Characterization of a DNA Polymerase Activity in Cultured Human Melanoma Cells That Copies Poly(2'-O-methylcytidylate)*

(Received for publication, February 1, 1979)

Gary F. Gerard, Paul M. Loewenstein,‡ and Maurice Green§

From the Institute for Molecular Virology, St. Louis University School of Medicine, St. Louis, Missouri 63110

While utilizing poly(2'-O-methylcytidylate)-oligo-deoxyguanylyl ate ([CM]p-[(dG)12-18]) to assay for DNA polymerase activity during fractionation of total cell extracts of cultured human, malignant cells, a new DNA polymerase activity called DNA polymerase Cm was identified in the human melanoma cell line A-375. This activity, which was not associated with particles with the density of RNA tumor viruses, was purified from the cytoplasmic fraction some 13,000-fold by sequential chromatography on DEAE-cellulose, phosphocellulose, CM-Sephadex, and poly(Cm)-Sepharose. Purified DNA polymerase Cm was identified in the human melanoma cell line A-375. This enzyme activity is not associated with particles and differs from retrovirus reverse transcriptase in several important respects; it also appears to be distinct from the cellular DNA polymerases thus far identified.

Postmitochondrial cytoplasmic and/or nuclear extracts of cultured human cells contain at least three different species of DNA polymerase, designated α, β, and γ (1, 2). At least one laboratory (3) has reported the presence in human cell nuclei of another species of DNA polymerase, polymerase N3, distinct from polymerases α, β, and γ. Mitochondria isolated from human cells also contain at least one DNA polymerase species that resembles DNA polymerase γ (2, 4). In addition to these enzymes, some human tumor cells contain a cytoplasmic, particle-associated DNA polymerase with biochemical (5-9) and sometimes immunological (5, 8, 9) properties identical with primate retrovirus reverse transcriptase. One of the properties that differentiates retrovirus reverse transcriptase and the human particle-associated DNA polymerase from DNA polymerase α, β, γ, and N3 is the ability to effectively copy the template-primer poly(2'-O-methylcytidylate)-oligo-deoxyguanylate ([CM]p-[(dG)12-18]) (7, 10-13). We report here on the isolation and characterization of a DNA polymerase activity with the capacity to copy poly(dG)12-18 that is found in extracts of the human melanoma cell line A-375. This enzyme activity is not associated with particles and differs from retrovirus reverse transcriptase in several other important respects; it also appears to be distinct from the cellular DNA polymerases thus far identified.

* This work was supported by Contract NO1 CP 61049 within the Virus Cancer Program of the National Cancer Institute. The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

‡ To whom reprint requests should be addressed.

§ United States Public Health Service Research Career Awardee (5K6-Al4739) of the National Institute of Allergies and Infectious Diseases.

MATERIALS AND METHODS

Reagents—[3H]Deoxyribonucleoside triphosphates, [α-32P]dCTP, and [α-32P]dATP were purchased from New England Nuclear. Calf thymus DNA (type IV) from the Sigma Chemical Co. was activated by the procedure of Schlabach et al. (13). The oligonucleotides (dG)12-18 and (dA)18 were obtained from Collaborative Research, Inc. (Cm), (A)m, (A)n, bovine serum albumin (albumin, Pentex), and ovalbumin (5x crystallized, electrophoretically pure) were purchased from Miles Laboratories. (Cm), and (dA), were from P-L Biochemicals, Inc. Nondet P-40 (NP-40) was obtained from Shell Chemical, London, England. Whatman DEAE-cellulose (DE22 and DE52), phosphocellulose (P-11), and DEAE-cellulose (DE81) paper discs were provided by Rhee Angel. Sephadex G-100 (superfine), CM-Sephadex (C-25), and Sepharose 4B were from Pharmacia. Antisera to simian sarcoma virus and RD-114 virus-purified reverse transcriptase, obtained by inoculation of rabbits (14), were provided by Dr. J. Aaronson, National Cancer Institute. Purified IgG from antisera against human DNA polymerase α prepared in rats (15) was a gift from Dr. R. Gallo, National Cancer Institute. Normal rat IgG was purchased from Miles Laboratories.

Preparation of Poly(Cm) -Sephadex and Heparin-Sepharose—Poly(2'-O-methylcytidylate) (50 Å2 units) was coupled to 10 ml of cyanogen bromide-activated Sepharose 4B as described (18). Routinely, 60% of the input Å2 units were bound to the activated Sepharose. Heparin (Sigma, type I) was coupled to cyanogen bromide-activated Sepharose 4B in 0.1 M NaHCO3.

Viruses—Simian sarcoma virus complex (SSV/SSAV) was produced in chronically infected human NC-37 cells and baboon endogenous virus in the dog thymus BK-CT cell line. Purified preparations of these viruses were provided by the Pfizer Laboratories, Maywood, N.J., through the auspices of the Office of Program Resources and Logistics of National Cancer Institute and the assistance of Dr. J. Gruber.

The abbreviations used are: (Cm)p, poly(2'-O-methylcytidylate); albumin, bovine serum albumin; SSV/SSAV, simian sarcoma virus complex; Tis, 2'-amino-2-(hydroxymethyl)-1, 3-propanediol; EDTA, (ethylenedinitrilotetraacetic acid; IgG, immunoglobulin G.

