PRIMER-TEMPLATE SPECIFICITY OF DNA POLYMERASES BOUND TO MITOCHONDRIA AND NUCLEI

L. SORIANO and Y. CROISILLE

Institut d'Embryologie et Tétratologie expérimentales du CNRS, 49 bis, avenue de la Belle Gabrielle, 94130 Nogent-sur-Marne, France

Received 9 November 1972
Original illustrations received 5 March 1973

1. Introduction

Recently DNA polymerases of eukaryotes have been the subject of intensive investigation as a consequence of the importance of research on RNA-dependent DNA polymerases in normal [1, 2] and in tumor cells and tissues [3–6]. Attention has also been focused on the activity of DNA polymerases in regenerating tissues [7] and in developing systems [8–11].

Margolis [12] has studied the distribution patterns of DNA-dependent DNA polymerase in different regions of brain of chick embryo, and one of us has shown [13] that mitochondria and nuclei of chick embryonic brain contain bound enzymes which catalyze the polymerization of dGMP when poly dG:poly dC or poly dG:poly rC are provided as primer templates.

In the present paper we report the characterization of partially purified poly dT:poly rA-dependent DNA polymerase (bound to nuclei) and oligo dT:poly rA-dependent DNA polymerase (bound to mitochondria), both of them polymerizing thymidylic acid. Moreover, we have studied poly dG:poly dC and poly dG:poly rC-dependent activities polymerizing deoxyguanylic acid, in both mitochondria and nuclei.

2. Materials and methods

2.1. *Preparation of the enzymes*

Cerebral hemispheres and optic lobes are removed from eleven-day old chick embryos as previously described [13]. Mitochondria which are isolated according to Chappell and Hansford [14] are suspended in buffer A (50 mM Tris-HCl pH 7.8, 250 mM KCl, 1 mM EDTA, 1 mM dithiothreitol) and Nonidet P40 (Shell) is added to a final concentration of 0.4% (v/v). The extract is incubated at 37° for 15 min and centrifuged at 15,000 *g* for 15 min. After precipitation of the supernatant with 66% (w/v) ammonium sulphate the mixture is centrifuged at 30,000 *g* for 20 min and the precipitate is suspended in a small volume of buffer A containing 20% of glycerol (v/v), dialyzed against 400 vol of this buffer and then stored at −20° in the presence of 50% of glycerol (v/v) until use for enzyme purification.

Nuclei are isolated according to Maggio et al. [15]. They are suspended in buffer A and treated with detergent and ammonium sulphate in exactly the same way as mitochondria.

2.2. *Ion-exchange chromatography*

Crude extracts are dialyzed against 400 vol of buffer B (150 mM Tris-HCl pH 8.3, 50 mM KCl, 0.5 mM EDTA, 5 mM $MgCl_2$, 1 mM dithiothreitol, 5% glycerol, v/v). Then they are adsorbed on a column (10 mm × 260 mm, volume 20 ml) of DEAE-cellulose (exchange capacity 0.82 meq/g) which is equilibrated with buffer B. After washing with 6 column volumes of the equilibrating buffer, a linear gradient (400 ml 50–350 mM KCl) is applied. 10 ml fractions are collected at a flow rate of 15–20 ml/hr. All steps are performed at 5°.

Mitochondrial fraction I (fig. 1) and nuclear fraction I (fig. 2) eluted with the washing buffer are concentrated by ultrafiltration (Amicon membrane UM

20 E) and are adjusted to 50% (v/v) with glycerol and stored at −20° until use for enzyme assays. Mitochondrial fraction II (fig. 1) which is eluted 150–175 mM KCl on DEAE-cellulose is concentrated by ultrafiltration. After dialysis against 400 vol of buffer C (the same as buffer B except that it contains 150 mM KCl) it is applied to a column (10 mm × 260 mm, volume 20 ml) of phosphocellulose (exchange capacity 7.4 meq/g) which is equilibrated with buffer C. After washing with 6 column volumes of the equilibrating buffer, a linear gradient (400 ml 150–800 mM KCl is applied and 10 ml fractions are collected at a flow rate of 10–15 ml/hr. Fractions eluted with the washing buffer (mitochondrial fraction III) and eluted at 300–320 mM KCl (mitochondrial fraction IV) (fig. 3) are concentrated and stored at −20° in the presence of 50% (v/v) of glycerol. Concentrations of proteins are determined either by the method of Lowry et al. [16] or the absorbancy at 280 nm by the relationship 0.65/mg/ml [17].

