ONCORNAVIRAL-LIKE DNA POLYMERASE ACTIVITY IN A CASE OF CHILDHOOD MYELOFIBROTIC SYNDROME*

P. CHANDRA, Linda K. STEEL, H. LAUBE and B. KORNHUBER

Gustav-Embden-Zentrum der Biologischen Chemie, Abteilung für Molekularbiologie; and Kinderklinik der Universität, Frankfurt/Main-70, West Germany

Received 1 July 1975

1. Introduction

The first documentation on the myelofibrotic syndrome was published by Heuck in 1879 [1] who reported two cases, exhibiting a very high leucocyte count and fibrosis of the bone marrow. These, then, seemed to be the cases of 'splenic' leukemia as it was then understood. Neumann [2] interpreted that the marrow might have been hyperplastic before becoming necrotic and might, therefore, have originated the leukemic process. However, a different view was taken by Donhauser [3]. His patient had splenomegaly; the blood showed little anemia and the leucocyte count was only 11 550; and the autopsy demonstrated myelofibrosis and myeloid metaplasia. In the following years the myelofibrotic syndrome was postulated by several investigators [4–8] to have a distinct nature of its own and is different from leukemia. Myelofibrotic syndrome typically occurs in middle-aged and elderly persons [9–10] although a few cases have been found in children [11].

A variety of etiological factors responsible for myelofibrosis have been reported in literature (see review by Hunstein and Hauswaldt, [12]). However, no indications of a virus etiology of the disease exist as yet. In the present report we will describe the existence of a DNA-polymerase in a post-operative spleen specimen from a 2½ year old girl having myelofibrotic syndrome; this enzyme resembles in many of its biochemical parameters the DNA-polymerases of RNA tumor viruses. The purification of this enzyme and some of its biochemical characteristics are described here.

*Correspondence should be sent to: Professor Dr P. Chandra, Gustav-Embden-Zentrum der Biologischen Chemie, Abteilung für Molekularbiologie, Klinikum der J. W. Goethe-Universität, Theodor-Stern-Kai 7, Frankfurt-Main 70, West Germany.

2. Materials and methods

Labelled deoxynucleoside triphosphates were obtained from NEN-Chemicals GmbH, Germany, unlabelled deoxynucleoside triphosphates from Calbiochem or Boehringer Mannheim, Tutzing. Polycytidylic acid (PC) was supplied by Miles Laboratories, Elkhart, Indiana, U.S.A., and oligo (dG) by Collaborative Research, Massachusetts.

Spleen from a patient having osteo-myelofibrosis (approx. 1.45 kg) was obtained from a 2½ year old girl; sectioned and frozen at −70°C. All operations were performed at 0–4°C. 100 g of spleen was combined with 500 ml of buffer containing 0.05 M Tris–HCl (pH 7.5), 0.02 M dithiothreitol, 0.0005 M EDTA, 0.25 M saccharose and 0.005 M $MgCl_2$ (Buffer B); and homogenized under icewater cooling in a Waring blendor for 8 min at low speed and 4 min at high speed. The suspension was then filtered through a monolayer of nylon stocking (Fraction I), and the filtrate centrifuged for 10 min at 900 *g* in a Sorvall centrifuge using an SS-34 rotor. The pellet, containing nuclei, was discarded and the supernatant was centrifuged at 10 000 *g* for 15 min. The mitochondrial pellet was discarded. Following a modified procedure of Mayer et al. [13], the supernatant was placed on a 25% w/w saccharose cushion and centrifuged at 171 000 *g* for 2 h in a Damon IEC ultracentrifuge using a 404 rotor.

The microsomal pellets were solubilized in 100 ml buffer containing 0.05 M Tris–HCl (pH 7.5), 0.001 M dithiothreitol, 0.001 M EDTA, 20% ethylene glycol (Buffer A) with 1.0 M NaCl and 0.5% Nonidet P-40, and stirred slowly for 1 h (Fraction II). Particulate debris was removed by centrifuging at 171 000 *g* for 1 h. Solubilized microsomal extracts were dialyzed against 4 l of Buffer A for 17 h.

