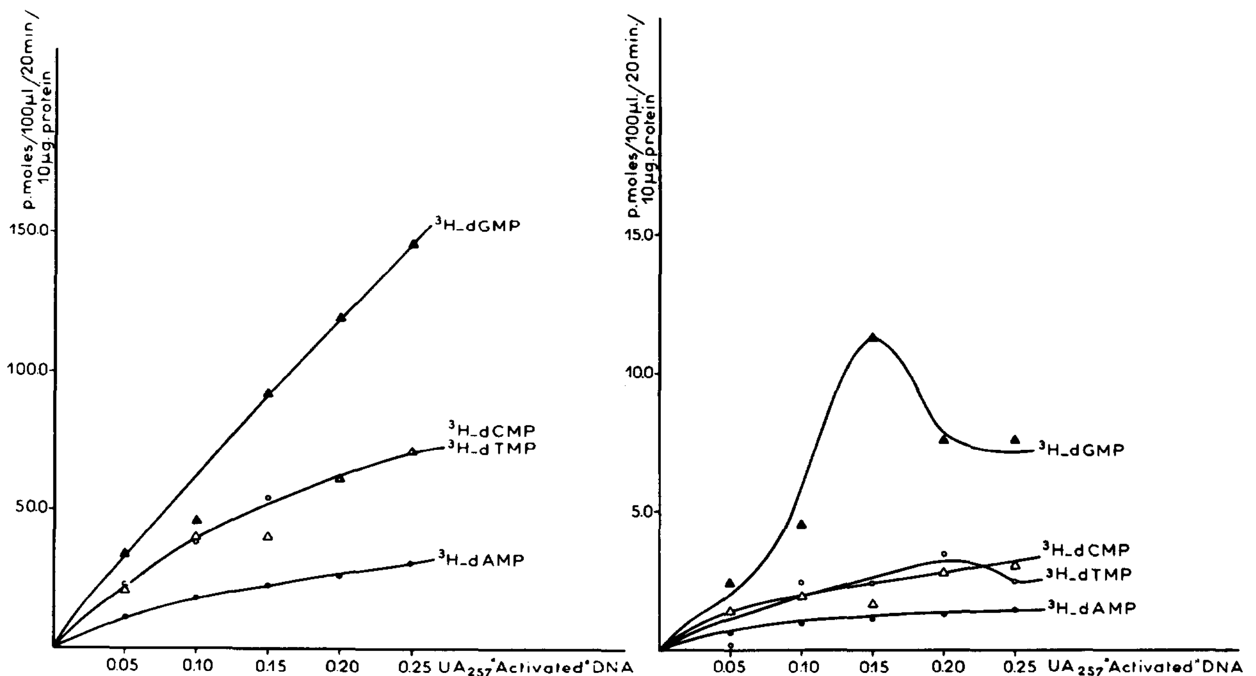


Figs. 1 and 2. "Activated" DNA-dependent DNA polymerase of nuclear (fig. 1) and mitochondrial (fig. 2) fractions. Stock solution of "activated" calf thymus DNA contains 13.72 $A_{257}$ units. $Mg^{2+}$ (2 mM) was used in the reaction mixture. For other details see Material and methods.

adenosine 5'-triphosphate (8-$^3$H), and thymidine 5'-triphosphate (methyl-$^3$H) were purchased from Schwarz-Mann, deoxyguanosine 5'-triphosphate (8-$^3$H) from Amersham, unlabeled deoxynucleoside triphosphates and polydeoxynucleotides (poly dA, poly T, poly dC, poly dG and poly dI:dC) from General Biochemicals, polyribonucleotides (poly A, poly U, poly I, poly C and poly G) from Biopolymers Inc.

Heteropolymers (poly dT:rA, poly dA:rU, poly dC:rG, poly dG:rC, poly dC:rI, poly dC:dG, poly rC:rG and poly rC:rI) were prepared by mixing equal amounts of each single-stranded homopolymer as by Spiegelman et al. [13].

