Purification, Biochemical Characterization and Serological Analysis of Cellular Deoxyribonucleic Acid Polymerases and a Reverse Transcriptase from Spleen of a Patient with Myelofibrotic Syndrome*

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The present study describes the separation and purification of a reverse transcriptase and cellular DNA polymerases from the human spleen of a patient with myelofibrotic syndrome. The specific requirements with respect to bivalent cations and template-primers for DNA polymerase- α , DNA polymerase- β and DNA polymerase- γ , as well as for the reverse transcriptase, are reported. Sedimentation-velocity measurements of the purified enzymes gave values of 150 000, 40 000, 100 000 and 70 000 daltons for DNA polymerase- α , DNA polymerase- β , DNA polymerase- γ and the reverse transcriptase respectively. Serological studies have shown that the reverse transcriptase from human spleen is not antigenically related to cellular DNA polymerase- α , - β or - γ , but is antigenically related to reverse transcriptase from simian sarcoma virus and gibbon-ape leukaemia virus.

Much attention has been centred on the involvement of RNA tumour viruses in human cancer. Direct investigation of human tumour cells or leukaemic plasma by the electron microscopy has not proved very useful. Particles resembling animal C-type RNA tumour viruses have been described (Dmochowski *et al.*, 1965; Gross, 1970), but the possibility that these observations are artifacts cannot be disputed since the tumours were not assayed for biological activity.

Since the initial discovery of an oncornaviral-like reverse transcriptase activity in human leukaemic cells by Gallo *et al.* (1970), many studies have been carried out to detect this enzymic activity in a variety of malignant tissues and virus-infected cells (for review see Gallo, 1974). The object of these studies has been to obtain evidence for the presence of an RNA tumour virus in certain neoplastic cells of man to determine if this enzyme can be useful as a marker for the detection of viral information in human malignant diseases and to study the relationship between cellular DNA polymerases and the viral reverse transcriptase. In many of these studies, strict criteria for the characterization of reverse transcriptase were not applied, since the enzyme was not purified to homogeneity. So far, only two reports are available (Gallo *et al.*, 1975; Witkin *et al.*, 1975) where human reverse transcriptase has been purified to homogeneity. The characterization of template specificity or ionic requirements do not necessarily constitute proof of the presence of a reverse transcriptase activity. One of the cellular DNA polymerases, DNA poly-

merase- γ , resembles, in several of its biochemical properties, the viral reverse transcriptase, and was confused by several investigators with this enzyme. Thus the designation of an enzyme as reverse transcriptase requires not only its biochemical characterization, but also its serological analysis.

Myelofibrotic syndrome has been reported to result from a variety of aetiological factors (see review by Hunstein & Hauswald, 1974). Pathological investigations indicate that the syndrome differs from leukaemia (Leonard *et al.*, 1957; Gilbert, 1970; Ward & Block, 1971), generally occurring in the middle-aged or elderly (Pitcock *et al.*, 1962; Silverstein *et al.*, 1967) although a few cases in children have been reported (Rosenberg & Taylor, 1958).

The first indication for a virus aetiology of myelofibrotic syndrome came from data reported by our laboratory (Chandra *et al.*, 1975). A DNA polymerase resembling the reverse transcriptases of RNA tumour viruses was isolated from a post-operative spleen specimen of a girl of 2½ years having myelofibrotic syndrome. The present paper describes the isolation and biochemical characterization of the cellular DNA polymerases and reverse transcriptase from this spleen specimen. The serological specificities of these enzymes and the immunological relatedness of our reverse transcriptase to those from other primate oncornaviruses are presented.

Experimental

Materials

Labelled deoxynucleoside triphosphates were from NEN-Chemicals G.m.b.H., Dreieichenhain, Germany; non-labelled deoxynucleoside triphos-

* Dedicated to Professor Erich Heinz on his 65th birthday.

phates were from Calbiochem A.-G., Lucerne, Switzerland, or Boehringer, Mannheim, Tuting, Germany. Anti-(reverse transcriptase) IgG* from gibbon-ape leukaemia virus, simian (woolly monkey) sarcoma virus, Rauscher leukaemia virus and avian myeloblastosis virus, and reverse transcriptases of gibbon-ape leukaemia virus and simian sarcoma virus were from Litton Bionetics, Bethesda, MD, U.S.A. The reverse transcriptase of avian myeloblastosis virus was from Schwarz/Mann, New York, NY, U.S.A. 'Activated' DNA (salmon sperm) was prepared by the method of Schlabach *et al.* (1971). DEAE-cellulose (DE23) and phosphocellulose P were from Serva, Heidelberg, West Germany. DEAE-cellulose (DE52; microgranular) was obtained from Whatman Biochemicals, Maidstone, Kent, U.K. DNA-cellulose was prepared from cellulose powder (2200ff; Machery, Nagel and Co., Düren, Germany) by the methods of Alberts & Herrick (1971). All operations were performed at 4°C. Salt concentrations were determined by conductivity.