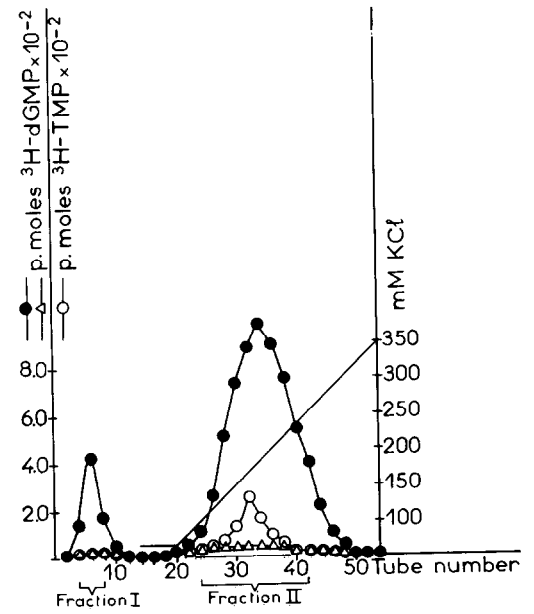

Fig. 1. DEAE-cellulose column chromatography of crude extract of mitochondrial fraction. 100 μl of each fraction are tested in a reaction mixture (150 μl) as described in Materials and methods, containing either 0.09 A_{260} units of poly dG:poly dC (●—●—●), 2 μg of oligo dT:poly rA (○—○—○) or 0.07 A_{252} units of poly dG:poly rC (△—△—△). Incubation time 20 min.

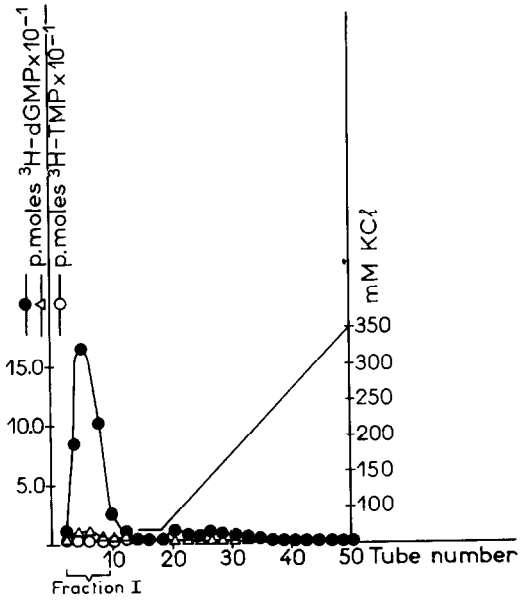

Fig. 2. DEAE-cellulose column chromatography of a crude extract of nuclear fraction. Conditions and symbols as in fig. 1.

2.3. *Homopolymer templates and substrates*

Poly dG is obtained from General Biochemicals, poly rC from Biopolymer, poly dG:poly dC from Miles Laboratories, poly dT:poly rA (molecular weight of each single strand 100,000), oligo dT:poly rA (oligo dT has a chain length of 12–18 nucleotides) are supplied by Collaborative Research. Poly dG:poly rC is prepared by mixing equimolar amounts of each single-stranded homopolymer as described previously by Spiegelman et al. [18]. [8-^{3}H]Deoxyguanosine 5′-triphosphate and [methyl-^{3}H]thymidine 5′-triphosphate are obtained from Radiochemical Centre, Amersham.