### 2.5. *Standard DNA polymerase assay*

Assays for the RNA-instructed and DNA-instructed DNA polymerase activities, were performed in a 100 µl incubation mixture containing: 40 mM Tris-HCl pH 8.3, 60 mM KCl, 2 mM dithiothreitol, 300 nM each unlabeled deoxynucleoside triphosphates, either 2 mM $MgCl_2$ or 0.1 mM $MnCl_2$, one of the following $^3$H-deoxynucleoside triphosphates: $^3$H-TTP, $2.9 \times 10^{-9}$ M (specific activity 178 cpm/pmole); $^3$H-dATP, $3.2 \times 10^{-9}$ M (specific activity 165 cpm/pmole); $^3$H-dCTP, $2.0 \times 10^{-9}$ M (specific activity 226 cpm/pmole); $^3$H-dGTP, $16.5 \times 10^{-9}$ M (specific activity 27 cpm/pmole); enzyme protein 5–10 µg, synthetic and natural templates at 0.14 $A_{257}$ units, unless otherwise stated. After incubation for 20 min at 37°, 20 µl aliquots were withdrawn and processed for determination of acid-precipitable radioactivity as described previously [14].

## 3. Results

### 3.1. *Natural templates*

"Activated" calf thymus DNA was used as primer and template because it was 10–20 times more active than the corresponding untreated native DNA, using either nuclear or mitochondrial fractions as source of

Table 1
DNA-dependent-DNA polymerase activities.

| Template | Substrate | Mitochondrial fraction | Nuclear fraction |
|---|---|---|---|
| dT:rA | $^3$H-dATP | 0.63 | 4.15 |
| dA:rU | $^3$H-dTTP | 0.20 | 1.60 |
| dC:rG | $^3$H-dGTP | 46.44 | 149.93 |
| dC:rI | $^3$H-dGTP | 87.44 | 169.17 |
| dC:dI | $^3$H-dGTP | 112.55 | 225.14 |
| dC:dG | $^3$H-dGTP | 188.07 | 243.07 |
| dG:rC | $^3$H-dCTP | 0.15 | 3.56 |
| dC | $^3$H-dGTP | 7.90 | 34.13 |
| dG | $^3$H-dCTP | 0.20 | 3.27 |
| | $^3$H-dTTP | 2.51 | 54.77 |
| | $^3$H-dATP | 1.35 | 22.70 |
| "Activated" DNA | $^3$H-dCTP | 1.20 | 38.53 |
| | $^3$H-dGTP | 12.43 | 92.50 |

Table 2
RNA-dependent-DNA polymerase activities.

| Template | Substrate | Mitochondrial fraction | Nuclear fraction |
|---|---|---|---|
| dT:rA | $^3$H-dTTP | 0.03 | 0.20 |
| dA:rU | $^3$H-dATP | 0.02 | 0.31 |
| dG:rC | $^3$H-dGTP | 2.01 | 163.66 |
| rG:rC | $^3$H-dGTP | 0.03 | 7.20 |
| rC | $^3$H-dGTP | 0.00 | 0.01 |
| dC:rG | $^3$H-dCTP | 0.46 | 2.75 |
| dC:rI | $^3$H-dCTP | 0.34 | 0.67 |
| rC:rI | $^3$H-dCTP | 0.02 | 5.40 |
| | $^3$H-dTTP | 0.58 | 6.24 |
| | $^3$H-dATP | 0.21 | 3.50 |
| Skin RNA | $^3$H-dCTP | 0.20 | 6.45 |
| | $^3$H-dGTP | 0.32 | 10.00 |

Tables 1 and 2. Polymerization is expressed as pmoles $^3$H-dNTP/100 $\mu$l/20 min/10 $\mu$g protein/0.14 $A_{257}$ units template. In table 2 activities of mitochondrial and nuclear enzymes using skin RNA were calculated from the relationship 1 $\mu$g RNA = 0.025 $A_{257}$ units.

enzyme. Figs. 1 and 2 show the kinetics of enzyme activity with nuclear and mitochondrial fractions using "activated" DNA and the four labeled deoxynucleoside triphosphates. It is observed that $^3$H-dCMP and $^3$H-TMP are polymerized at the same rate with both enzymes, whereas the incorporation of $^3$H-dAMP and $^3$H-dGMP is quite different. However, it is interesting to point out that the nuclear enzyme is 8–30 times more active than the mitochondrial one in catalyzing the polymerization of the four substrates (table 1).