G. F. Gerard, P. M. Loewenstein, and M. Green, unpublished data.
Cells—The human cell lines, A-375 and A-1188, derived from a melanoma metastasis and a lung carcinoma (19, 20), were provided by Dr. S. Aaronson, National Cancer Institute. A large number of clones of the A-375 cell line were established by Dr. N. Takemori of this laboratory. Clone 1 was used in these studies. A-375, clone 1, and A-1188 cells were mycoplasma free (21). Human embryonic fibroblast cultures were obtained from the laboratory of Dr. S. Aaronson and were purchased from Flow Laboratories and designated Flow 5000. A low passage, human, diploid, skin fibroblast cell line, A-1040, was provided by Dr. A. Freeman (Torrey Pines Research Center, La Jolla, Calif.).

Tissue Culture Methods—A-375 and A-1188 cells were grown in roller culture in Dulbecco's modified Eagle's medium supplemented with 10% heated fetal calf serum. The medium for A-1040 cells contained 1% sodium pyruvate. Cells were harvested by scraping, washed with phosphate-buffered saline, pelleted, and stored at -70°C.

Assays of DNA Polymerase Activities—DNA polymerase assays were carried out in 100-μl reaction mixtures at 37°C by monitoring the formation of labeled DNA. The details of each assay are described in figure legends and table footnotes.

Purification of DNA Polymerase Cm—Unless indicated otherwise, all operations were performed at 4°C. Buffer A is 50 mM Tris- HCl (pH 7.8), 1 mM dithiothreitol, and 10% glycerol. Buffer B is 50 mM Tris- HCl (pH 7.8), 1 mM dithiothreitol, 0.5% NP-40, and 10% glycerol. Buffer C is 20 mM Tris- HCl (pH 7.8), 1 mM dithiothreitol, 0.02% NP-40, and 10% glycerol. Buffer D is 20 mM Tris- HCl (pH 8.0), 1 mM dithiothreitol, 200 mM NaCl, 0.2% NP-40, and 10% glycerol.

Fifty grams of frozen cells were thawed, suspended in 2 volumes of 50 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, and 1 mM EDTA, and broken in a Dounce homogenizer (total cell extract). The nuclei were separated from the cytoplasm by centrifugation (1,000 g for 10 min). The nuclear pellet was further Dounce homogenized with 70 ml of 10 mM Tris-HCl (pH 7.8), 1 mM dithiothreitol, 0.5% NP-40, and 10% glycerol. Buffer D is 20 mM Tris-HCl (pH 8.0), 1 mM dithiothreitol, 200 mM NaCl, 0.2% NP-40, and 10% glycerol.

The cytoplasmic fraction and the Triton wash supernatant were separated from the cytoplasm by centrifugation (1,000 g for 10 min). The nuclei were broken in a Dounce homogenizer (total cell extract). The nuclei were isolated by centrifugation (100,000 g for 1 h). The peak fractions from phosphocellulose chromatography and dialysis. The sample was then treated in either of two ways. For analysis of samples treated in the second way and incubated with DNA polymerase Cm, poly(Cm)-Sepharose, concentrated by dialysis, and dialyzed against Buffer C. The gradients were centrifuged for 27 h at 350,000 g. The sedimentation coefficient was estimated from ovalbumin and albumin as standards.

Because of the extreme instability of DNA polymerase Cm activity during affinity chromatography, it was essential to add albumin to the column eluate during poly(Cm)-Sepharose chromatography. In order to determine the protein concentration of the poly(Cm)-Sepharose-purified enzyme, column buffer was removed by adsorption of an aliquot of the enzyme to a small poly(Cm)-Sepharose column (0.9 × 1.5 cm) in Buffer C, and the column was washed extensively with Buffer C to remove albumin, and DNA polymerase Cm activity was quantitatively eluted from the column with Buffer C plus 0.25 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluate showed this to be an effective method for removing albumin.

Purification of DNA Polymerase β—DNA polymerase β activity was extracted from the final nuclear pellet derived from 50 g of A-375 cells and nucleic acids were removed by the method of Spadari and Weissbach (23). The nucleic acid-free material was then applied to a DE52 column as described for DNA polymerase Cm. DNA polymerase β activity, which did not bind to the DEAE-cellulose column, was fractionated on a phosphocellulose column as described for DNA polymerase Cm. DNA polymerase β eluted at 0.24 M NaCl. The peak fractions from phosphocellulose chromatography and dialysis. The sample was then treated in the second way. For analysis of samples treated in the second way and incubated with DNA polymerase Cm, poly(Cm)-Sepharose, concentrated by dialysis, and dialyzed against Buffer C. The gradients were centrifuged for 27 h at 350,000 g. The sedimentation coefficient was estimated from ovalbumin and albumin as standards.

Because of the extreme instability of DNA polymerase Cm activity during affinity chromatography, it was essential to add albumin to the column eluate during poly(Cm)-Sepharose chromatography. In order to determine the protein concentration of the poly(Cm)-Sepharose-purified enzyme, column buffer was removed by adsorption of an aliquot of the enzyme to a small poly(Cm)-Sepharose column (0.9 × 1.5 cm) in Buffer C, and the column was washed extensively with Buffer C to remove albumin, and DNA polymerase Cm activity was quantitatively eluted from the column with Buffer C plus 0.25 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluate showed this to be an effective method for removing albumin.

