MOLECULAR HETEROGENEITY OF DNA POLYMERASE α FROM P815 MOUSE MASTOCYTOMA CELLS

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1. Introduction

Of the 3 DNA polymerases (α,β,γ) found in eukaryotic cells, DNA polymerase α appears the most likely to be directly involved in the process of DNA replication: its level of activity parallels the rate of DNA synthesis in vivo [1,2] and it seems to be associated with replication complexes [3,4].

Several groups have described the occurrence of multiple forms of DNA polymerase α during purification of material from a variety of sources by ion-exchange chromatography, gel filtration or velocity-sedimentation techniques [5–8]. Moreover, a new form of DNA polymerase α in the nuclei of HeLa cells has been described upon treatment with cycloheximide [9]. This form α′ makes a simple artificial generation of molecular heterogeneity by purification techniques, as suggested [10] seem unlikely. Heterogeneity is more probably an intrinsic property of DNA polymerase α, which may be correlated with its biological function(s) during replication of eukaryotic cells.

The involvement of DNA polymerases in the very complex mechanism of replication, requiring at least enzymes, template, primer, protein factors and ions, is still far from being clearly understood. In an attempt to assess the relevance of such heterogeneity within this process of replication, we selected the separation of the various enzyme forms by their affinity for pseudo-templates or primers bound to sepharose columns. This affinity might theoretically be more closely related to a biological function than adsorption on ion-exchange columns or separation by velocity sedimentation.

In this report data on the separation and partial analysis of two forms of DNA polymerase α from P 815 mouse mastocytoma cells are presented, the two peaks of activity being distinguished by their affinity for poly(dT) on poly(dT)–CL-Sepharose columns. The two forms both exhibit properties characteristic of DNA polymerases α (sensitivity to *N*-ethylmaleimide, dependency on dithiothreitol, molecular size range); on the other hand they differ in their relative ability to copy various synthetic templates, in the ionic conditions necessary to their maximal activity, and in the degree to which they are protected against heat inactivation by dithiothreitol; from the results obtained it is concluded that there are differences in the molecular structure and properties of the two forms.

2. Materials and methods

2.1. *Materials*

Dithiothreitol, *N*-ethylmaleimide, lactic acid dehydrogenase (EC 1.1.1.27) and DNA from salmon sperm were purchased from Calbiochem (Lucerne), the protease inhibitor phenylmethane sulphonyl-fluoride from Sigma (St Louis), poly- and oligonucleotides from P. L. Biochemicals (Milwaukee) or Collaborative Research (Waltham) and non-ionic detergent P-40 from Shell Chemical Co. (Zurich). Tritiated dTTP (30 Ci/mmol) was obtained from the Radiochemical Centre (Amersham) and unlabelled nucleotide triphosphates from Boehringer (Mannheim).

The polynucleotide poly(dT) was bound to CL-Sepharose-4B (Pharmacia, Zurich) as described for preparation of poly(rC)–Sepharose-4B [12].

2.2. *Growth of cells*

P815 mouse mastocytoma cells were a gift from Dr J. Gautschi of University of Berne. They were propagated in suspension cultures in MEM spinner medium containing 10% foetal calf serum (Seromed, Munich).

2.3. *Purification of DNA polymerases*

Partial separation of the 3 classes of DNA polymerase α,β and γ (EC 2.7.7.7) was achieved by fractionation of the exponentially growing P815 cells into chromatin, nucleoplasm and cytoplasm, followed by ion-exchange chromatography on phosphocellulose (PE-11) and DEAE cellulose (DE-52; Whatman, Maidstone) in column buffer (K-phosphate (pH 7.5), 10% glycerol, 0.1 mM EDTA, 1 mM dithiothreitol), essentially as in [11]. The procedure will be detailed elsewhere. Active fractions were pooled, dialysed and subjected to affinity chromatography, as described in the text.

2.4. *Assay for DNA polymerase α activity*

Standard reactions were carried out in final vol. 50 μl containing 50 mM Tris-HCl (pH 7.5), 0.1 mM dATP, 0.1 mM dCTP, 0.1 mM dGTP and 0.06 mM dTTP (spec. radioact. 0.33 Ci/mmol), 0.1 mM EDTA, 3.5 mM $MgCl_2$, 125 μg/ml activated DNA, 80 μg/ml bovine serum albumin and 1 mM dithiothreitol. Reactions were initiated with the enzyme fraction, terminated after 30 min at 37°C (standard assay conditions) with trichloroacetic acid (TCA) and analysed as in [12].