To check RNA-directed DNA polymerase activities, purified RNA from chick embryonic skin was used. Fig. 3 shows different levels of polymerization of the four deoxyribonucleoside triphosphates used, and a similar pattern to "activated" DNA-dependent nuclear enzymic fraction is to be seen. The mitochondrial fraction (not shown) shows very low levels of incorporation (table 2).

### 3.2. *Synthetic templates*

Several synthetic templates were tested as is shown in tables 1 and 2. One can see (table 1) that the transcription of the deoxythymidylate and the deoxyadenylate strands of poly dT:rA and poly dA:rU is very poor with both mitochondrial and nuclear enzymes. On the other hand, mitochondrial and nuclear enzymes catalyse very efficiently the polymerization of dGMP when either poly dC:rG, poly dC:rI, poly dC:dI or poly dC:dG are used as templates.

With regard to RNA-dependent DNA polymerase activities (table 2) it is shown that nuclear and mitochondrial fractions have a hybrid-dependent DNA polymerase which is directed by the ribocytidylate strand of poly dG:rC. A moderate polymerization of dGMP with poly rC:rG is also to be observed when nuclear extract is used to catalyse the reaction. Poly rC is not at all a template for the reaction.

Very low levels of dTMP polymerization (with poly dT:rA) and dAMP polymerization (with poly dA:rU) are observed with these nuclear and mitochondrial enzymic preparations.

Fig. 4 shows the kinetics of dGMP incorporation directed by poly dC:rG as a function of the time of incubation and using either $Mg^{2+}$ or $Mn^{2+}$ to activate the binding of the enzyme to the template, when using the mitochondrial fraction. With 2 mM $Mg^{2+}$ the optimal activity is reached after 20 min. With longer incubation times the uptake drops, probably owing to the presence of traces of a nuclease acting on the polymerized product [6].

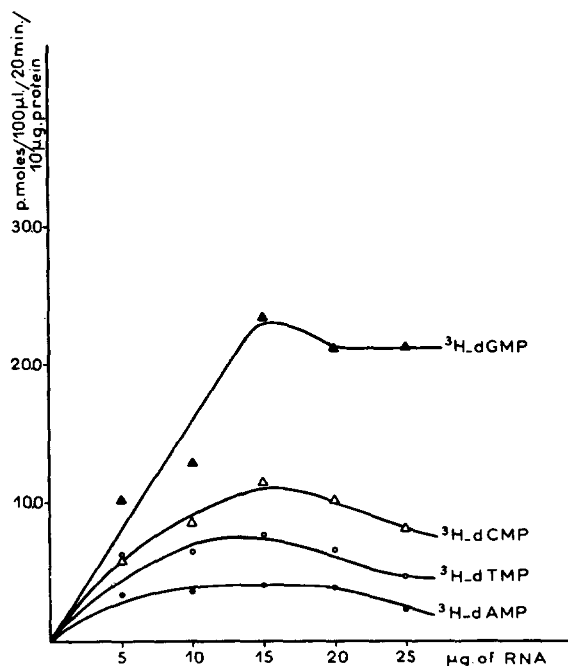


Fig. 3. RNA-dependent DNA polymerase of nuclear fraction RNA from 11-day chick embryo skin was extracted 3 times with hot phenol (55°) in 10 mM Tris-HCl pH 7.4 and 0.5% (v/v) sodium dodecylsulphate. RNA sample was made 10 mM with respect to Tris-HCl pH 7.2, 3 mM with respect to $MgCl_2$ and incubated with 5 µg/ml of deoxyribonuclease I (Sigma) for 20 min at 30°. The mixture was extracted twice with phenol, and the aqueous phase, after 2 re-extractions with chloroform-amyl alcohol (6:1 v/v), was made 1% (v/v) with respect to NaCl. 2½ volumes of cold ethanol was added, and the mixture was allowed to stand for 3–4 hr at −20°. After centrifugation, the pellet was dissolved in 50 mM NaCl containing 1% (w/v) of CTA (cetyltrimethyl ammonium bromide, Sigma). After washing 3 times with 70% alcohol containing 100 mM NaCl, the pellet was dissolved in a small volume of cold water and stored at −20° until use. Standard assay conditions were as described in Material and methods; $Mn^{2+}$ (0.1 mM) was used in the reaction mixture.