Purification of DNA Polymerase β—DNA polymerase β activity was extracted from the final nuclear pellet derived from 50 g of A-375 cells and nucleic acids were removed by the method of Spadari and Weissbach (23). The nucleic acid-free material was then applied to a DE52 column as described for DNA polymerase Cm. DNA polymerase β activity, which did not bind to the DEAE-cellulose column, was fractionated on a phosphocellulose column as described for DNA polymerase Cm. DNA polymerase β eluted at 0.24 M NaCl. The peak fractions from phosphocellulose chromatography and dialysis. The sample was then treated in the second way. For analysis of samples treated in the second way and incubated with DNA polymerase Cm, poly(Cm)-Sepharose, concentrated by dialysis, and dialyzed against Buffer C. The gradients were centrifuged for 27 h at 350,000 g. The sedimentation coefficient was estimated from ovalbumin and albumin as standards.

Because of the extreme instability of DNA polymerase Cm activity during affinity chromatography, it was essential to add albumin to the column eluate during poly(Cm)-Sepharose chromatography. In order to determine the protein concentration of the poly(Cm)-Sepharose-purified enzyme, column buffer was removed by adsorption of an aliquot of the enzyme to a small poly(Cm)-Sepharose column (0.9 × 1.5 cm) in Buffer C, and the column was washed extensively with Buffer C to remove albumin, and DNA polymerase Cm activity was quantitatively eluted from the column with Buffer C plus 0.25 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluate showed this to be an effective method for removing albumin.

Purification of DNA Polymerase β—DNA polymerase β activity was extracted from the final nuclear pellet derived from 50 g of A-375 cells and nucleic acids were removed by the method of Spadari and Weissbach (23). The nucleic acid-free material was then applied to a DE52 column as described for DNA polymerase Cm. DNA polymerase β activity, which did not bind to the DEAE-cellulose column, was fractionated on a phosphocellulose column as described for DNA polymerase Cm. DNA polymerase β eluted at 0.24 M NaCl. The peak fractions from phosphocellulose chromatography and dialysis. The sample was then treated in the second way. For analysis of samples treated in the second way and incubated with DNA polymerase Cm, poly(Cm)-Sepharose, concentrated by dialysis, and dialyzed against Buffer C. The gradients were centrifuged for 27 h at 350,000 g. The sedimentation coefficient was estimated from ovalbumin and albumin as standards.

Because of the extreme instability of DNA polymerase Cm activity during affinity chromatography, it was essential to add albumin to the column eluate during poly(Cm)-Sepharose chromatography. In order to determine the protein concentration of the poly(Cm)-Sepharose-purified enzyme, column buffer was removed by adsorption of an aliquot of the enzyme to a small poly(Cm)-Sepharose column (0.9 × 1.5 cm) in Buffer C, and the column was washed extensively with Buffer C to remove albumin, and DNA polymerase Cm activity was quantitatively eluted from the column with Buffer C plus 0.25 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluate showed this to be an effective method for removing albumin.

Purification of DNA Polymerase β—DNA polymerase β activity was extracted from the final nuclear pellet derived from 50 g of A-375 cells and nucleic acids were removed by the method of Spadari and Weissbach (23). The nucleic acid-free material was then applied to a DE52 column as described for DNA polymerase Cm. DNA polymerase β activity, which did not bind to the DEAE-cellulose column, was fractionated on a phosphocellulose column as described for DNA polymerase Cm. DNA polymerase β eluted at 0.24 M NaCl. The peak fractions from phosphocellulose chromatography and dialysis. The sample was then treated in the second way. For analysis of samples treated in the second way and incubated with DNA polymerase Cm, poly(Cm)-Sepharose, concentrated by dialysis, and dialyzed against Buffer C. The gradients were centrifuged for 27 h at 350,000 g. The sedimentation coefficient was estimated from ovalbumin and albumin as standards.

Because of the extreme instability of DNA polymerase Cm activity during affinity chromatography, it was essential to add albumin to the column eluate during poly(Cm)-Sepharose chromatography. In order to determine the protein concentration of the poly(Cm)-Sepharose-purified enzyme, column buffer was removed by adsorption of an aliquot of the enzyme to a small poly(Cm)-Sepharose column (0.9 × 1.5 cm) in Buffer C, and the column was washed extensively with Buffer C to remove albumin, and DNA polymerase Cm activity was quantitatively eluted from the column with Buffer C plus 0.25 M NaCl. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the eluate showed this to be an effective method for removing albumin.
bind to CM-Sephadex and eluted from poly(Cm)-Sepharose at 0.1 M NaCl.

Purification of DNA Polymerase α—DNA polymerase α was purified from the cytoplasmic fraction of A-375 cells by sequential chromatography on DEAE-cellulose, phosphocellulose, and heparin-Sepharose (24). DEAE-cellulose chromatography was performed as already described except that the column was eluted with a linear gradient of 0 to 0.5 M NaCl in Buffer D. The volume of the gradient exceeded that of the column by 10-fold. DNA polymerase α was pooled to exclude as much γ-polymerase activity as possible and was further purified on phosphocellulose as already described for DNA polymerase Cm. Finally, DNA polymerase α was separated from most contaminating γ-polymerase activity by chromatography on a 5-ml heparin-Sepharose column eluted with a linear gradient of 0 to 0.8 M KCl in Buffer D. DNA polymerase α eluted at 0.28 M NaCl. Heparin-Sepharose-purified DNA polymerase α copied activated calf thymus DNA 55-fold more efficiently than (A)n-(dT)12-18 under standard assay conditions, indicating minimal contamination with DNA polymerase γ. When subjected to gel filtration in Sephadex G-100, DNA polymerase α eluted in the void volume of the column.