Spleen (1.45 kg) from a patient with osteomyelofibrosis was obtained from a girl of 2½ years. The patient was subjected to splenectomy as part of therapeutic treatment. The spleen was immediately sectioned and frozen at -70°C.

DNA polymerase extraction

Spleen (100 g) was mixed with 500 ml of buffer [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.5 mM-EDTA/5 mM-MgCl₂] and homogenized under ice/water 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. The filtered homogenate was centrifuged for 10 min at 900 g in a Sorvall centrifuge; the supernatant was decanted and the pellet was discarded. The supernatant was centrifuged at 12 000 g for 15 min; the mitochondrial pellet was discarded, and the supernatant was layered on to a 25% (w/w) saccharose cushion [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.5 mM-EDTA/5 mM-MgCl₂/25% saccharose] and centrifuged at 171 000 g in a Damon IEC ultracentrifuge for 2 h. The microsomal pellet obtained was combined with 100 ml of buffer [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.1 mM-EDTA/0.3% (v/v) Nonidet P-40/1.0 M-KCl/10% (v/v) glycerol], homogenized with a hand-driven Teflon homogenizer and stirred

slowly for 1 h at 4°C. The homogenate was centrifuged at 17 000 g for 15 min, the supernatant saved and the pellet re-extracted as before by combining with 50 ml of the same buffer, hand homogenization and slow stirring for 1 h. The re-extracted pellet was centrifuged at 17 000 g for 15 min; the pellet (particulate debris) was discarded and the supernatant combined with the previous 17 000 g supernatant. The combined 17 000 g supernatants, containing solubilized microsomal extracts, were centrifuged at 171 000 g for 1 h. The resulting supernatant was decanted and saved; the pellet was re-extracted with 50 ml of the same buffer, hand-homogenized, and centrifuged at 171 000 g for 1 h. The resulting pellet was discarded and the supernatant was combined with the supernatant of the previous 171 000 g centrifugation and dialysed against buffer A [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/10% (v/v) glycerol] until the KCl concentration was less than 0.05 M.

DEAE-cellulose (DE23) chromatography

DEAE-cellulose (DE23; fibrous form) was equilibrated with buffer A and poured into a column (diam., 2.7 cm; vol., 1 ml of wet-packed cellulose per 5–6 mg of protein). The dialysed 171 000 g supernatant, containing microsomal extracts, was adsorbed on the column and eluted with 3 column volumes of buffer A containing 0.3 M-KCl. The void volume and KCl washings were pooled and concentrated three-fold by dialysis against 500 ml of poly(ethylene glycol) buffer [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/1 mM-EDTA/0.05 M-KCl/20% (v/v) glycerol, in 30% (v/v) poly(ethylene glycol)]. The dialysis residue was then dialysed against buffer B [0.05 M-Tris/HCl (pH 7.9)/1 mM-dithiothreitol/10% (v/v) glycerol].

DEAE-cellulose (DE52) chromatography

DEAE-cellulose (preswollen DE52; microgranular) was equilibrated with buffer B, then treated with bovine serum albumin according to the method of Sedwick *et al.* (1972a). The cellulose was then washed with buffer B until no more bovine serum albumin was eluted (A_{280} of the effluent), and poured into a column (diam., 1.6 cm; vol., 1 ml of wet-packed cellulose per 5 mg of protein). The concentrated and dialysed fraction from the DEAE-cellulose (DE23) column was adsorbed on the column and washed with buffer B containing 0.05 M-KCl until no more protein was eluted (A_{280}). The void volume and KCl washings were pooled and saved. The column was then washed with 3 column volumes of buffer B containing 0.3 M-KCl.

The 0.05 M- and 0.3 M-KCl washings were separately concentrated 2- to 3-fold against 500 ml of poly(ethylene glycol) buffer, and dialysed against buffer C [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.02% (v/v) Nonidet P-40/20% (v/v) glycerol] containing 0.05 M-KCl.

* Abbreviations: IgG, γ -globulin 7S fraction prepared from serum; poly(dA)·(dT)₁₀, DNA-DNA hybrid of polydeoxyadenylic acid and oligodeoxythymidylic acid (chain length 10); poly[d(A-T)], co-polymer of deoxyadenylic acid and deoxythymidylic acid; poly(rA)·(dT)₁₂, RNA-DNA hybrid of a polyriboadenylic acid and oligodeoxythymidylic acid (chain length 12); poly(rC)·(dG)₁₂₋₁₈, RNA-DNA hybrid of a polyribocytidylic acid and oligodeoxyguanylic acid (chain length 12–18).

Phosphocellulose chromatography

Phosphocellulose (P) was prepared by the method of Burgess (1969), equilibrated with buffer C and then treated with bovine serum albumin according to the method of Sedwick *et al.* (1972a). The phosphocellulose was then washed with buffer C containing 0.05 M-KCl until no more bovine serum albumin was eluted (A_{280} of the effluent). Two columns (diam., 1.6 cm; vol., 20 ml of wet-packed phosphocellulose) were prepared. The concentrated and dialysed 0.05 M-KCl washing from the DE52-cellulose column was applied to one column. After adsorption, the column was washed with 20 ml of buffer C containing 0.07 M-KCl, and eluted with a 200 ml linear gradient with respect to concentration (0.1–0.7 M-KCl) in buffer C. Then 100 2 ml fractions were collected and every second tube was assayed for DNA polymerase activity.

