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Purification on poly(U)–Sepharose 4B of human breast cancer cell line T-47D DNA polymerases

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ABSTRACT

The purification of DNA polymerases (RNA-directed DNA polymerases and DNA-directed DNA polymerases) on poly(U)–Sepharose 4B from a breast tumour cell line (T-47D) is reported. The elution of these enzymes was followed in each fraction by activity measurements with the four primer-templates poly(rA)–oligo$(dT)_{12-18}$, poly(dA) oligo$(dT)_{12-18}$, poly(rC)–oligo$(dG)_{12-18}$ and poly(rCm)–oligo $(dG)_{12-18}$. The control of the polymerase purification by chromatography was performed by sodium dodecyl sulphate–polyacrylamide gel electrophoresis of the pooled active enzymatic fractions.

INTRODUCTION

DNA polymerase (RNA-directed DNA and DNA-directed DNA polymerase) activities have been detected in Rous sarcoma [1] and in different human neoplasias, such as leukaemias [2–4] and breast tumours [5].

The DNA polymerases have been purified using glycerol gradient centrifugation [6], a combination of sucrose gradient and ion-exchange column chromatography [7] or extraction of retroviruses with a detergent followed by an oligo$(dT)_{12-18}$–cellulose column and non-dissociating gel electrophoresis [8]. These procedures are cumbersome and their yields are often poor. This paper demonstrates that highly pure DNA polymerase can be readily prepared by a single chromatographic step on a polyuridylic acid [poly(U)]–Sepharose 4B column.

EXPERIMENTAL

Chemical and reagents

Poly(riboadenylic acid)–oligo(deoxythymidylic acid)$_{12-18}$ [poly(rA)–oligo-$(dT)_{12-18}$], poly(deoxyadenylic acid)–oligo(deoxythymidylic acid)$_{12-18}$ [poly(dA)–oligo$(dT)_{12-18}$], poly(ribocytidylic acid)–oligo(deoxyguanylic acid)$_{12-18}$ [poly(rC)–oligo$(dG)_{12-18}$], poly(2′-O-methylribocytidylic acid)–oligo(deoxyguanylic acid)$_{12-18}$-

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[poly(rCm)–oligo(dG)$_{12-18}$] and poly(uridylic acid) [poly(U)]–Sepharose 4B were purchased from Pharmacia (Uppsala, Sweden). Nonidet P 40 (NP 40) and trichloroacetic acid (TCA) were purchased from Sigma (St. Louis, MO, U.S.A.). Deoxy[^{3}H]-methylthymidine 5′-triphosphate ([^{3}H]-dTTP) and deoxy[8-^{3}H]guanosine 5′-triphosphate([^{3}H]-dGTP) were purchased from Amersham International (Amersham, U.K.). Filters were obtained from Millipore (Bedford, MA, U.S.A.) and T_{75} flasks from Corning (Corning, NY, U.S.A.). RPMI 1640 medium was purchased from Gibco Europe (Renfrewshire, U.K.). Foetal calf serum (FCS) and phosphate-buffered saline (PBS) were obtained from Boehringer (Mannheim, Germany).

Cell culture and harvesting

T-47D cell line originated from a pleural effusion of a patient with intraductal and invasive breast carcinoma [9]. Cells were grown in closed plastic T_{75} flasks in growth medium composed of RPMI 1640 buffered with sodium hydrogencarbonate (2 g/l) supplemented with 2 m*M* glutamine, gentamycin (20 μg/ml) and 10% heat-inactived FCS. Cells were grown in a humidified incubator with 5% carbon dioxide at 37°C. Cultures were re-fed every 2–3 days. Culturing (after growth for 1 week) involved trypsin digestion (7.5 mg/ml PBS per flask), to obtain monocellular suspensions, followed by the plating of 3×10^6 cells per flask. Cultures were confluent after 5–6 days (*ca.* 10^7 cells per flask). Ten flasks were used.