RESULTS

Purification of DNA Polymerase Cm Activity from Cultured Human Melanoma Cells (A-375 Cell Line)

Total cell extracts of a number of established, malignant human cell lines were examined for soluble DNA polymerase activity responsive to the template-primer (Cm)n-(dG)12-18 (data not shown). Among the cell lines examined, a melanoma line, A-375 (20), contained the most of such DNA polymerase activity, i.e. 400 pmol of [3H]GTP incorporated/h/g of cells. During subcellular fractionation (“Materials and Methods”), greater than 80% of the (Cm)n-(dG)12-18-responding activity was found in the cytoplasmic extract (Table I). This activity was not retained by DEAE-cellulose in low salt (data not shown) and eluted from phosphocellulose at 0.18 M NaCl (Fig. 1). We have called this enzyme activity DNA polymerase Cm because of its ability to copy (Cm)n-(dG)12-18 and because of its apparent lack of identity with other human DNA polymerases thus far identified (see below). Assay of the same phosphocellulose column with (A)n-(dT)12-18 revealed three peaks of activity (Fig. 1), one of which eluted at slightly higher salt than the peak with (Cm)n-(dG)12-18. The other two peaks eluted at high salt (0.22 M and 0.44 M NaCl) and did not respond to (Cm)n-(dG)12-18 as template-primer.

The peak fractions of DNA polymerase Cm activity from phosphocellulose (Fig. 1) were pooled, dialyzed to remove salt, and subjected to CM-Sephadex chromatography (Fig. 2A). The majority of the DNA polymerase Cm activity adsorbed to the column and eluted as a single peak at 70 mM NaCl. Routinely, 10 to 30% of the activity responsive to (Cm)n-(dG)12-18 did not bind to the column. This material was not characterized further. A second minor peak of DNA polymerase responsive to (Cm)n-(dG)12-18 was sometimes observed eluting at 50 mM NaCl (Fig. 2A). This activity when present was pooled along with the major peak for further purification. Subsequent affinity chromatography and sedimentation analysis of this pooled DNA polymerase Cm activity revealed only uncharacterized activity. The peak fractions of DNA polymerase Cm activity from the CM-Sephadex column were pooled and subjected to CM-Sephadex chromatography as described under "Materials and Methods." Twenty-microliter samples of alternate column fractions (6 ml) were assayed for DNA polymerase activity during 60-min incubations with (A)n-(dT)12-18. Cm and (Cm)n-(dG)12-18 (●) as described in the legend to Fig. 1 except that assays with (Cm)n-(dG)12-18 contained 0.8 mM MnCl2. With (A)n-(dT)12-18 as template-primer (●), reaction mixtures contained the same constituents as in assays with (A)n-(dT)12-18 except that (dA)2-(dT)12-18 was substituted for (A)n-(dT)12-18. Twenty-microliter samples of alternate column fractions (5 ml) were assayed as described in A above.

Table I

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume (%)</th>
<th>Protein (mg)</th>
<th>Units/a</th>
<th>Specific activity (pmol/min per mg protein)</th>
<th>Purification Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total cell extract</td>
<td>100</td>
<td>5,500</td>
<td>21</td>
<td>&lt;0.01</td>
<td>1</td>
</tr>
<tr>
<td>Cytoplasmic extract</td>
<td>169</td>
<td>2,550</td>
<td>17</td>
<td>&lt;0.01</td>
<td>1.5</td>
</tr>
<tr>
<td>100,000 g supernatant</td>
<td>162</td>
<td>2,140</td>
<td>15</td>
<td>&lt;0.01</td>
<td>1.8</td>
</tr>
<tr>
<td>DEAE-cellulose</td>
<td>230</td>
<td>644</td>
<td>18</td>
<td>0.03</td>
<td>7</td>
</tr>
<tr>
<td>Phosphocellulose</td>
<td>65</td>
<td>50</td>
<td>9.7</td>
<td>0.19</td>
<td>49</td>
</tr>
<tr>
<td>CM-Sephadex concentrate</td>
<td>4.5</td>
<td>8.3</td>
<td>4.5</td>
<td>0.54</td>
<td>116</td>
</tr>
<tr>
<td>Poly(Cm)-Sepharose concentrate</td>
<td>0.07</td>
<td>3.6</td>
<td>3.6</td>
<td>51.4</td>
<td>12,860</td>
</tr>
</tbody>
</table>

a A unit is defined as the incorporation of 1 nmol of [3H]GTP into polydeoxyxynucleotide/h at 37°C with (Cm)n-(dG)12-18 as template-primer.

b Albumin was removed prior to determination of protein in this sample.
a single species of enzyme (Figs. 2B and 3B). We, therefore, conclude that A-375 cells contain one major species of DNA polymerase Cm activity, and assume the variable appearance of the minor peak to be an artifact of CM-Sephadex fractionation.