The concentrated and dialysed 0.3 M-KCl washings from the DEAE-cellulose (DE52) column was adsorbed on the second phosphocellulose column, washed with 20 ml of buffer C containing 0.07 M-KCl and eluted with a 200 ml linear gradient of 0.1–0.5 M-KCl in buffer C. Then 100 2 ml fractions were collected and every second tube was assayed for DNA polymerase activity. Peak activities were pooled and concentrated by dialysis against poly(ethylene glycol) buffer, then dialysed against buffer D [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/0.05 M-KCl/20% (v/v) glycerol containing 0.10 mg of bovine serum albumin/ml of buffer].

DNA-cellulose chromatography

DNA-cellulose was prepared from powdered cellulose by the procedure of Alberts & Herrick (1971), and stored in 0.01 M-Tris (pH 7.4)/1 mM-EDTA containing 0.15 M-NaCl at -20°C . The prepared DNA-cellulose was poured into a column (diam., 1.0 cm) and allowed to settle at room temperature (25°C) with deaeration (vol., 5 ml of wet-packed cellulose). The column was then placed in a cold-room (4°C) and equilibrated with buffer D until the effluent had the same pH and conductivity as the buffer.

A sample (5 ml) of the concentrated and dialysed peaks from the second phosphocellulose column was equilibrated with buffer D by the addition of 7.5 ml of buffer D containing an additional 0.067 mg of bovine serum albumin/ml of buffer. The sample was slowly adsorbed on the DNA-cellulose column (2 ml/h). After adsorption, the column was washed with 5 ml of buffer D and eluted with a 50 ml linear gradient of 0.075–0.4 M-KCl in buffer D. Then 100 0.5 ml fractions were collected and every second tube was assayed for DNA polymerase activity.

DNA polymerase assay system

Stages in the isolation and purification of the DNA polymerases from the spleen of a patient with

myelofibrotic syndrome were characterized by their response to various template-primer systems. Fractions were assayed for the presence of DNA polymerase activity by adding 0.025 ml of the test fraction to a volume of 0.225 ml containing 0.05 M-Tris/HCl (pH 7.5 or 7.8), 1 mM-dithiothreitol, 0.01 M-MgCl₂ or 1 mM-MnCl₂, 0.06 M- or 0.125 M-KCl, 1 μCi of [³H]dGTP [192 c.p.m./pmol; for oligo(dG)·poly(rC)-primed reactions] or 1 μCi of [³H]dTTP (1279 c.p.m./pmol), unlabelled 0.4 mM-dATP, -dCTP and -dGTP [0.4 mM-dTTP for oligo(dG)·poly(rC)-primed reactions] and 1 μg of the indicated template-primer (2 μg in the case of 'activated' DNA). Reaction mixtures were incubated for 60 min at 37°C (MgCl₂ present) or 30°C (MnCl₂ present) and stopped by the addition of 0.36 mg of bovine serum albumin and 3 ml of cold 10% (w/v) trichloroacetic acid. Acid-precipitable material was collected on Whatman glass-fibre paper discs, washed three times with 5 ml of 5% (w/v) trichloroacetic acid, dried at 100°C for 30 min, then suspended in 10 ml of toluene scintillator fluid (Quickszint; Zinsser, Frankfurt, Germany) and counted for radioactivity in a liquid-scintillation spectrometer.

Molecular-weight determination

Molecular-weight determination of the spleen reverse transcriptase was performed by glycerol-gradient centrifugation by using a modification of the procedure of Martin & Ames (1961). Protein concentrations were measured by the method of Lowry *et al.* (1951) with bovine serum albumin as standard. The enzyme was dialysed against buffer F [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/1 mM-EDTA/0.20 M-NaCl] and 1 ml of the dialysis residue (140 μg) was layered on a pre-formed 5–20% (v/v) glycerol gradient (9 ml, in buffer F). Three external protein standards, ovalbumin (mol.wt. 45000), bovine serum albumin (mol.wt. 67000) and aldolase (mol.wt. 147000), were prepared at concentrations of 1 mg/ml of buffer F and similarly layered on parallel gradients. Gradients were centrifuged at 4°C for 24 h at 200000g, then fractionated in 0.3 ml portions from the top by using an ISCO density-gradient fractionator and the A_{280} of each protein standard sample was determined. DNA polymerase activity of the fractionated reverse transcriptase samples (0.3 ml) was assayed by the standard DNA polymerase assay system. A portion (0.04 ml) of each sample was assayed with poly(rC)·oligo(dG) as template-primer, in the presence of 1 mM-MnCl₂, 0.06 M-KCl and other reaction substrates, as described above, in a final incubation volume of 0.25 ml (pH 7.5). Mixtures were incubated for 1 h at 30°C and processed by the methods described above for assay systems.