2.5. *Template/primer preparations*

Salmon sperm DNA was activated and synthetic template/primers were prepared following the procedures in [12].

3. Results

3.1. *Separation of 2 forms of DNA polymerase α*

After purification by phosphocellulose and DEAE-cellulose, DNA polymerase α activity is apparently free of polymerase β and of most of polymerase γ, as judged by inhibition by *N*-ethylmaleimide and template/salt preferences. Further analysis of polymerase α material on poly-(dT)–CL-Sepharose yields 2 peaks of activity upon development of the column with a 0–400 mM linear gradient of KCl (fig.1A).

Whereas the 2 activities of DNA polymerase α elute at 100 and 200 mM KCl, respectively, DNA polymerase γ activity eluted under these conditions as a single peak at a molarity of about 240 mM KCl; thus, contaminating material with a similar activity pattern from previous purification steps would not overlap (results not shown).

The most active fractions of each of the two

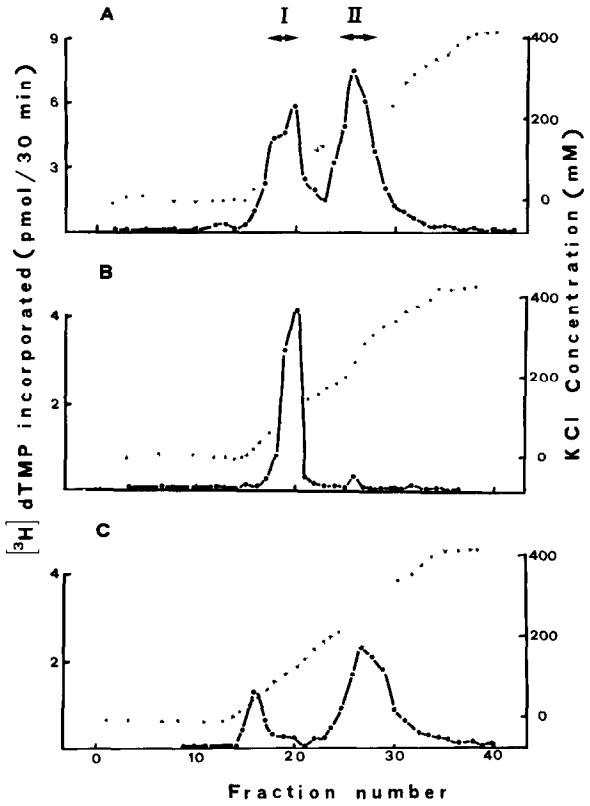

Fig.1. Poly(dT)–CL-Sepharose affinity chromatography of DNA polymerase α. The fractions from DEAE-cellulose chromatography containing enzyme activity were applied to the column in K-phosphate buffer (10 mM, pH 7.9) with 10% glycerol, 0.1 mM EDTA and 1 mM dithiothreitol, washed and then eluted with a linear gradient (0–400 mM KCl) in the same buffer. (A) Chromatography of active fractions from DE-52; (B) rechromatography of peak I-material; (C) re-chromatography of peak II-material. The concentration of KCl in each fraction was estimated from conductivity measurements in a Philips PW 9501 conductivity meter.

peaks were pooled, dialysed against column buffer and then rechromatographed under the same conditions as in the original separation. Whereas peak I material elutes at the same position (fig.1B), peak II material again yields 2 peaks of activity (fig.1C), comparable to those found with the original material: forms I and II as in fig.1A; this 2 peak pattern remains if affinity chromatography is performed in the presence of the proteinase inhibitor phenylmethane sulphonylfluoride (5×10^{-4} M).

3.2. *Comparison of the 2 forms*

3.2.1. Enzymatic activity

Since incorporation of tritiated dTMP into TCA-insoluble material catalysed by either form remains linear for about 2 h (fig.2) the 2 preparations are unlikely to differ in contaminating nucleases and proteinases, and can therefore be incubated for 30 min (or even 60 min). Comparison of the catalytic properties of the 2 forms reveals a difference when they are assayed in the presence of varying concentrations of monovalent (K^+; fig.3A) or divalent (Mn^{2+}; fig.3B) cations.