The pooled peak fractions of DNA polymerase Cm activity from CM-Sephadex were dialyzed and chromatographed on poly(Cm)-Sepharose (Fig. 2B). All of the DNA polymerase Cm activity was adsorbed and eluted as a single peak at 90 mM NaCl. DNA polymerase activity with (A),..(dT)12-ls, (Cm),.(dG)12-18, and (dA),.(dT)12-18 co-eluted with activity responsive to (Cm),.(dG)12-18. The presence of albumin (500 µg/ml) was essential during affinity chromatography to stabilize DNA polymerase Cm activity. It should be emphasized that SSV(SSAV) or Moloney murine leukemia virus reverse transcriptase when subjected to poly(Cm)-Sepharose chromatography elutes as a single peak at 0.32 M NaCl.

Summary of Purification

Table I summarizes the purification of DNA polymerase Cm from A-375 cells. After poly(Cm)-Sepharose, the enzyme had a specific activity of 51 units/mg of protein with (Cm),.(dG)12-18 as template-primer and was purified some 13,000-fold. Poly(Cm)-Sepharose-purified enzyme concentrated by dialysis and then dialyzed against Buffer C plus 40% glycerol when stored at -70°C in the presence of 10 mg/ml of albumin lost 50% and 80% of its activity in 1 and 5 weeks, respectively.

Unless indicated otherwise, the studies described subsequently were performed with poly(Cm)-Sepharose-purified enzyme.

Properties of Purified DNA Polymerase Cm

All attempts to determine the molecular weight of poly(Cm)-Sepharose-purified DNA polymerase Cm by Sephadex G-100 gel filtration failed due to inactivation of the enzyme. However, DNA polymerase Cm was stable during Sephadex G-100 gel filtration if the procedure was performed after phosphocellulose chromatography. Fig. 3A shows an elution profile of DNA polymerase Cm from Sephadex G-100 in 0.2 M NaCl. The enzyme eluted at a volume consistent with a molecular weight of 50,000.

Poly(Cm)-Sepharose-purified DNA polymerase Cm had a sedimentation coefficient of 3.4 S determined by sucrose gradient sedimentation in 0.2 M NaCl (Fig. 3B). The sedimentation coefficient of DNA polymerase Cm purified through phosphocellulose was identical. The enzyme tended to aggregate and lose activity if sedimentation was carried out in the absence of salt. Assuming a globular shape for DNA polymerase Cm, a sedimentation coefficient of 3.4 S corresponds to a molecular weight of 40,000 (25). The fact that DNA polymerase Cm eluted ahead of ovalbumin from Sephadex G-100 but sedimented more slowly than ovalbumin suggests that DNA polymerase Cm has a higher axial ratio than ovalbumin.

Divalent and monovalent cation and pH optima were determined for DNA polymerase Cm with (Cm),.(dG)12-18, (A),.(dT)12-18, and (dA),.(dT)12-18 (data not shown). With all three template-primers, the best divalent metal ion for activity was Mn2+. The optimum with each template-primer was similar and centered around 0.8 to 1.2 mM Mn2+. With (Cm),.(dG)12-18 and (dA),.(dT)12-18, reaction rates with 2 mM Mg2+ would not substitute for Mn2+, while with (dA),.(dT)12-18, reaction rates with 2 mM Mg2+ were 1/4 those with 1 mM Mn2+.

The optimal NaCl concentration for activity with each template-primer was different. With (Cm),.(dG)12-18, (A),.(dT)12-18, and (dA),.(dT)12-18, the optima were 15 mM, 50 mM, and 80 to 100 mM, respectively. The higher salt optima with the A-containing templates probably reflect the requirement for a certain amount of salt to maintain hydrogen bonding between template and primer. Regardless of the template-primer, DNA polymerase Cm was almost totally inhibited at 200 mM NaCl. The pH optimum with each template-primer in 20 mM Tris-HCl was 8.2.

The ability of DNA polymerase Cm to copy synthetic template-primers and activated calf thymus DNA is summarized in Table II. Reaction conditions determined to be optimal for activity with various synthetic template-primers were employed. Activated calf thymus DNA was assayed in the presence of 10 mM MgCl2. The best template-primer for DNA polymerase Cm is (A),.(dT)12-18. The enzyme used (A),.(dT)12-18 some 2.8-fold better than (dA),.(dT)12-18 and 20-fold better than (Cm),.(dG)12-18 or (Cm),.(dG)12-18. DNA polymerase Cm used activated DNA as a template as expected of a true DNA polymerase, but had little or no terminal transferase activity with (dG)12-18 or (dT)12-18. Incorporation was linear for at least 2 h with (A),.(dT)12-18, (Cm),.(dG)12-18, and (dA),.(dT)12-18 (data not shown).

Comparison of Purified DNA Polymerase Cm to Other DNA Polymerases

Comparison to Retrovirus Reverse Transcriptases—In all of the instances where a DNA polymerase resembling retroviral reverse transcriptase has been isolated from human cells, the enzyme was associated with particles (5–9). Repeated attempts to identify particle-associated reverse transcriptase activity utilizing (Cm),.(dG)12-18 (26) or the "simultaneous
DNA Polymerase Activity in Human Cells That Copies (Cm)

TABLE II
Comparison of template specificity of DNA polymerase Cm and β purified through the poly(Cm)-Sepharose chromatography step