As a further measure of the molecular weight of the spleen reverse transcriptase, the isolated enzyme and the same protein standards were subjected to

Scheme 1. Methods used to separate and isolate the DNA polymerases from myelofibrotic spleen [adapted from Lewis *et al.* (1974a,b)]

disc gel electrophoresis by the procedures of Davis (1964) and Ornstein (1964).

Molecular weights of DNA polymerase- α , - β and - γ were determined by sucrose-gradient centrifugation by a modification of the procedure of Martin & Ames (1961). The enzymes were dialysed for 16 h

against buffer G [0.05 M-Tris/HCl (pH 7.5)/1 mM-dithiothreitol/1 mM-EDTA/0.35 M-KCl] and 1 ml of each dialysis residue (120 μ g of DNA polymerase- α , 135 μ g of DNA polymerase- β and 100 μ g of DNA polymerase- γ) was layered on to a pre-formed 5-20% (w/v) sucrose gradient (9 ml, in buffer G).

Protein standards (1 mg/ml of buffer G) were similarly layered on to parallel gradients. Gradients were centrifuged for 24 h at 200000g (4°C) and fractionated from the top in 0.25 ml samples. The A_{280} of protein standard samples was determined. A portion (0.04 ml) of each sample of the enzyme fractionations was assayed in the DNA polymerase assay system, with 'activated' DNA template-primer and 0.01 M-MgCl₂ for DNA polymerase- α samples, poly(rA)·(dT)₁₂ and 1 mM-MnCl₂ for DNA polymerase- γ samples, and poly(dA)·(dT)₁₀ and 1 mM-MnCl₂ for DNA polymerase- β samples. No KCl was added to the system because of the high KCl concentration in the gradients. Incubation and processing procedures are as described for DNA polymerase assay systems.

Preparation of antisera

Antiserum against the purified spleen reverse transcriptase was prepared in two female goats. Each was initially given 70 μ g of enzyme emulsified with Freund's adjuvant and injected intramuscularly. Three subsequent booster injections of 35 μ g of enzyme and adjuvant were given intramuscularly at 2-week intervals. Then 10 days after the last injection, blood was collected and the serum recovered. IgG was obtained by (NH₄)₂SO₄ precipitation and purified on a column of Sephadex G-200. Normal goat serum IgG was used as a control in the enzyme neutralization assays.

Antibody inhibition studies

The DNA polymerases isolated from spleen were challenged with antibody prepared in goats to spleen

reverse transcriptase. A portion (0.025 ml) of the enzyme was incubated for 40 min at 4°C with an equal volume of antibody (4–64 μ g of IgG protein). The remaining enzyme activity was measured with the DNA polymerase assay system in a total incubation volume of 0.15 ml.

DNA polymerases from other sources were similarly challenged with the spleen anti-(reverse transcriptase) IgG. Enzyme (0.025 ml) was incubated for 16 h at 4°C with an equal volume (4–64 μ g) of pre-immune or immune IgG, and the remaining activity was measured by the DNA polymerase assay system with a total incubation volume of 0.15 ml.

The isolated spleen reverse transcriptase was challenged with anti-(reverse transcriptase) IgG from other sources. Enzyme (0.025 ml) was incubated for 16 h with 4–32 μ g of various anti-(reverse transcriptase) antisera or pre-immune IgG. Reactions were pre-formed in a total incubation volume of 0.15 ml, increasing the labelled deoxynucleoside triphosphate to 2 μ Ci. Other reaction conditions were as described for the DNA polymerase assay system.

Results

Purification of cellular DNA polymerase- α , - β and - γ and reverse transcriptase

Scheme 1 (adapted from Lewis *et al.* 1974a,b) outlines the overall technique of DNA polymerase separation from myelofibrotic human spleen. DEAE-cellulose (DE23) chromatography of the solubilized microsomal extracts removed most of the nucleic

Fig. 1. Phosphocellulose chromatography of material eluted from the DEAE-cellulose (DE52) column with the 0.05 M-KCl wash

Assay conditions are as described in the Experimental section, utilizing poly(rA)·(dT)₁₂ (●), poly(dA)·(dT)₁₀ (○) or poly[d(A-T)] (△) as template-primer with Mn²⁺ ions and 0.06 M-KCl present. Resolution of the reverse transcriptase (eluting at 0.23 M-KCl) and DNA polymerase- β (eluting at 0.51 M-KCl) was achieved. ----, KCl concentration gradient.

acids, as determined by the A_{254} of input and effluent materials.

DEAE-cellulose (DE52) chromatography of the effluent from DEAE-cellulose (DE23) chromatography resulted in the elution of DNA polymerase- β and reverse transcriptase in the column void volume and 0.05M-KCl wash, as determined by assay of samples in the DNA polymerase assay system. DNA polymerase assay of a sample of the 0.3M-KCl wash showed the activities of DNA polymerase- α and - γ .