Preparation of NP 40 extract

The growth medium was removed from each flask and cells were washed in the flask with PBS. Protein solubilization was performed with 3 ml of 0.5% NP 40 in TBS [150 m*M* NaCl–50 m*M* Tris–0.02% NaN_3 (pH 7.0)] for 15 min at 4°C. The insoluble material was removed by centrifugation at 30 000 *g* for 30 min. The collected extract was used immediately.

Affinity chromatography on poly(U)–Sepharose 4B

The NP 40 extract was poured onto a poly(U)–Sepharose 4B column (10 × 0.9 cm I.D.), previously equilibrated with 2–3 volumes of buffer A [50 m*M* Tris–HCl (pH 7.5)–0.1 *M* EDTA–3 m*M* dithiothreitol–0.02% (v/v) NP 40–10% (v/v) glycerol]. NP 40 extract precycling was performed for DNA–polymerase adsorption on the affinity gel. The enzyme was eluted with a linear gradient from 0 to 0.8 *M* KCl in buffer A at 10 ml/h. Fractions (1.5 ml) were collected with an automatic fraction collector and aliquots (100 μl) were taken and assayed for DNA polymerase activity [10].

Sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE)

Control of the purification of the DNA polymerases was performed by SDS-PAGE after their elution on poly(U)–Sepharose. Fractions exhibiting an enzymatic activity were pooled, then concentrated by ultrafiltration (Millipore CX 10 immersible ultrafilters, cut-off 10 000 dalton) to a final volume of 500 μl.

A sample of 50 μl was denatured by heating for 2 min at 100°C with addition of 50 μl SDS gel sample buffer [60 m*M* Tris–HCl (pH 6.8)–11.2% glycerol–3% SDS–0.01% bromophenol blue–5% β-mercaptoethanol]. The sample was then analysed on a 12.5% SDS-polyacrylamide gel together with molecular mass standards (phosphorylase *b*, M_r 94 000; bovine serum albumin, M_r 67 000; ovalbumin, M_r 43 000;

carbonic anhydrase, M_r 30 000; soybean trypsin inhibitor, M_r 20 100; α-lactalbumin, M_r 14 400; Pharmacia). Electrophoresis was performed at 20 mA for 3 h. The gel was then fixed in 20% TCA (0.5 h), stained with 0.04% (w/v) Serva Blue W (Serva, Heidelberg, Germany) in water (0.5 h), destained with water (1 h) and 20% glycerol (12 h) and dried on a slab dryer (Protean II; Bio-Rad Labs.) under vacuum.

Assays for DNA–polymerase activity

Before assay with the primer templates poly(rA)–oligo(dT)$_{12\text{-}18}$ and poly(dA)–oligo(dT)$_{12\text{-}18}$, samples were dialysed for 5 h against buffer B [40 m*M* Tris–HCl (pH 7.8)–60 m*M* KCl–2.2 m*M* dithiothreitol–10 m*M* $MgCl_2$–0.1% Triton X-100]. Before assay with the primer-templates poly(rCm)–oligo(dG)$_{12\text{-}18}$ and poly(rC)–oligo(dG)$_{12\text{-}18}$, samples were dialysed in buffer C, which was buffer B with 0.8 m*M* $MnCl_2$ in place of 10 m*M* $MgCl_2$ [11]. The assay was run to a final volume of 130 μl containing 8 μCi of [^{3}H]-dTTP (specific activity 2.07 TBq/mmol) and 1.6 μg of poly(rA)–oligo(dT)$_{12\text{-}18}$ or poly(dA) oligo(dT)$_{12\text{-}18}$, or 8 μCi of [^{3}H]-dGTP (specific activity 518 GBq/mmol) with 1.6 μg of poly(rCm)–oligo(dG)$_{12\text{-}18}$ or poly(rC)–oligo(dG)$_{12\text{-}18}$. Aliquots of the solubilized proteins (100 μl) and buffer B or C (14 μl) were then added. Controls were performed in the same reaction medium without a primer-template. Each assay was performed in triplicate. The reaction was conducted for 30 min at 37°C and stopped by precipitation of the polymerized material with a mixture of 5% TCA, 0.05 *M* sodium pyrophosphate and 0.5 *M* HCl (4 ml). Precipitates were collected by filtration on 0.22-μm filters, washed, dried and their radioactivity measured in a liquid scintillation counter (Packard Tri-Carb 4530) after dissolution in Ready Solv MP (Beckman) scintillation medium.