<table>
<thead>
<tr>
<th>Template-Primer</th>
<th>Substrates</th>
<th>NaCl</th>
<th>pmol of Labeled deoxyribonucleotide incorporated/30 min/μl of enzyme</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>Cm</td>
</tr>
<tr>
<td>(Cm)5-(dG)12-18</td>
<td>[3H]dATP, dCTP</td>
<td>15</td>
<td>0.40</td>
</tr>
<tr>
<td>(Cm)5-(dG)12-18</td>
<td>[3H]dGTP</td>
<td>15</td>
<td>0.38</td>
</tr>
<tr>
<td>(dA)9-(dA)12-18</td>
<td>[3H]dATP, dCTP</td>
<td>50</td>
<td>8.12</td>
</tr>
<tr>
<td>(dA)9-(dA)12-18</td>
<td>[3H]dGTP</td>
<td>50</td>
<td>16.5</td>
</tr>
<tr>
<td>Activated calf thymus DNA</td>
<td>[3H]dATP, dCTP</td>
<td>50</td>
<td>0.38</td>
</tr>
<tr>
<td>(dT)12-18-(A)12</td>
<td>[3H]dATP, dCTP</td>
<td>80</td>
<td>0.54</td>
</tr>
<tr>
<td>(dT)12-18-(A)12</td>
<td>[3H]dGTP</td>
<td>15</td>
<td>0.02</td>
</tr>
<tr>
<td>(dT)12-18-(A)12</td>
<td>[3H]dATP, dCTP</td>
<td>15</td>
<td>0.02</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Reaction mixture description</th>
<th>Labeled substrate</th>
<th>pmol of Labeled deoxyribonucleotide incorporated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Complete</td>
<td>[3H]dATP, dCTP</td>
<td>7.5</td>
</tr>
<tr>
<td>-Mg2+ or -DNA</td>
<td>[3H]dATP, dCTP</td>
<td>0</td>
</tr>
<tr>
<td>-NaCl</td>
<td>[3H]dATP, dCTP</td>
<td>3.7</td>
</tr>
<tr>
<td>-MgCl2 + 0.8 mM MnCl2</td>
<td>[3H]dATP, dCTP</td>
<td>0.3</td>
</tr>
<tr>
<td>-Bovine serum albumin</td>
<td>[3H]dATP, dCTP</td>
<td>5.9</td>
</tr>
<tr>
<td>-dGTP</td>
<td>[3H]dATP, dCTP</td>
<td>7.2</td>
</tr>
<tr>
<td>-dGTP, -dTTP, -dCTP</td>
<td>[3H]dATP, dCTP</td>
<td>6.5</td>
</tr>
<tr>
<td>Complete</td>
<td>[3H]dGTP</td>
<td>9.1</td>
</tr>
<tr>
<td>Complete</td>
<td>[3P]dCTP</td>
<td>3.8</td>
</tr>
</tbody>
</table>

* Aliquot (6 μl) of poly(Cm)-Sepharose-purified DNA polymerase Cm were assayed for 30 min. The complete reaction mixture contained 20 mM Tris-Cl (pH 8.2), 2 mM dithiothreitol, 0.8 mM NaCl, 60 μg/ml of albumin, 10 mM MgCl2, 100 μg/ml of activated calf thymus DNA, and 50 μM concentration of each of three unlabeled deoxyribonucleoside triphosphates, and 50 μM concentration of one labeled triphosphate ([3H]dATP, 1300 cpm/pmol; [3H]dCTP, 2700 cpm/pmol; or [3P]dCTP, 1600 cpm/pmol).

not shown.

In a competitive radioimmune assay designed to detect with maximum sensitivity interspecies cross-reactivity between reverse transcriptases (30), no reverse transcriptase-related protein (limit of sensitivity 25 ng/ml of protein in reverse transcriptase) could be detected in a DNA polymerase Cm preparation purified through the CM-Sepharose step. These results indicate a complete absence of antigenic relationship between A-375 cell DNA polymerase Cm and the reverse transcriptases of some primate and most mammalian subprimate retroviruses.

Comparison to Cellular DNA Polymerases—The reaction requirements of DNA polymerase Cm with activated calf thymus DNA as template-primer are shown in Table III. The enzyme resembles other DNA polymerases in requiring a template-primer and a divalent metal ion for activity. Interestingly, A-375 cell DNA polymerase Cm resembles DNA polymerase β purified from other cultured human cells (1, 2, 31) in its ability to incorporate a single deoxyribonucleoside triphosphate in the presence of activated calf thymus DNA (Table III). To help clarify the relationship between DNA polymerase Cm and DNA polymerase β in A-375 cells, both enzymes were purified by the same procedure ("Materials and Methods," see Table I) and were compared with respect to substrate specificity under identical assay conditions (Table II) and sensitivity to inhibition by N-ethylmaleimide. DNA polymerase β was unable to copy either (Cm)5-(dG)12-18 or (Cm)5-(dG)12-18 and used (dA)9-(dA)12-18 twice as efficiently as (dA)9-(dA)12-18 (Table II), consistent with previously published data (10, 23, 28, 32). These results are in contrast to those already described for DNA polymerase Cm (Table II), which copied (Cm)5-(dG)12-18 and (Cm)5-(dG)12-18 and utilized (dA)9-(dA)12-18 and (dA)9-(dA)12-18 better than (dA)9-(dA)12-18 (Table II). A-375 DNA polymerase Cm and β also clearly differ in sensitivity to N-ethylmaleimide at all concentrations tested between 0.001 mM and 10 mM. Consistent with results from other laboratories (1, 2), β-polymerase activity was completely resistant to inhibition with either (dA)9-(dA)12-18 or (A)9-(dT)12-18 as template-primer. Cm-polymerase was inhibited 50% with either (Cm)5-(dG)12-18 or (dA)9-(dT)12-18 as template-primer at approximately 0.01 mM N-ethylmaleimide and was inhibited 90 to 100% at 0.7 mM.