When the 0.05M-KCl wash was chromatographed on phosphocellulose, adequate separation of DNA polymerase- β and reverse transcriptase was achieved. DNA polymerase assay of fractions (Fig. 1) indicated two distinct peaks of activity: reverse transcriptase was eluted at 0.23–0.29M-KCl concentration and DNA polymerase- β was eluted at 0.51M-KCl.

Phosphocellulose chromatography of the 0.3M-KCl-wash from DEAE-cellulose (DE52) chromatography resulted in two overlapping peaks of activity

Fig. 2. Phosphocellulose chromatography of the material eluted off the DEAE-cellulose (DE52) column with the 0.3M-KCl wash

Assay conditions are as described in the Experimental section, with 'activated' DNA (Mg^{2+} ions and 0.06M-KCl present) (Δ) or poly(rA)·d(T)₁₂ (Mn^{2+} ions and 0.125M-KCl present) ($\bullet$). ----, KCl concentration gradient.

Fig. 3. DNA-cellulose chromatography of the 0.34M-KCl peak (DNA polymerase- α and - γ) from the phosphocellulose column

DNA polymerase activity was measured as described in the Experimental section, with 'activated' DNA (Mg^{2+} ions and 0.06M-KCl present) (Δ) or poly(rA)·d(T)₁₂ (Mn^{2+} ions and 0.125M-KCl present) ($\bullet$). DNA polymerase- α (eluted at a concentration of 0.13M-KCl) and DNA polymerase- γ (eluted at a concentration of 0.19M-KCl) were adequately separated. ----, KCl concentration gradient.

Fig. 4. Velocity sedimentation of the reverse transcriptase isolated from spleen and three protein standards on a glycerol gradient

Details of procedure are given in the Experimental section. About 30 equal fractions were collected from the top of the tube for each gradient. (a) A_{280} of protein standard fractions. ○, Ovalbumin (mol.wt. 45000); △, bovine serum albumin (mol.wt. 67000); □, aldolase (mol.wt. 147000). (b) [^3H]dGMP incorporation of the enzyme measured in the DNA polymerase assay system (details given in the text). ----, Glycerol concentration in the gradient.

Fig. 5. Velocity sedimentation of the cellular DNA polymerases isolated from spleen on sucrose gradients

About 40 equal fractions were collected from the top of the tube for each gradient. Samples of DNA polymerase- α were assayed with 'activated' DNA and Mg^{2+} ions present, samples of DNA polymerase- β assayed with poly(dA)·(dT)₁₀ and Mn^{2+} ions present, and samples of DNA polymerase- γ assayed with poly(rA)·(dT)₁₂ and Mn^{2+} ions present. Other conditions are as described in the Experimental section. ----, Sucrose concentration gradient. □, Poly(dA)·(dT)₁₀ present; ○, 'activated' DNA present; △, poly(rA)·(dT)₁₂ present. A, Ovalbumin (mol.wt. 45000) standard marker; B, bovine serum albumin (mol.wt. 67000) standard marker; C, aldolase (mol.wt. 147000) standard marker.

(Fig. 2). DNA polymerase- α and - γ were eluted at a concentration of 0.27–0.40M-KCl. The peaks were pooled and further chromatographed on DNA-cellulose. DNA polymerase assay of samples from the DNA-cellulose column is shown in Fig. 3. Resolution of the two enzymes was achieved: DNA polymerase- α was eluted at a concentration of 0.13M-KCl and DNA polymerase- γ was eluted at 0.19M-KCl.

Molecular-weight determination

Results of the glycerol-gradient centrifugation of the purified spleen reverse transcriptase and three protein standard markers are shown in Fig. 4. The apparent molecular weight of the enzyme, assuming a globular shape, is approx. 70000. Disc gel electrophoreses of the enzyme demonstrated a mobility corresponding to that of bovine serum albumin, further verifying the glycerol-gradient value of 70000 daltons for the molecule. This determination agrees with the reported molecular weight of reverse transcriptases isolated from other mammalian type-C viruses (Grandgenett *et al.*, 1972; Tronick *et al.*, 1972; Abrell & Gallo, 1973; Gallo *et al.*, 1975; Mondal *et al.*, 1975; Witkin *et al.*, 1975). However, in contrast with the reverse transcriptase of leukaemic cells, where two size classes of 70000 and 130000 daltons have been reported (Mondal *et al.*, 1975), only one molecular species of 70000 daltons could be detected in the human myelofibrotic spleen.

Molecular-weight determination of the cellular DNA polymerases is shown in Fig. 5. The sucrose-gradient centrifugation of DNA polymerase- β indicates a mol.wt. of approx. 40000. Other laboratories (Smith & Gallo, 1972; Sedwick *et al.*, 1972a; Chang,

1973; Lewis *et al.*, 1974a) have reported mol.wts. of 40000–45000 for the enzyme. DNA polymerase- γ sucrose-gradient centrifugation indicated a mol.wt. of 100000–110000. Lewis *et al.* (1974a) have reported a value of approx. 10^5 daltons for this enzyme isolated from human lymphoblastoid cells. DNA polymerase- α demonstrated an approx. mol.wt. of 150000. Similar findings for this polymerase have been reported by Sedwick *et al.* (1972a), Smith & Gallo (1972), Lewis *et al.* (1974b) and Loeb (1974).