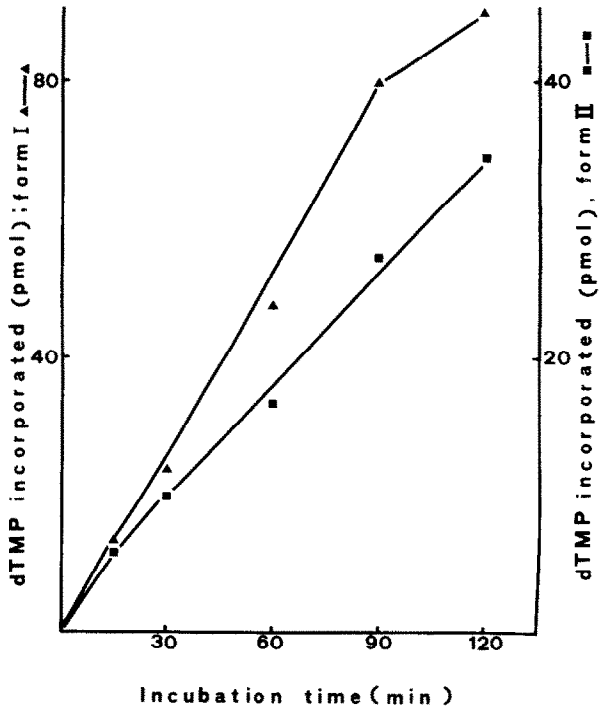

Fig.2. Kinetics of form I and form II DNA polymerase α activities. Standard conditions of incubation were used. (▲) Form I material; (■) form II material.

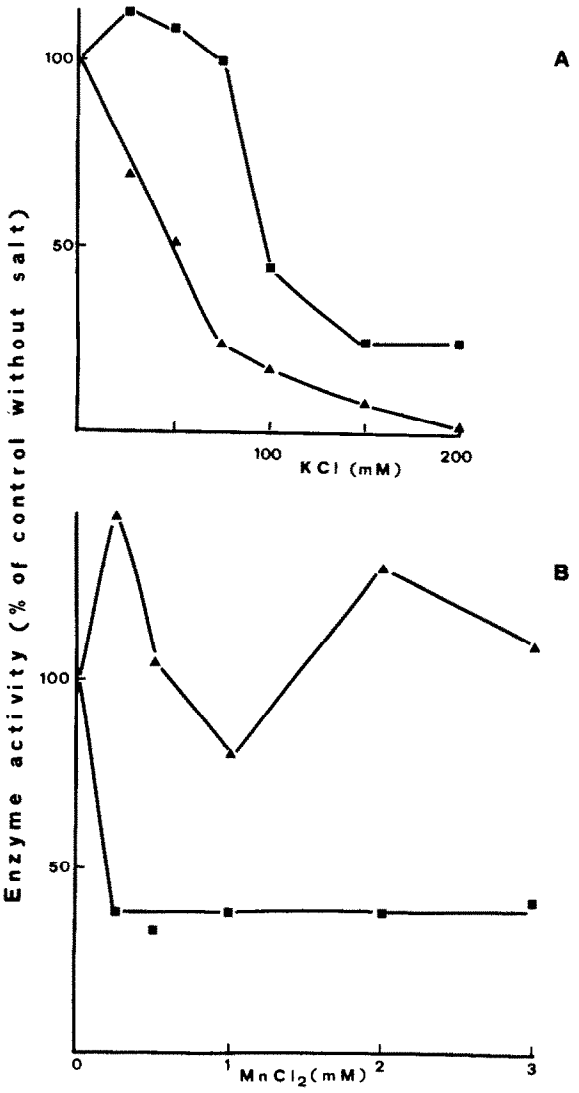

Fig.3. Salt-dependencies of form I and form II DNA polymerase α activities. Standard conditions of incubation were used. (▲) Form I material; (■) form II material. (A) Effect of increasing concentrations of KCl (in the presence of 3.5 mM $MgCl_2$) and 50 mM NaCl originating from the template/primer preparation; (B) effect of increasing concentrations of $MnCl_2$ (with 0.5 mM $MgCl_2$ and in the presence of 50 mM NaCl).

3.2.2. Sedimentation analysis

Analysis by velocity sedimentation on glycerol gradients (7–30% in high-salt buffer containing 50 mM Tris·HCl (pH 7.9), 500 mM KCl, 0.1 mM EDTA, 2 mM dithiothreitol, 0.02% non-ionic detergent P-40, 200 μg/ml bovine serum albumin and 7% glycerol) shows a slight difference in migration (18 h, 300 000 × *g*; SW-56): form I migrates a little faster than form II, indicating, at first sight, a slightly larger molecular weight (results not shown). If form II were indeed smaller than form I, the pattern of re-chromatography shown in fig.1C would be more difficult to explain; however, since molecular asymmetry of DNA polymerase α has already been suggested [5], here again a difference in configuration or quaternary structure could cause the altered sedimentation properties.