2.4. *DNA polymerase assays*

In a final volume of 100–150 μl the reaction mixture contains: 40 mM Tris-HCl pH 8.3, 60 mM KCl, 1 mM dithiothreitol, either 2 mM $MgCl_2$ (when poly dG:poly dC-dependent DNA polymerase activity is tested) or 1 mM $MnCl_2$ (when oligo dT:poly rA, poly dT:poly rA or poly dG:poly rC-dependent DNA polymerase activities are tested), one of the two [^{3}H]deoxynucleoside triphosphates: 2.9 nmoles [^{3}H]TTP (specific activity 178 cpm/pmole) or

16.5 nmoles of [^{3}H]dGTP (specific activity 27 cpm/pmole), one of the synthetic primer-templates and the enzyme fractions at the concentrations indicated in the legend of the figures. All incubations are at 37°. The time of incubation is indicated for each particular experiment. 20–30 μl aliquots are withdrawn and processed for determination of acid-precipitable radioactivity [19].

3. Results

3.1. *Chromatography of polymerases*

Fig. 1 shows that more than 80% of poly dG:poly dC-dependent activity and all detected oligo dT:poly rA-dependent activity of mitochondrial fraction are eluted at 150–175 mM KCl on DEAE-cellulose. Low levels of poly dG:poly rC-dependent activity are detected.

In the nuclear fraction (fig. 2) DEAE-cellulose chromatography shows that roughly 90% of poly dG:poly dC-dependent activity is not adsorbed by the column and is eluted with the washing buffer. Oligo dT:poly rA-dependent activity is not at all detected. As in the mitochondrial fraction, poly dG:poly rC-dependent activity is very low. Fig. 3 shows the elution profile of mitochondrial fraction II adsorbed on a phosphocellulose column. Two peaks are observed, each of them showing poly dG:poly dC-dependent and oligo dT:poly rA-dependent activities.

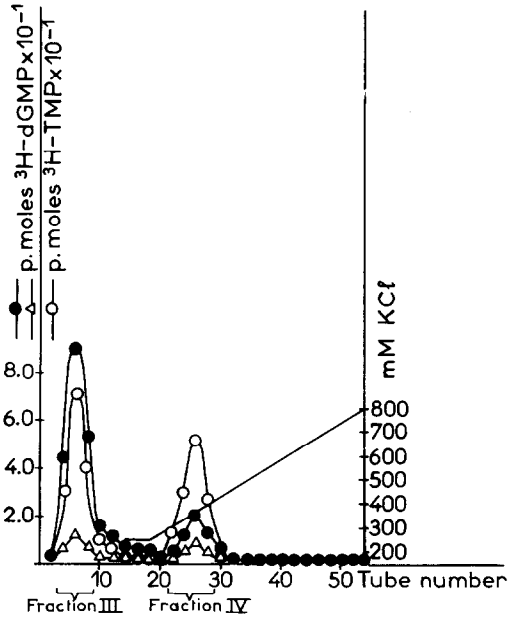

Fig. 3. Phosphocellulose column chromatography of mitochondrial fraction II (eluted at 150–175 mM KCl on DEAE-cellulose and processed as indicated in Materials and methods). Conditions and symbols as in fig. 1.

Table 1
Reaction mixture in 100 μl.

Enzyme	Proteines μg/test	Incubation Time minutes	oligo dT: poly rA μg	poly dT: poly rA A_{260} units	p. moles ^{3}H-TMP incorporated
Mitochondrial fraction IV	24	60	6	-	17.6
Nuclear fraction I	24	60	6	-	2.1
Mitochondrial fraction IV	24	60	-	0.32	0.3
Nuclear fraction I	24	60	-	0.32	908.0

Conditions as described in Materials and methods.

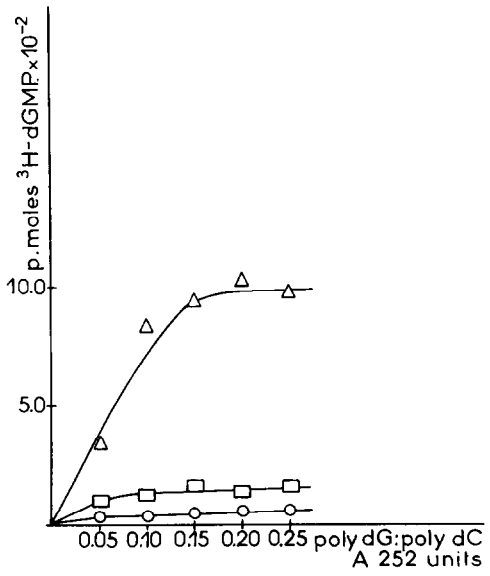

Fig. 4.