Bivalent cationic, ionic and template requirements

Table 1 summarizes the preferences of the cellular polymerases and reverse transcriptase for Mg^{2+} or Mn^{2+} cations in the presence of various template-primers. DNA polymerase- α required 'activated' DNA in the presence of Mg^{2+} cation, and poly-[d(A-T)] with either bivalent cation. This specificity for deoxyribose-template systems is a characteristic of the response *in vitro* of this enzyme, i.e. it preferentially transcribes poly(dA), and poly(rA) or poly(rC) are poorly utilized (Sedwick *et al.*, 1972a; Loeb, 1974; Lewis *et al.*, 1974a). DNA polymerase- α was also sensitive to high ionic strength and pH. The enzyme functioned best at pH 7.5 and at 60mM-KCl. This agrees with the findings of Bollum (1960), who found DNA polymerase- α to function optimally at pH 7.0–7.5 and at low ionic strength.

DNA polymerase- β preferentially transcribed poly(dA)·(dT)₁₀, but transcribed poly(rA) poorly and poly(rC)·oligo(dG) not at all. Smith & Gallo (1972) and Lewis *et al.* (1974a) have found similar results for this enzyme isolated from human lymphocyte cells. DNA polymerase- β showed a preference for higher ionic strength and pH, which agrees with

Table 1. *Bivalent cation and template specificities of the cellular DNA polymerases and reverse transcriptase isolated from human myelofibrotic spleen*

Reactions were performed as described for the DNA polymerase assay system. Reactions containing the reverse transcriptase enzyme were carried out at pH 7.5, with DNA polymerase- α at pH 7.5 and DNA polymerase- β and - γ at pH 7.8. All reactions with DNA polymerase- α and those with the template-primer 'activated' DNA contained 0.06M-KCl; all others contained 0.125M-KCl.

Template	Bivalent cation	³ H]Deoxynucleoside 5'-phosphate incorporated (pmol/mg of protein)			
		Reverse transcriptase	DNA polymerase- α	DNA polymerase- β	DNA polymerase- γ
Poly(rA)·(dT) ₁₂	Mn ²⁺	1355.0	16.9	13.1	1015.0
	Mg ²⁺	1714.0	10.1	6.3	770.1
Poly(dA)·(dT) ₁₀	Mn ²⁺	7.9	0.1	572.4	0.1
	Mg ²⁺	0.1	37.1	553.5	424.6
Poly[d(A-T)]	Mn ²⁺	38.5	503.2	94.4	710.3
	Mg ²⁺	224.5	597.6	173.0	188.7
Poly(rC)·oligo(dG)	Mn ²⁺	2060.1	24.0	0.1	56.1
	Mg ²⁺	1628.3	0.1	0.1	37.4
'Activated' DNA	Mg ²⁺	30.8	1844.4	36.7	728.6

the higher pH optima and stimulation by high ionic strength reported by Sedwick *et al.* (1972b).

The template-primer specificities of DNA polymerase- γ are similar to those reported by Fridlender *et al.* (1972), Loeb (1974) and Lewis *et al.* (1974a). Assay in the presence of Mn^{2+} cations exhibits a preference for poly(rA)·(dT)₁₂. Poly(dA)·(dT)₁₀ is utilized in the presence of Mg^{2+} cations and poly-

(rC)·oligo(dG) is poorly utilized with either bivalent cation. Less activity was observed at pH7.5 than at pH7.8, agreeing with the pH optimum of 7.6–8.0 reported by Fridlender *et al.* (1972). DNA polymerase- γ also showed a preference for 0.125M-KCl rather than 0.06M-KCl.

The reverse transcriptase from myelofibrotic spleen showed a strong preference for poly(rC)·oligo(dG) and poly(rA)·(dT)₁₂, characteristic for mammalian C-type oncornavirus DNA polymerase (Baltimore & Smoler, 1971). Optimal activities were obtained when the KCl concentration was 60–125mM at pH7.5. Variations in the Mn^{2+} cation concentration (Fig. 6) showed optimal activities between 0.8 and 1.0mM. The Mg^{2+} cation concentration was also tested (0–18mM) and the optimal concentration was found to be 8–12mM.

Serological specificities

Table 2 summarizes the responses of the cellular DNA polymerases and reverse transcriptase when challenged with various concentrations of the anti-(spleen reverse transcriptase) IgG. DNA polymerase- α and - β showed no inhibition at concentrations of 2–64 μ g of IgG, whereas DNA polymerase- γ was inhibited at a very low concentration (15% with 64 μ g of IgG). Splenic reverse transcriptase was strongly inhibited by the anti-(spleen reverse transcriptase) IgG (78.1% inhibition with 64 μ g of IgG). These results support the idea that the isolated reverse transcriptase is a unique, immunologically distinct, DNA polymerase of the human spleen from a patient with myelofibrotic syndrome.