3.3.3. Heat stability in the presence of SH-reagents

Whereas the 2 forms are indistinguishable in the degree to which they are inhibited by *N*-ethylmaleimide or activated by dithiothreitol, preincubation at various temperatures in the absence of template with different concentrations of dithiothreitol reveals that form I is more heat-stable than form II (table 1). Moreover, form I is much better protected by 5 mM dithiothreitol from heat inactivation than form II. These observations confirm the possible existence of structural differences between the 2 forms.

3.3.4. Template/primer preferences

The template/primer preferences of forms I and II were analysed in the presence of Mg^{2+} or Mn^{2+} as divalent cations (table 2). The difference observed using poly(dA)·oligo(dT) is striking and may indicate that the structural differences between the 2 forms, as suggested above, could be linked to a template or primer recognition site.

Table 2
Template/primer preferences for forms I and II

Template/primer	Enzyme activity[a] (%)			
	Form I		Form II	
	Mg^{2+}[b]	Mn^{2+}[c]	Mg^{2+}[b]	Mn^{2+}[c]
Activated DNA	100	88	100	56
Poly(dA)·oligo(dT)	13	61	117	80
Poly d(A–T)	103	109	159	103
Poly(rA)·oligo(dT)	1[d]	2	2	5

[a] Enzyme activity with activated DNA as template/primer and Mg^{2+} as cation was defined as 100% (= 23.8 pmol for form I, 6 pmol for form II)
[b] 3.5 mM $MgCl_2$
[c] 0.25 mM $MnCl_2$
[d] Background level 1%

Table 1
Heat stability of forms I and II in the presence of SH-reagents

30 min pre-incubation temp.	Enzyme activity[a] (%)									
	Form I					Form II				
	DTT[b]			NEM[c]		DTT[b]			NEM[c]	
	0.2 mM	1 mM	5 mM	0.2 mM	1 mM	0.2 mM	1 mM	5 mM	0.2 mM	1 mM
Without pre-incubation	66	100	115	15	5	59	100	130	6	6
37°C	40	113	107			13	37	65		
40°C		60	87				35	50		
45°C		33					22			

[a] Enzyme activity under standard conditions (without pre-incubation) was defined as 100% (30 pmol for form I, 10 pmol for form II; 30 min/37°C)
[b] Dithiothreitol
[c] *N*-Ethylmaleimide

4. Discussion

When the main peak of activity of DNA polymerase α obtained by chromatography on DEAE-cellulose is analysed by affinity chromatography, 2 forms of this enzyme can be separated on the basis of their affinity for a synthetic template, poly(dT), bound to CL-Sepharose. Structural and/or functional differences between the 2 forms are assessed by:

(i) Salt requirements for optimal activity;
(ii) Slight but constant differences in sedimentation velocity (S = 7.3 for form I, 6.5 for form II using the methods described;
(iii) Heat stability in the presence of SH-reagents;
(iv) Enzyme activity with poly(dA)-oligo(dT).

However, at the degree of purity (about 14 000-fold) of the material applied to the polynucleotide affinity columns the preparations are not yet homogeneous; thus, the possibility cannot be excluded that the 2 forms are generated artificially by their passage through the column, or that their different properties are due to co-purifying molecules, such as endonucleases [13], nucleic acid-unwinding proteins [14], or others [15]. Whatever the reasons, it is interesting that the generation of multiple forms is observed only with DNA polymerase α (not with β or γ) and that such molecular heterogeneity of DNA polymerase α has also been found after separation on other nucleic acid–CL-Sepharose columns (manuscript in preparation) or by other means than affinity chromatography [5–8]. Comparison of the forms of DNA polymerase α observed with mouse mastocytoma P815 cells and those described in connection with other systems is in progress. Molecular heterogeneity of a DNA polymerase evidenced by nucleic acid affinity chromatography may reflect structural and/or functional properties. The identification of 2 distinct forms of DNA polymerase α, their affinity for various template and/or primer molecules bound to CL–Sepharose and the evaluation of the generation of form I from form II may therefore lead to further elucidation of the involvement of this polymerase in the process of replication of eukaryotic cells.

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