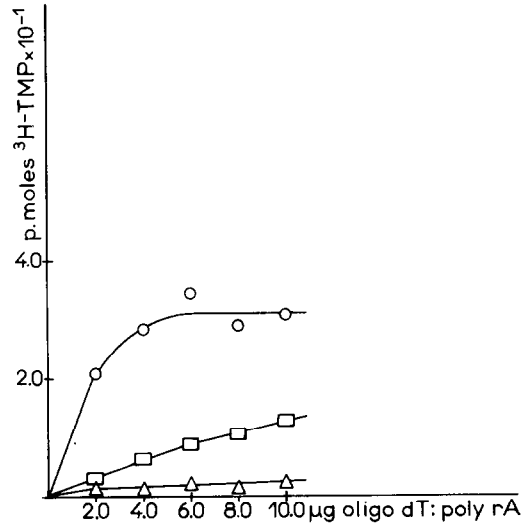

Fig. 5.

3.2. *Kinetics of enzyme activity*

The kinetics of poly dG:poly dC-dependent DNA polymerase as a function of template concentration (fig. 4) show that in nuclear fraction I (eluted with the washing buffer on DEAE-cellulose) this activity is about 10 times higher than in either mitochondrial fraction III (eluted with the washing buffer on phosphocellulose) or in mitochondrial fraction IV (eluted at 300–320 mM KCl on phosphocellulose). Fig. 5 shows that oligo dT:poly rA-dependent activity is present in mitochondrial fractions IV and III but not in nuclear fraction I. In contrast fig. 6 shows that poly dT:poly rA-dependent activity is particularly high in nuclear fraction I and it is barely present in mitochondrial fractions III and IV.

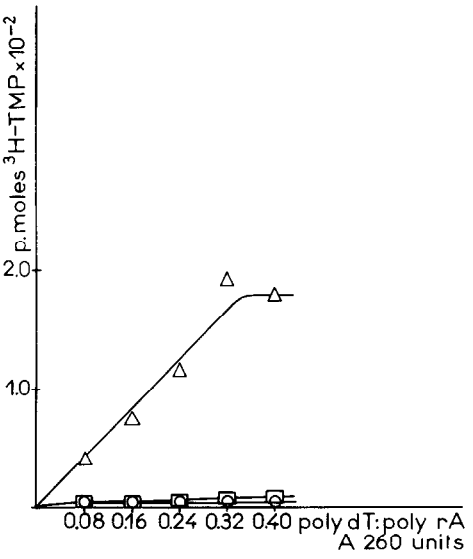

Fig. 4, 5 and 6. Kinetics of DNA polymerase activities as a function of template concentration. Assays are performed in 100 µl of reaction mixture as described in Materials and methods. They contain the indicated amounts of poly dG:poly dC (fig. 4), oligo dT:poly rA (fig. 5) and poly dT:poly rA (fig. 6), and 10 µg of each tested fraction: mitochondrial fraction III (□—□—□), mitochondrial fraction IV (○—○—○) and nuclear fraction I (△—△—△). Incubation time 20 min.

Fig. 7 shows the reciprocal influence of the two enzymes (nuclear fraction I and mitochondrial fraction IV) when one of them is incubated with increasing amounts of the other in the presence of the appropriate primer-template. With high concentrations of the mitochondrial fraction IV a clear inhibitory effect on the nuclear fraction I polymerizing activity can be observed. The reverse influence is less apparent.

4. Discussion

The different chromatographic behavior of nuclear and mitochondrial poly dG:poly dC-dependent DNA