To test the relationship between A-375 DNA polymerase Cm and DNA polymerase α, the effect on both enzymes of...
anti-DNA polymerase α IgG (15) was determined. While A-375 cell α-polymerase was inhibited 90% at the highest anti-α IgG concentration tested (72 μg/ml of protein) with activated calf thymus DNA as template-primer, Cm-polymerase was inhibited only 8% with (A)₉-(dT)₁₂-₁₈ as template-primer. Cm-polymerase and α-polymerase were inhibited 11% and 14%, respectively, by a similar concentration of normal IgG. Therefore, the activity of DNA polymerase Cm is not inhibited by anti-DNA polymerase α IgG, suggesting a lack of antigenic relatedness.

Distribution in Other Cultured Human Cells

Several other cultured human malignant cell lines, as well as an adult normal line and an embryonic line, have been examined by us for the presence of DNA polymerase Cm. In these experiments, total extracts (33) from 10 g of cells were subjected to DEAE-cellulose filtration and chromatography as already described ("Materials and Methods"). The DEAE-cellulose flow through material and that which eluted with 0.5 M KCl from DEAE-cellulose were subjected separately to phosphocellulose chromatography on columns (0.9 x 20 cm). The phosphocellulose columns were assayed for DNA polymerase α, β, γ, and Cm activity. A line (A-1188) derived from a lung carcinoma had about one-half the DNA polymerase Cm activity of A-375 cells. The adult human, diploid, skin fibroblast cell line, A-1040, and the embryonic cell line, Flow 5000, were devoid of detectable DNA polymerase Cm activity (data not shown).

DISCUSSION

Examination of cultured human malignant cells for DNA polymerase activity capable of copying (Cm)₉-(dG)₁₂-₁₈ has led to the identification and characterization of an apparently new DNA polymerase activity in the cultured human melanoma cell line A-375. Because of its ability to copy (Cm)₉-(dG)₁₂-₁₈, we have called the enzyme activity DNA polymerase Cm. The ability to effectively copy (Cm)₉-(dG)₁₂-₁₈ is thought to be unique to reverse transcriptase (11). By definition, however, a putative reverse transcriptase must also be capable of copying heteropolymeric RNA. The preparations of A-375 cell DNA polymerase Cm thus far tested do not manifest this ability. DNA polymerase Cm differs from retrovirus reverse transcriptase and the human particle-associated reverse transcriptases reported in the literature (5-9) in several other important respects. (i) DNA polymerase Cm is smaller than mammalian retrovirus reverse transcriptases, which have molecular weights in the monomeric form of 70,000 to 80,000 (5-9, 12, 34-37). (ii) Preparations of particle-associated reverse transcriptase isolated from cells taken from patients with several types of malignancies (5, 7-9) were found to be immunologically related to primate reverse transcriptase, particularly SSV(SSAV) reverse transcriptase. In contrast, no antigenic relationship could be established between A-375 Cm-polymerase and any reverse transcriptase tested, including SSV(SSAV), RD-114, and mammalian subprimate reverse transcriptase. As has been emphasized (38), however, immunological relationships between putative human reverse transcriptase isolates and retrovirus reverse transcriptases have been established for only a minority of the human enzyme preparations tested. (iii) A-375 Cm-polymerase elutes at substantially lower salt than SSV(SSAV) reverse transcriptase from the affinity matrix poly(Cm)-Sepharose (Fig. 2B), indicating a substantial difference between these enzymes in binding affinity for this polynucleotide. (iv) DNA polymerase Cm in A-375 cells is not associated with intracytoplasmic particles, as have been all of the putative human reverse transcriphtases isolated thus far (5-9). A distinction must, therefore, be made between DNA polymerase Cm detected in A-375 cells with (Cm)₉-(dG)₁₂-₁₈ and the particle-associated DNA polymerase detected with (Cm)₉-(dG)₁₂-₁₈ in various human malignant tissues (7, 28, 39). Such human particles have been shown to have many of the biochemical and physical properties of retrovirus type C virions (5-7, 12, 38), e.g. they contain high molecular weight RNA. It is assumed that the reverse transcriptase within such human particles ultimately serves its function by copying the particle RNA. A lack of association between DNA polymerase Cm and intracytoplasmic particles suggests that Cm-polymerase might serve a different function within the cell. (v) DNA polymerase Cm can utilize a synthetic oligoribonucleotide to prime DNA-directed DNA synthesis (Table II), while mammalian reverse transcriptase does not (28).

DNA polymerase Cm resembles DNA polymerase β in size (1, 2), its molecular weight being 40,000 to 50,000, in behavior on DEAE-cellulose and poly(Cm)-Sepharose, and in ability to incorporate one deoxyribonucleoside triphosphate in the presence of activated cell DNA. Cm-polymerase differs from A-375 cell β-polymerase, however, in a number of properties. The properties include: (i) the salt concentration required for elution from phosphocellulose, Cm-polymerase elutes at 0.18 M and β-polymerase at 0.44 M NaCl (Fig. 1); (ii) sensitivity to inhibition by salt and N-ethylmaleimide, β-polymerase is completely resistant at concentrations of NaCl and N-ethylmaleimide that inhibit Cm-polymerase 90% to 100%; (iii) subcellular distribution determined with aqueous fractionation techniques, Cm-polymerase is found almost totally in the cytoplasmic fraction, while β-polymerase is found primarily in the nuclear fraction; (iv) size as determined by Sephadex G-100 gel filtration, Cm-polymerase consistently eluted before and β-polymerase after ovalbumin from Sephadex G-100 (Fig. 3); and (v) template specificity (Table II).