Table 3 shows the results of antibody inhibition

Fig. 6. Effect of bivalent cation concentration on the incorporation of [³H]dUMP by reverse transcriptase isolated from myelofibrotic spleen

DNA polymerase activity was measured with poly-(rA)·(dT)₁₂ or poly(rC)·oligo(dG) as template-primer, 0.125M-KCl, and various concentrations of the divalent cation Mn^{2+} in the system; other conditions are as described in Experimental section. ○, Poly(rA)·(dT)₁₂ present; ●, poly(rC)·oligo(dG) present.

Table 2. Response of the DNA polymerases isolated from spleen to myelofibrotic spleen anti-(reverse transcriptase) IgG Myelofibrotic spleen anti-(reverse transcriptase) IgG and pre-immune (control) IgG were prepared to concentrations of 2, 4, 8, 16, 32 and 64 μ g in 0.05M-Tris/HCl, pH7.8. A portion (0.025ml) of each polymerase was preincubated with an equal volume of IgG for 40min at 4°C (total preincubation volume 0.50ml). Assay mixtures with 'activated' DNA template-primer (2 μ g) contained 0.01M-MgCl₂, 1 μ Ci of [³H]dTTP, 0.05M-Tris/HCl (pH7.5)/1mM-dithiothreitol/0.06M-KCl and unlabelled 0.004M-dATP, -dCTP and -dGTP. Assays utilizing poly[d(A-T)] and poly(rA)·(dT)₁₂ (1 μ g) contained 1mM-MnCl₂, 1 μ Ci of [³H]dTTP, 0.05M-Tris/HCl (pH7.8), 1mM-dithiothreitol, 0.125M-KCl and 0.004M-dATP, -dCTP and -dGTP. Assay mixtures with poly(rC)·oligo(dG) as template-primer (1 μ g) contained 0.001M-MnCl₂, 1 μ Ci of [³H]dGTP, 0.05M-Tris/HCl (pH7.5), 1mM-dithiothreitol, 0.125M-KCl and 0.004M-dATP, -dCTP and -dTTP. Total incubation volume 0.15ml. Other methods as described in text. nt, Not tested.

DNA Polymerase	Template-primer	Spleen anti-(reverse transcriptase) IgG (μ g)	...	Percentage of inhibition *					
				2	4	8	16	32	64
α	'Activated' DNA			nt	nt	0	0	0	0
β	Poly[d(A-T)]			nt	nt	0	0	0	0
γ	Poly(rA)·(dT) ₁₂			nt	nt	0	1.7	12.8	15.0
Reverse transcriptase	Poly(rC)·oligo(dG)			8.8	19.8	35.7	49.0	71.2	78.1

* Data expressed as the percentage of inhibition of the DNA polymerase tested with immune IgG compared with the same quantity of pre-immune (control) IgG.

Table 3. *Responses of reverse transcriptase from other sources to the myelofibrotic spleen anti-(reverse transcriptase) IgG* Pre-immune (control) and immune anti-(myelofibrotic spleen reverse transcriptase) IgG were prepared at concentrations of 4–64 μg per 0.025 ml of 0.05M-Tris/HCl, pH7.5. Gibbon-ape leukaemia virus reverse transcriptase and avian myeloblastosis virus reverse transcriptase were prepared at concentrations of 1 μg per 0.025 ml of 0.05M-Tris/HCl, pH7.5. Simian sarcoma (woolly monkey) virus reverse transcriptase was prepared at a concentration of 0.032 μg per 0.025 ml of Tris/HCl, pH7.5, and myelofibrotic spleen reverse transcriptase was prepared at a concentration of 0.5 μg per 0.025 ml of Tris/HCl, pH7.5. Each reverse transcriptase was incubated for 16h at 4°C with an equal volume (4–64 μg) of pre-immune or immune IgG. Remaining activity was measured in a ^3H -DNA polymerase assay as described in the text (total incubation volume 0.15 ml).

Source of reverse transcriptase	Template-primer	Spleen anti-(reverse transcriptase) IgG (μg) ...	Percentage of inhibition*				
			4	8	16	32	64
Simian sarcoma virus	Poly(rA)·(dT) ₁₂		4.2	11.8	30.2	54.0	51.0
Gibbon-ape leukaemia virus	Poly(rA)·(dT) ₁₂		0	0	5.7	12.6	22.2
Avian myeloblastosis virus	Poly(rA)·(dT) ₁₂		0	0	1.1	6.7	7.7
Human spleen	Poly(rC)·oligo(dG)		19.8	35.7	49.0	71.2	78.1

* Data expressed as the percentage of inhibition of the reverse transcriptase tested with immune IgG compared with the same quantity of pre-immune (control) IgG.