In a batch method, previously described by Smith et al. [14], the extract was adjusted to 0.25 M KCl and added to 150 ml of fibrous DEAE-cellulose (Serva, capacity 0.69) which had been equilibrated with Buffer A + 0.25 M KCl (1.5 ml gravity-packed wet vol per gram starting tissue). All salt concentrations were determined with a conductivity meter. The slurry was gently stirred for 1 h and centrifuged at 4100 *g* for 10 min. The supernatant was decanted and saved, and the DEAE-cellulose was resuspended with 100 ml of Buffer A + 0.30 M KCl, gently stirred for 1 h and centrifuged at 4100 *g* for 10 min. The supernatant was decanted, combined with the previous supernatant, and dialyzed against 4 l of Buffer A for 18 h (Fraction III). The resultant extract was equilibrated to 0.075 M KCl and applied in batch method to 150 ml of granular DEAE-cellulose (Servacel, capacity 0.99), equilibrated with Buffer A + 0.075 M KCl (1.5 ml gravity-packed wet vol per gram starting tissue). The slurry was gently stirred for 1 h, then centrifuged at 4100 *g* for 10 min. The supernatant was decanted and saved (Fraction IV A). The packed DEAE-cellulose was resuspended with 100 ml Buffer A, adjusted to 0.3 M KCl, and gently stirred for 1 h. The slurry was centrifuged at 4100 *g* for 10 min and the supernatant was saved (Fraction IV B). Supernatant fractions IV A and IV B were dialyzed separately against Buffer A + 30% diethylene glycol for 21 h. Fraction IV B was stored at –70°C.

Using the procedure previously described by Gallo et al. [15], dialyzed Fraction IV A was adjusted to 0.05 M KCl and layered on to a phosphocellulose (Serva, type P, capacity 0.85) column (0.9 × 30 cm) equilibriated with Buffer A + 0.05 M KCl (column vol was 0.75 ml gravity-packed wet vol per gram starting tissue; vol 75 ml). After the fraction was absorbed, a linear gradient from 0.1 M–0.75 M KCl in Buffer A + 0.02% Nonidet P-40 and 0.1 mg/ml bovine serum albumin in the elution buffers was applied (1250 ml × 1250 ml). Fractions were collected in 4 ml aliquots and assays were performed on every tenth fraction.

The assay system for Fractions I – V (total vol 0.25 ml) contained: 0.002 M $MgCl_2$ or 0.001 M $MnCl_2$, 0.08 M Tris–HCl (pH 7.8), 0.0016 M dithiothreitol (DTT), 0.4 mM each of unlabelled dATP, dCTP, dGTP (for assays containing poly (dA-dT) and poly rA·p$(dT)_{12}$) and dTTP (for assays containing poly rC·oligo dG); 1 μCi [^{3}H]-dTTP (1279 cpm/pmole) or 1 μCi [^{3}H]-dGTP (192 cpm/pmole), 1 μg of the indicated template, and 0.025 ml of the test fraction. All reactions were performed at 37°C for 60 min. Acid precipitable material was collected on Whatman glass fiber paper discs (GF/C, 2.5 cm ϕ), and counted in a liquid scintillation spectrometer. Protein was estimated by the method of Lowry et al. [16].

3. Results and discussion

The DNA polymerase activity in the solubilized high speed pellet (171 000 *g*) was extracted with salt and detergent and purified as described in materials and methods. The activities of this enzyme at various steps of purification, using different template-primers are documented in table 1. The single peak of activity eluted from the phosphocellulose column at 0.29 M salt concentration (Fraction V), represents a 625-fold purification over the enzymatic activity of the crude homogenate using poly rC·oligo dG as the template-primer. With poly rA·p$(dT)_{12}$ the purification was 485-fold, and the poly (dA-dT) catalyzed activity exhibited a 215-fold purification over the homogenate.

The purification procedure employed here, except some minor modifications, is based on the method of Mayer, Smith and Gallo [13–15]. However, in our experiments the peak activity was eluted at 0.29 M KCl, whereas, these authors reported the elution of their peak activity at 0.26 M KCl. This minor shift of elution peak has been observed by these authors in their recent experiments also (Dr R. C. Gallo, National Cancer Insitiute, USA; personal communication).