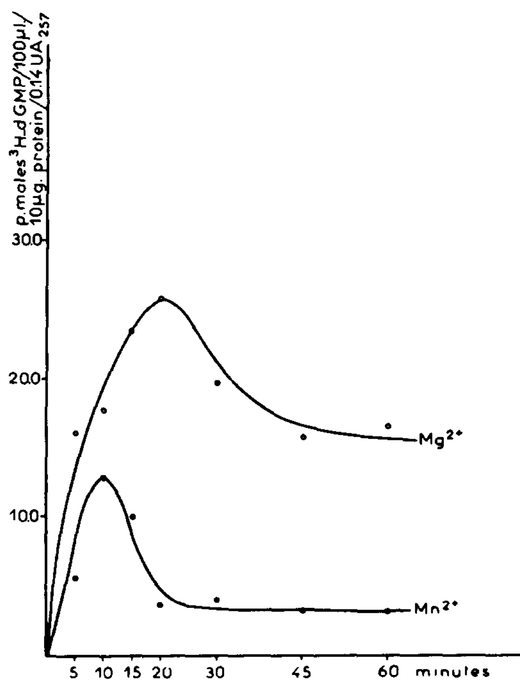


Fig. 4. Poly dC:rG-dependent DNA polymerase of mitochondrial fraction as a function of time of incubation using either $Mg^{2+}$ (2 mM) or $Mn^{2+}$ (0.1 mM) as divalent cations. Template was added at a concentration of 0.14 $A_{257}$ units.

## 4. Discussion

It has been shown that mitochondrial and nuclear preparations are able to catalyse the polymerization of dGMP when synthetic hybrids and DNA duplexes (poly dC:rG, poly dC:rI, poly dC:dI, poly dC:dG and poly dG:rC) are used as primers and templates, but not the polymerization of TMP or dAMP when poly dT:rA or poly dA:rU, respectively, are used as templates. A similar situation was observed with preparations of enzyme from avian myeloblastosis virus [13] and with enzyme from visna virus [15], which both show a striking preference for dGTP as substrate. This recalls an analogous situation observed with QB replicase [16] and DNA-dependent RNA polymerase [17] both of which synthesize poly G if provided with poly C as template.

Like DNA polymerases from chick embryo [6], DNA polymerases from embryonic brain prefer as templates synthetic hybrids and DNA duplexes, which are clearly superior by several orders of magnitude to either the embryonic skin or "activated" calf thymus DNA.

It is known that DNA polymerases are apparently unable to initiate DNA synthesis "de novo" on a single stranded template, but require a primer containing a free 3'-OH [18–20]. The ability of poly dG:rC to act as a template for mitochondrial and nuclear DNA polymerases while poly rC alone is com-

pletely inactive (table 2), suggests that these enzymes may also need a free 3′-OH terminus on which to initiate synthesis. The poly dG in poly dG:rC would then be necessary in order to provide a 3′-OH. It seems also to be the case for poly dC:rG when enzymes catalyze the incorporation of dGMP; here 3′-OH would be provided by poly rG, since the polymerization of dGMP is significantly diminished when poly dC alone is used as template.

One can conclude that:

1) RNA-dependent and DNA-dependent DNA polymerases from chick embryonic brain are several times more active in the nuclear fraction than in the mitochondrial one, when using natural or synthetic templates.

2) The enzyme (or enzymes) copies the deoxyribocytidylate of synthetic templates (e.g. poly dC:dG) much more efficiently than the ribocytidylate of poly dG:rC.

3) The enzyme(s) is unable to transcribe either the riboadenylate strand of poly dT:rA or the uridylate strand of poly dA:rU. This represents an important difference with respect to other embryonic systems [6, 7] in which TMP is polymerized when poly dT:rA is provided. It is possible that in our experiments isolation of enzymes from tissue organelles results in the activation or blocking of specific sites on the enzyme molecule concerned with the transcription of specific sequences.

4) It is not known what is the function of RNA-dependent DNA polymerase during embryonic development. It was recently suggested [21] that this enzyme plays a role in gene amplification during early oocyte maturation. However, the present, as well as earlier [6, 7] experiments show that the enzyme activity is present at all stages of embryonic development.

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