Table 4. *Effect of anti-(reverse transcriptase) IgG from other sources on the reverse transcriptase isolated from human spleen* Anti-(reverse transcriptase)IgG from avian myeloblastosis virus, Rauscher leukaemia virus, gibbon-ape leukaemia virus, simian sarcoma virus and human myelofibrotic spleen were prepared at concentrations of 4–32 μg per 0.025 ml of Tris/HCl, pH7.5. Reverse transcriptase (0.025 ml, 0.5 μg) from human myelofibrotic spleen was incubated for 16h at 4°C with an equal volume of pre-immune or immune anti-(reverse transcriptase) IgG. DNA polymerase assay of the mixtures utilized poly(rC)·oligo(dG) as template-primer, Mn^{2+} as bivalent cation and 2 μCi of [^3H]dGTP; all other conditions are as described in the text, in a total incubation volume of 0.15 ml.

Source of anti-(reverse transcriptase) IgG	Anti-(reverse transcriptase) IgG (μg) ...	Reverse transcriptase of human spleen Percentage of inhibition*			
		4	8	16	32
Avian myeloblastosis virus		0	3.3	7.4	6.4
Rauscher leukaemia virus		0	1.3	12.7	14.0
Gibbon-ape leukaemia virus		9.7	42.4	55.8	60.4
Simian sarcoma virus		30.1	61.4	75.4	76.3
Human spleen		19.8	35.7	49.0	78.1

* Data expressed as the percentage of inhibition of the reverse transcriptase tested with immune IgG compared with the same quantity of pre-immune (control) IgG.

studies with reverse transcriptases isolated from simian sarcoma virus, gibbon-ape leukaemia virus and avian myeloblastosis virus utilizing antisera against the spleen reverse transcriptase. The data show inhibition of the simian sarcoma virus and gibbon-ape leukaemia virus enzymes of 51% and 22.2% respectively, at 64 μg of IgG. Avian myeloblastosis virus reverse transcriptase was not significantly inhibited. It would appear that the reverse transcriptase isolated from human spleen is antigenically related to the simian sarcoma virus enzyme, and the gibbon-ape leukaemia virus enzyme to a lesser extent. Table 4 supports this idea; anti-reverse transcriptase IgG from simian sarcoma virus inhibits the splenic reverse transcriptase by 76.3% with 64 μg of

IgG, whereas gibbon-ape leukaemia virus anti-reverse transcriptase IgG inhibits the splenic reverse transcriptase by 60.4% with 64 μg of IgG. The anti-(reverse transcriptase) IgG from two non-primate sources, avian myeloblastosis virus and Rauscher leukaemia virus, has a very low inhibitory effect.

Data from other laboratories further support these observations. Reverse transcriptase isolated from human acute-myeloblastic-leukaemia cells has shown cross-reactivity only with gibbon-ape leukaemia virus and simian sarcoma virus (Todaro & Gallo, 1973). Simian sarcoma virus was found to be the closest to, but not identical with, HL23V-1, a human leukaemia virus purified from long-term cultures of myeloid cells from a leukaemia patient (Teich *et al.*, 1975).

Nooter *et al.* (1975) have described the isolation of a virus related to simian sarcoma virus from human leukaemia cells.

Discussion

The results reported in the present paper are useful for two reasons. First, it is the first DNA polymerase isolated from a human malignant tissue that strictly meets the biochemical and immunological criteria defining a reverse transcriptase; the other enzyme that also meets these requirements is the reverse transcriptase from human leukaemic cells (Gallo *et al.*, 1975). The fact that 3 weeks after surgery this child developed acute myelogenous leukaemia makes this study even more interesting, indicating the prognostic role of this enzyme in human malignancy. Secondly, the application of antibodies to this human reverse transcriptase is the first approach to establish the serological relatedness of the human enzyme to reverse transcriptases from the primate tumour viruses or endogenous viruses. The fact that our antibodies do not inhibit the DNA polymerase activity of baboon endogenous virus indicates that the oncornaviral information in the human spleen was not derived from the endogenous virus, but was rather a consequence of some infective pathway. This is analogous to the observations by Mayer *et al.* (1974), who were able to inhibit the reverse transcriptase activity from Rhesus monkey placenta by antibodies to reverse transcriptase from baboon endogenous virus, but failed to inhibit the same activity by antibodies to reverse transcriptases from primate RNA tumour viruses. Mayer *et al.* (1974) have implied that the reverse transcriptase activity of Rhesus monkey placenta must have been derived from an endogenous virus, i.e. class I viruses according to the nomenclature of Gillespie & Gallo (1975).

Gallo *et al.* (R. C. Gallo, unpublished work) have isolated, from plasma membranes of blood and bone-marrow cells of patients with acute myelogenous leukaemia, natural antibodies that do not react with the reverse transcriptases from RNA tumour viruses of primate origin, but consistently react very strongly with reverse transcriptase from HL-23 virus, isolated from an acute myelogenous leukaemia patient. They have found that these antibodies had a very strong neutralizing effect on our reverse transcriptase; this was comparable with the inhibition of their HL-23 virus reverse transcriptase by these antibodies. This suggests that their reverse transcriptase from an acute myelogenous leukaemia patient, and our reverse transcriptase from a patient with a pre-leukaemic disease, are antigenically almost identical.

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