RESULTS AND DISCUSSION

We have demonstrated the purification of DNA polymerases (RNA-directed DNA polymerase and DNA-directed DNA polymerase) on poly(U)–Sepharose 4B (gives a high yield of purified enzyme) in comparison with more conventional methods, such as sucrose gradient centrifugation followed by phosphocellulose [8] or oligo(dT)$_{12\text{-}18}$–cellulose [12] columns, which are time consuming (and give a limited amount of purified enzyme).

The cellular DNA polymerases can be obtained with poly(U)–Sepharose 4B from different cell sources such as cell lines, surgical biopsies or peripheral blood. The method allows the identification of the DNA polymerases in the eluted fractions using a synthetic primer-template. Ross *et al.* [13] have demonstrated that poly(rA)–oligo(dT)$_{12\text{-}18}$ was copied by DNA polymerases. Later, it was demonstrated that poly(dA)–oligo(dT)$_{12\text{-}18}$ was copied by DNA–DNA polymerases and RNA–DNA polymerases [14]. Other workers have demonstrated that RNA–DNA polymerase activity was more specific with poly(rC)–oligo(dG)$_{12\text{-}18}$ than with poly(rA)–oligo(dT)$_{12\text{-}18}$ [15]. Gerard *et al.* [7] have demonstrated that poly(rCm)–oligo(dG)$_{12\text{-}18}$ was highly specific for RNA–DNA polymerases.

In this work, we determined in each fraction the DNA polymerase activities from T-47D cells after chromatography of the extract on poly(U)–Sepharose 4B, with the four primer-templates poly(rA)–oligo(dT)$_{12\text{-}18}$, poly(dA)–oligo(dT)$_{12\text{-}18}$, poly(rC)–oligo(dG)$_{12\text{-}18}$ and poly(rCm)–oligo(dG)$_{12\text{-}18}$, in order to establish an activity

spectrum allowing enzyme characterization. The reactions were conducted as follows. After incubation of the ^{3}H-labelled nucleotide with primer-template complex, the high-molecular-mass polydeoxyribonucleotides obtained, insoluble in 5% TCA, were separated from the TCA-soluble labelled starting materials by filtration. The incorporated radioactivity was determined by dissolution in a scintillation liquid and counting.

The results for the primer-templates poly(rA)–oligo(dT)$_{12-18}$ and poly(dA)–oligo(dT)$_{12-18}$ are plotted in Fig. 1. Each value corresponds to the arithmetic mean of the three different assays for each eluted fraction. Significant incorporation of [^{3}H]-dTTP into poly(dA)–oligo(dT)$_{12-18}$, corresponding to a DNA–DNA polymerase activity, was measured. Fractions 8–19 were concentrated and submitted to SDS-PAGE as described above. Between fractions 10 and 15 was measured a low incorporation of [^{3}H]-dTTP into poly(rA)–oligo(dT)$_{12-18}$, corresponding to a low specificity of DNA–DNA polymerase against this primer-template, which confirms the findings of Ross *et al.* [13]. Conversely, no incorporation of [^{3}H]-dGTP with the primer-templates poly(rC)–oligo(dG)$_{12-18}$ and poly(rCm)–oligo(dG)$_{12-18}$, which usually corresponds to an RNA- directed DNA polymerase activity, was measured. This result shows the absence of RNA–DNA polymerase in the solubilized proteins from non-stimulated T-47D cells. This enzyme, characteristic of the retroviruses and retrovirus-like particles, was not found because the cells were not stimulated by 17β-estradiol and progesterone, which are necessary for the activation of the retrovirus life cycle [9].