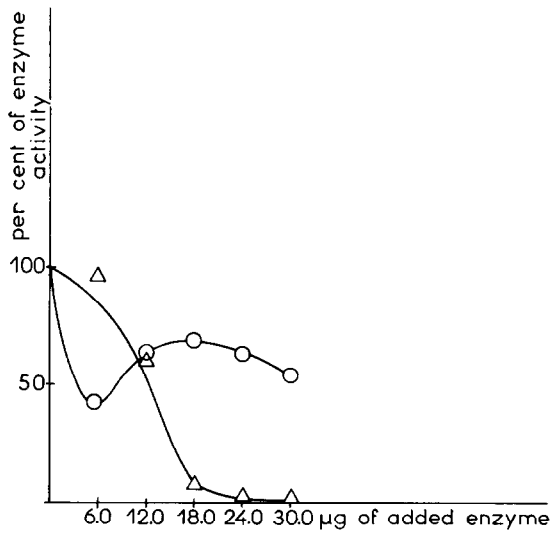

Fig. 7. Reciprocal influence of mitochondrial fraction IV and nuclear fraction I on the TMP polymerizing activities. (○—○—○) 150 µl of reaction mixture contains 6 µg of oligo dT:poly rA, 6 µg of mitochondrial fraction IV, and the indicated amounts of nuclear fraction I. Incubation time 60 min. (△—△—△) 150 µl of reaction mixture contains 0.24 A_{260} units of poly dT:poly rA, 6 µg of nuclear fraction I and the indicated amounts of mitochondrial fraction IV. Incubation time 20 min.

polymerases on DEAE-cellulose, seems to indicate that two different molecular entities catalyzing dGMP polymerization are present, one in mitochondria and the other in nuclei of chick embryonic brain. Similar results have been obtained previously [20] with DNA-dependent DNA polymerases from rat liver mitochondria and nuclei.

Attempts to separate poly dG:poly dC-dependent and oligo dT:poly rA-dependent activities in mitochondrial fraction II by phosphocellulose chromatography were unsuccessful (fig. 3). However, it should be mentioned that highly purified chick-embryonic RNA-dependent DNA polymerase [9] retains the capacity to polymerize dATP when poly dA:poly dT is provided.

A result which deserves some comment is the oligo dT:poly rA dependence of the mitochondrial enzyme and the poly dT:poly rA dependence of the nuclear enzyme. It was pointed out [21] that viral oncogenic reverse transcriptase preferentially recognizes oligo dT:poly rA as synthetic primer-template, whereas the cellular polymerases give reactions with oligo dT:poly dA and with poly dT:poly rA [11]. Moreover, an oligo dT:poly rA-dependent activity was detected in mitochondria of Rous sarcoma cells [22]. Our findings show that oligo dT:poly rA-dependent activity is associated with mitochondria from non-infected cells.

The results concerning poly dT:poly rA-dependent and poly dG:poly dC-dependent polymerase activities in the nuclear fraction are in agreement with those of Chang and Bollum [23] with low-molecular weight purified nuclear enzyme from rabbit bone-marrow. This enzyme utilizes poly dT:poly rA fairly well as primer-template but can not recognize oligo dT:poly rA. On the other hand it should be mentioned that, as in our nuclear enzyme preparation which catalyzes the polymerization of dGMP when poly dG:poly dC is provided as primer-template, this low-molecular weight nuclear enzyme can polymerize dGMP in the presence of oligo dG:poly dC. These results could indicate that these two nuclear enzymes are similar in their template requirements.

The inhibitory effect of high doses of mitochondrial fraction IV on the TMP polymerizing activity of nuclear fraction I in the presence of poly dT:poly rA could be explained by the presence in the mitochondrial preparation of a nuclease acting on the end product. On the other hand, the kinetics of enzyme activity as a function of time of incubation (unpublished results) prove that poly dT:poly rA-dependent activity of nuclear fraction I reaches a maximum after 20 min of incubation. With longer incubation times, the synthesis drops, probably owing to the presence in our partially purified nuclear enzyme of a hybridase [24, 25] which degrades the poly rA strand of the template. Experiments are in progress to test these possibilities.

We think that our enzyme preparations differ from the endogenous RNA-directed DNA polymerase activity found in uninfected chicken embryos by Kang and Temin [26] for two reasons: first our extracts do not show endogenous enzyme activity, and second Kang's enzyme is isolated from the high-speed centrifugation pellet (190,000 *g* for 1 hr) and is distinct from the exogenous DNA-directed DNA polymerase.

All these *in vitro* experiments show that reverse transcription seems to be responsible for poly dT synthesis. Since it has been shown recently that oligoribonucleotide sequences are the actual primer for DNA replication [27] one can suppose the existence of

small riboadenylate sequences hydrogen-bonded with poly dT to prime poly dA synthesis. However, it remains to be demonstrated that this is what happens *in vivo*.