It is interesting to note that though the enzyme uses all three templates very efficiently throughout the purification procedures, the maximum activity of the phosphocellulose enzyme is shown in the

Table 1
DNA-polymerase activities at various purification steps[a]

Fraction No.	Purification step	Enzyme activities with various templates Incorporation of the labelled precursor (pmoles/mg protein)		
		Poly (dA-dT)	Poly rA·p(dT)$_{12}$	Poly rC·oligo dG
I	Crude homogenate	0.908 (0.438)[b]	0.442	1.571
II	171 000 *g* resuspended pellet	29.821 (9.620)	12.713	8.520 (5.619)
III	After fibrous DEAE-Cellulose	51.575	13.407	16.935
IV	Fraction IV A After granular DEAE-Cellulose	21.225	23.430	48.610
	Fraction IV B	99.466	25.280	2.403
V	DNA-polymerase activity of fraction IV A after phosphocellulose	193.933	214.400	983.120

[a] Assay conditions as described in Materials and methods, using Mg^{++} ions and [^{3}H]-dTTP for poly (dA-dT) and poly rA·p(dT)$_{12}$, and Mn^{++} ions and [^{3}H]-dGTP for poly rC·oligo dG.
[b] Figures in brackets are for endogenous incorporation values.

presence of poly rC·oligo dG. Furthermore, the pelleted crude enzyme (Fraction II) shows an endogenous incorporation of [^{3}H]-TMP and [^{3}H]-dGMP (shown in brackets in table 1); the endogenous activity was lost in the presence of pancreatic RNase (data not shown).

The use of several synthetic template-primers for monitoring viral RNA-dependent DNA polymerase (RDDP) has increased because of their relative specificity. Among these templates, poly rC·oligo dG and poly rA·oligo dT appear to be relatively specific for the DNA polymerases associated with oncornaviruses. Recently, Lewis et al. [17] have isolated a cellular DNA-polymerase III (γ-DNA polymerase) which has a very high affinity for the template-primer poly rA·oligo dT. The transcription of this template by the γ-polymerase requires however, Mn^{++} ions; whereas, in our experiments the transcription of poly rA is about 5 times better with Mg^{++} ions than with Mn^{++} ions (table 2). Gallo et al. [15] have reported that

Table 2
Template and ionic requirements of the purified DNA-polymerase[a]

Template used	^{3}H-labelled precursor	Incorporation of the labelled precursor (pmoles/mg protein)	
		Mg^{++}	Mn^{++}
Poly (dA-dT)	dTTP	193.93	15.72
Poly rA·p(dT)$_{12}$	dTTP	214.40	47.69
Poly rC·oligo dG	dGTP	361.00	983.12

[a] Assay conditions as described in Materials and methods.

Table 3
Biochemical properties of the purified enzyme[a]

Additions	Incorporation of the labelled precursor (pmoles/mg protein)
Endogenous system	0.0
Complete (poly rC·oligo dG)	1054.70
Minus Mn^{++}	1.63
Plus RNase (80 μg/ml)	9.54

[a]Assay conditions as described in Materials and methods, using poly rC·oligo dG as template, and Mn^{++} for ionic requirements and [^{3}H]-dGTP as labelled precursor.

oligo dG·poly rC can be used, but weakly, by the γ-polymerase. In our system, the maximum response was observed with poly rC·oligo dG. Again here we find an ionic preference, i.e. the enzyme has far better activity in the presence of Mn^{++} ions than with Mg^{++} ions. This ionic preference resembles the observations made by Howk et al. [18], Abrell and Gallo [19] and Dion et al. [20] for DNA polymerase activities associated to C-type oncornaviruses.

The complete dependency of the polymerase activity on Mn^{++} ions is shown in table 3; the omission of Mn^{++} ions in the complete reaction mixture leads to a total inhibition of [^{3}H]-dGMP incorporation into DNA. Addition of pancreatic RNase to the reaction mixture causes a total loss of enzyme activity, indicating the utilization of poly rC strand. This observation is important in the sense, that our enzyme is unable to incorporate [^{3}H]-dGMP in the presence of oligo dG alone. This type of activity, catalyzed by the enzyme terminal transferases, has been shown to occur in thymus [21–23], in white blood cells of patients with acute lymphoblastic leukemia [24] and with chronic myelogenous leukemia [25].

The data in table 4 rule out the possibility that our enzyme preparation has any terminal transferase activity. It is known that terminal transferase works best with the primer alone [21–25], and the presence of unlabelled deoxynucleoside triphosphates in the reaction mixture inhibits the terminal transferase activity [25]. The data reported in Table 4 give just the opposite picture, i.e. the higher activity is obtained in the presence of primer hybridized to template poly

Table 4
Incorporation of [^{3}H]-dGMP in the presence of poly rC·oligo dG and oligo dG[a]

Template used	Additions	Incorporation of the labelled precursor (pmoles/mg protein)
Poly rC·oligo dG	[^{3}H]-dGTP + (dTTP, dCTP, dATP)	561.4
Oligo dG	[^{3}H]-dGTP + (dTTP, dCTP, dATP)	24.5
Oligo dG	[^{3}H]-dGTP (alone)	0.0

[a]Assay conditions as described in Materials and methods.

rC, and the [^{3}H]-dGMP incorporation in the absence of other deoxynucleoside triphosphates is zero.