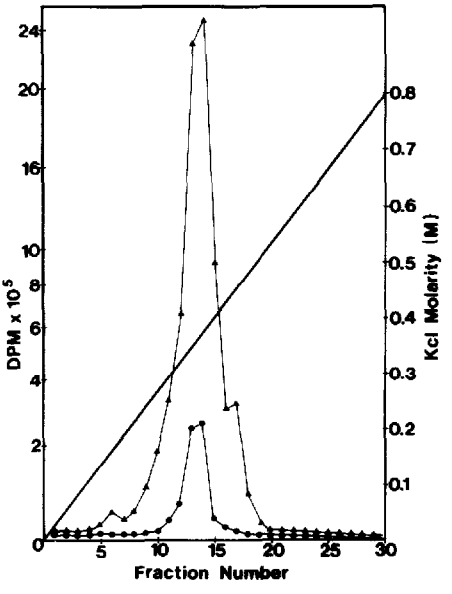

Fig. 1. Elution pattern on poly(U)–Sepharose 4B of DNA polymerases obtained from T-47D tumour cells. Flow-rate, 10 ml/h with a linear gradient of KCl from 0 to 0.8 *M* in buffer A. Aliquots (100 μl) of each 1.5-ml fraction were assayed for incorporation of [^{3}H]-dTTP into (▲) poly(dA)–oligo(dT)$_{12-18}$ and (●) poly(rA)–oligo(dT)$_{12-18}$.

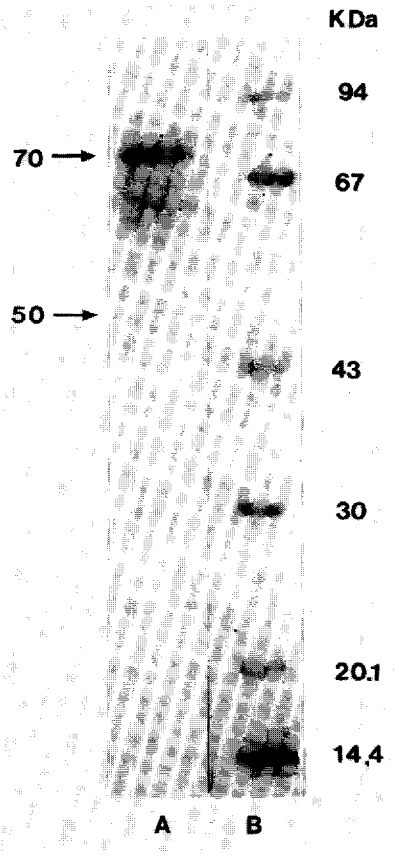

Fig. 2. SDS-PAGE of DNA polymerases after purification on poly(U)–Sepharose 4B (A). Molecular mass standards were phosphorylase *b* (M_r 94 000), bovine serum albumin (M_r 67 000), ovalbumin (M_r 43 000), carbonic anhydrase (M_r 30 000), soybean trypsin inhibitor (M_r 20 100), α-lactalbumin (M_r 14 400) (B). Staining was performed with Serva Blue W. KDa = Kilodalton.

The purification yield on poly(U)–Sepharose of DNA polymerases from 10^8 cells as starting material was about 54% when the incorporation of radioactivity was measured with the primer-template poly(rA)–oligo(dT)$_{12-18}$ and about 67% when the incorporation of radioactivity was measured with poly(dA)–oligo(dT)$_{12-18}$. Moreover, the degree of purity of DNA polymerases after purification on poly(U)–Sepharose was demonstrated after SDS-PAGE of pooled active enzymatic fractions. One major band was obtained at 70 000 dalton, and less intense bands between 70 000 and 50 000 dalton (Fig. 2). These results are in agreement with the findings of Hesselwood *et al.* [16] on calf thymus α-DNA polymerase and De Recondo *et al.* [17] on rat liver. Similar results were obtained by Grummt *et al.* [18], who demonstrated that calf thymus α-DNA polymerase was constituted of an association of seven polypeptide chains between 64 000 and 52 000 dalton.

In conclusion, poly(U)–Sepharose 4B chromatography enables DNA poly-

merases (RNA- and DNA-directed DNA polymerases) to be isolated from cell extracts with a good yield and high purity. Their enzymatic activities can be determined in the eluted fractions by measuring the incorporation of [^{3}H]nucleotides in their corresponding primer-templates. In this way, DNA polymerases can be obtained from cells and from retroviruses secreted in the culture medium.

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