In conclusion, it seems that two different synthetic template-dependent DNA polymerases can be distinguished in chick embryonic brain: a mitochondrial-bound enzyme which is only able to recognize oligo dT as primer in the complex oligo dT:poly rA, and a nuclear-bound enzyme which preferentially recognizes poly dT as primer in the hybrid poly dT:poly rA (table 1).

For the time being, we do not know whether the above mentioned specificities are a feature of the particular embryological system we are dealing with, or a general characteristic of animal cells.

Acknowledgements

We are grateful to Professor E. Wollf for encouragement and generous support; to Professor F. Chapeville for helpful discussions and critical reading of the manuscript and to Doctor J. Smith for advice in preparation of the manuscript. The competent technical assistance of L. Addade and M. Haucomat is acknowledged.

References

[1] R.C. Gallo, S.S. Yang and R.C. Ting, Nature 228 (1970) 927.
[2] P.E. Penner, L.H. Cohen and L.A. Loeb, Biochem. Biophys. Res. Commun. 42 (1971) 1228.
[3] T.W. Reid and D.M. Albert, Biochem. Biophys. Res. Commun. 46 (1972) 383.
[4] I. Suzuka and M. Muto, Biochem. Biophys. Res. Commun. 46 (1972) 1874.
[5] W-K. Yang, C-K. Koh and L.C. Waters, Biochem. Biophys. Res. Commun. 47 (1972) 505.
[6] B. Fridlender, M.Fry, A. Bolden and A. Weissbach, Proc. Natl. Acad. Sci. U.S. 69 (1972) 452.
[7] A.M. De Recondo, J.A. Lepesant, O. Fichot, L. Grasset, J.M. Rosignol and M. Cazillis, J. Biol. Chem., in press.
[8] J.G. Stavrianopoulos, J.D. Karlas and E. Chargaff, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2207.
[9] J.G. Stavrianopoulos, J.D. Karkas and E. Chargaff, Proc. Natl. Acad. Sci. U.S. 69 (1972) 2609.
[10] A. Ficq and J. Brachet, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2774.
[11] I. Slater and D.W. Slater, Nature New Biol. 237 (1972) 81.
[12] F.L. Margolis, J. Neurochem. 16 (1969) 447.
[13] L. Soriano, FEBS Letters 22 (1972) 310.
[14] J.B. Chappell and R.G. Hansford, in: Subcellular components, eds. G.D. Birnie and S. Fox (Butterworths, London, 1969) p. 49.
[15] R. Maggio, P. Siekevitz and G.E. Palade, J. Cell Biol. 18 (1963) 267.
[16] O.H. Lowry, N.J. Rosebrough, A.L. Farr and R.S. Randall, J. Biol. Chem. 193 (1951) 265.
[17] J.P. Richardson, Proc. Natl. Acad. Sci. U.S. 55 (1966) 1616.
[18] S. Spiegelman, A. Burny, M.R. Das, J. Keydar, J. Schlom, M. Travnicek and K. Watson, Nature 227 (1970) 563.
[19] S. Spiegelman, A. Burny, M.R. Das, J. Keydar, J. Schlom, M. Travnicek and K. Watson, Nature 227 (1970) 563.
[20] R.R. Meyer and M.V. Simpson, Proc. Natl. Acad. Sci. U.S. 61 (1968) 130.
[21] N.C. Goodman and S. Spiegelman, Proc. Natl. Acad. Sci. U.S. 68 (1971) 2203.
[22] J. Kara, M. Dvorak and H. Cerna, FEBS Letters 25 (1972) 33.
[23] L.M.S. Chang and F.J. Bollum, Biochem. 11 (1972) 1264.
[24] H. Stein and P. Hausen, Science 166 (1969) 393.
[25] P. Hausen and H. Stein, European J. Biochem. 14 (1970) 278.
[26] C.Y. Kang and H.M. Temin, Proc. Natl. Acad. Sci. U.S. 69 (1972) 1550.
[27] A. Sugino, S. Hirose and R. Okazaki, Proc. Natl. Acad. Sci. U.S. 69 (1972) 1863.