The data presented here indicate that the DNA-polymerase activity purified from a spleen of childhood myelofibrotic syndrome is clearly distinguishable from the γ-polymerase (DNA-polymerase III) by three criteria: (a) the purification procedure (Gallo et al. [15]); (b) template specificity and (c) ionic requirements. The absence of terminal transferase activity in the purified enzyme preparation is indicated by the RNase digestion experiments, and the inability of oligo dG alone to incorporate [^{3}H]-dGMP. The template requirements and the ionic specificity of the purified enzyme is identical to the oncornaviral reverse-transcriptase. Further experiments on its physiochemical characterization and its immunological relatedness to other primate RNA viruses are in progress.

Acknowledgements

We thank Dr Robert C. Gallo (National Cancer Institute, Bethesda, Maryland, USA) for valuable suggestions, and Frau A. Götz and Frau H. Heyland for excellent technical assistance. This work was supported by Grant KO 286/12 and 6 of the Deutsch Forschungsgemeinschaft, Kind-Philipp-Stiftung, and Vereinigung von Freunden und Förderern der John Wolfgang Goethe-Universität.

References

[1] Heuck, G. (1879) Virchows Arch. Path. Anat. 78, 475.
[2] Neumann, E. (1880) Klin. Wochensch. 17, 281.
[3] Donhauser, J. L. (1908) J. Exp. Med. 10, 559.
[4] Jordan, H. E. and Scott, J. K. (1941) Arch. Pathol. 31, 895.
[5] Rosenthal, N. and Erf, L. A. (1943) Arch. Intern. Med. 71, 793.
[6] Leonard, B. J., Israëls, M. C. and Wilkinson, J. F. (1957) J. Med. 26, 131.
[7] Gilbert, H. S. (1970) Mt. Senai J. Med. N. Y. 37, 426.
[8] Ward, H. P. and Block, M. H. (1971) Medicine 50, 357.
[9] Pitcock, J. A., Reinhard, E. H., Justus, B. W. and Mendelsohn, R. S. (1962) Ann. Intern. Med. 51, 73.
[10] Silverstein, M. N., Gomez, M. R., Remine, W. H. and Elveback, L. R. (1967) Arch. Intern. Med. 120, 546.
[11] Rosenberg, H. S. and Taylor, F. M. (1958) J. Pediatr. 52, 407.
[12] Hunstein, W. and Hauswaldt, Ch. (1974) Klin. Wochschr. 52, 305.
[13] Mayer, R. J., Smith, R. G. and Gallo, R. C. (1974) Science 185, 864.
[14] Smith, G. R., Abrell, J. W., Lewis, B. J. and Gallo, R. C. (1975) J. Biol. Chem., in press.
[15] Gallo, R. C., Gallagher, R. E., Miller, N. R., Mondal, H., Saxinger, W. D., Mayer, R. J., Smith, R. G. and Gillespie, D. H. (1975) in: Cold Spring Harbor Symp. on Quant. Biol.: Tumor Viruses, Vol. 34, in press.
[16] Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951) J. Biol. Chem. 193, 265.
[17] Lewis, B. J., Abrell, J. W., Smith, R. G. and Gallo, R. C. (1974) Science 183, 867.
[18] Howk, R. S., Rye, L. A., Killeen, L. A., Scolnick, E. M. and Parks, W. P. (1973) Proc. Natl. Acad. Sci. U.S. 70, 2177.
[19] Abrell, J. W. and Gallo, R. C. (1973) J. Virol. 12, 431.
[20] Dion, A. S., Vaidya, A. B. and Fout, G. S. (1974) Canc. Res. 34, 3509.
[21] Bollum, F. J. (1968) Methods in Enzymol. 12B, 591.
[22] Bollum, F. J. (1974) The Enzymes (Boyer, P. D., ed.) vol. 10, 145.
[23] Chang, L. M. S. (1971) Biochem. Biophys. Res. Commun. 44, 124.
[24] McCaffrey, R., Smoler, D. F. and Baltimore, D. (1973) Proc. Nat. Acad. Sci. U.S.A. 70, 521.
[25] Sarin, P. S. and Gallo, R. C. Biochim. Biophys. Acta, in press; personal communication.