These observations are consistent with several different interpretations concerning DNA polymerase Cm in A-375 cells. The first alternative is that DNA polymerase Cm is a new species of human cell DNA polymerase capable of copying (Cm)₉-(dG)₁₂-₁₈ but not heteropolymeric RNA. If this is the case, then by analogy with other vertebrate cell DNA polymerases (1, 2), the genetic information necessary to encode enzymes similar to DNA polymerase Cm should be present in the genome of other animal species. This alternative can be tested. The second alternative is that DNA polymerase Cm is a human reverse transcriptase antigenically distinct from reverse transcriptases found in mammalian retroviruses. According to this interpretation, DNA polymerase Cm is derived from expression of endogenous proviral information within A-375 cells. This information would be sufficiently different from viral information coding for subhuman primate reverse transcriptases to exclude detectable antigenic crossreactivity between enzymes. The lack of ability of DNA polymerase Cm to copy heteropolymeric RNA could reflect a lack of suitable in vitro assay conditions for demonstration of activity, the presence in enzyme preparations of inhibitory contaminants, or inactivation of certain catalytic capabilities during purification. Such inactivation and the small size of DNA polymerase Cm might be the result of proteolytic cleavage during isolation of a larger enzyme molecule. Moelling (36) has shown that proteolytic cleavage of murine RNA tumor virus reverse transcriptase can generate enzyme which maintains its capacity to copy homopolymeric RNA but has a reduced ability to transcribe heteropolymeric RNA. In this regard, we have attempted to purify DNA polymerase Cm from A-375 cells in the presence of the protease inhibitor...
phenylmethylsulfonyl fluoride. Enzyme with properties similar to those reported here was obtained after the phosphocellulose chromatography step. It is also possible that DNA polymerase a (40) and Cm-polymerase (41) have protein subunits with molecular weights in the 60,000 to 80,000 range. The negative serological analysis with anti-DNA polymerase a IgG argues against but does not exclude the possibility that Cm-polymerase is derived from a-polymerase. Finally, there is precedent for the presence in eukaryotic cells of a small protein factor(s) which binds to Cm-polymerase is derived from a-polymerase. Further studies on the biochemical and immunological properties of more highly purified preparations of DNA polymerase Cm and of its distribution in eukaryotic cells will be necessary to clarify these issues.

The specific activity of purified DNA polymerase Cm (Table II) as template-primer was 1000 units/mg of protein. Approximately 1.4 units of DNA polymerase Cm activity/g of A-375 cells was recovered after poly(Cm)-Sepharose chromatography. In order to place this level of DNA polymerase Cm activity in A-375 cells in perspective, comparison must be made to the levels in dividing human cells of some other DNA polymerase species purified to specific activities comparable to Cm-polymerase. The amount of DNA polymerase activity per gram of starting material recovered with a specific enzymatic activity in the range of 200 to 1000 units/mg of protein was 300 for KB cell a-polymerase (40), 30 for HeLa cell (41) and lymphocyte (27) Cm-polymerase, respectively, 2 for KB cell Cm-polymerase (43), 1 for A-375 cell Cm-polymerase, and 0.1 for particle-associated reverse transcriptase from human leukemic spleen cells (6).

Careful analysis of cell lines established from a melanoma metastasis (A-375) and a lung carcinoma (A-1188) has demonstrated the presence of DNA polymerase Cm. Preliminary screening of four other cell lines established from melanoma metastases or carcinomas indicates the presence of the enzyme, although in quantities smaller than in the cell lines mentioned above. Fibroblast lines established from human foreskin (A-1040) and a total embryo explant (Flow 5000) lacked detectable DNA polymerase Cm. These results suggest that perhaps DNA polymerase Cm is expressed only in malignant cells. Interestingly, however, a human cell line established from a malignancy, HeLa, which has been in culture for an extended period of time and has been used frequently in other laboratories as a source of human DNA polymerases, contained no detectable DNA polymerase Cm activity.

Acknowledgments—We are indebted to Dr. S. Aaronson for advice and generous gifts of A-375 and A-1188 cells and antiserum to SSVD and RD-114 reverse transcriptase, to Dr. J. Krakower for immunological analyses, to Dr. A. Weissbach for critical reading of the manuscript, and to Drs. Guroff and Gallo for gifts of antiserum to DNA polymerases. We thank M. Pursley, R. Eucholz, and G. Large for technical assistance and Dr. H. Thornton, S. Loewenstein, and A. Rankin for their assistance in the large scale growth of cells. We acknowledge the excellent work of N. Takemori in the isolation of clones of A-375 cells.

REFERENCES

Characterization of a DNA polymerase activity in cultured human melanoma cells that copies poly(2'-O-methylcytidylate).
G F Gerard, P M Loewenstein and M Green


Access the most updated version of this article at http://www.jbc.org/content/255/3/1015.citation

Alerts:
  • When this article is cited
  • When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/255/3/1015.citation.full.html#ref-list-1