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Deoxyribonucleic Acid Polymerases of BHK-21/C13 Cells

PARTIAL PURIFICATION AND CHARACTERIZATION OF THE ENZYMES

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DNA polymerase from BHK-21/C13 cells was separated into two species, DNA polymerase I corresponding to the heterogeneous enzyme with sedimentation coefficient of 6–8S, and DNA polymerase II, corresponding to the enzyme with sedimentation coefficient of 3.3S. DNA polymerase I was purified 114-fold and DNA polymerase II 154-fold by a simple extraction procedure followed by column chromatography on phosphocellulose and gel filtration through Sephadex G-100. The purified enzymes differed markedly in respect of pH optimum, stimulation and inhibition by K^+, K_m for the deoxyribonucleoside 5'-triphosphates, stability to heating at 45°C, and inhibition by *N*-ethylmaleimide. The preferred primer-template for both enzymes was 'activated' DNA (DNA submitted to limited degradation by pancreatic deoxyribonuclease); native or thermally denatured DNA templates were relatively very poorly copied. When certain synthetic templates were tested, substantial differences were revealed between the two enzymes. Poly[d(A-T)] was poorly used by polymerase I but was superior to 'activated' DNA for polymerase II. Poly[d(A)]·oligo[$d(pT)_{10}$] was used efficiently by polymerase I but not by polymerase II. Poly(A)·oligo[$d(pT)_{10}$] was not an effective primer-template although polymerase I could use it to a limited extent when Mn^{2+} replaced Mg^{2+} in the polymerase reaction and when the temperature of incubation was lowered from 37° to 30°C. When only one or two or three triphosphates were supplied in the reaction mixture, the activity of polymerase I was more severely diminished than that of polymerase II.

Two distinct types of deoxyribonucleotide-polymerizing enzyme have been isolated from mammalian cell systems. One is terminal deoxyribonucleotidyltransferase (EC 2.7.7.31; Yoneda & Bollum, 1965), an enzyme of unknown function and apparently peculiar to thymus tissue (Chang, 1971), and the other a heterogeneous group of replicative DNA-dependent deoxyribonucleotidyltransferases (DNA nucleotidyltransferase, EC 2.7.7.7; hereafter termed DNA polymerases). The possible roles of the latter in DNA biosynthesis, and their apparent subcellular distribution have recently been reviewed (Keir & Craig, 1973; Craig & Keir, 1974).

The replicative DNA polymerases can in turn be divided into two major categories, primarily on the basis of molecular weight. A low-molecular-weight enzyme (sedimentation coefficient 3.3S) has been extensively characterized, and purified apparently to homogeneity from calf thymus tissue (Chang, 1973) and from KB cells (Sedwick *et al.*, 1972). In addition to the 3.3S DNA polymerase, a high-molecular-weight species (sedimentation coefficient 6–8S), itself showing heterogeneity of molecular weight, has been isolated from a variety of proliferating cell lines and tissues (see Craig & Keir, 1974).

The present paper concerns the identification, purification and characterization of both the 3.3S and the 6–8S DNA polymerases in exponentially growing BHK-21/C13 cells.

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Experimental

Buffers

Buffer A was 10mM-Tris–HCl (pH7.5 at 4°C)–5mM-2-mercaptoethanol; buffer B was 0.2M-potassium phosphate (pH7.5)–5mM-2-mercaptoethanol; buffer C was 0.1M-potassium phosphate (pH7.5)–5mM-2-mercaptoethanol; TD buffer was 25mM-Tris–HCl (pH7.5 at 4°C)–0.14M-NaCl–0.05M-KCl–0.2M-Na_2HPO_4.

All Tris-containing buffers are expressed in molar concentrations with respect to Tris and were adjusted to the required pH with 4M-HCl. Ammediol (2-amino-2-methyl-1,3-propanediol) buffers were prepared similarly. All phosphate buffers are expressed in molar concentrations with respect to phosphate and were prepared by mixing appropriate proportions of equimolar solutions of K_2HPO_4 and KH_2PO_4.

Materials

Calf serum was purchased from Flow Laboratories Ltd., Irvine, Ayrshire, U.K., or from Bio-Cult Laboratories Ltd., Glasgow, U.K.; tryptose phosphate broth was from Difco Laboratories Inc., Detroit, Mich., U.S.A.; amino acids and vitamins for Eagle's medium and cytochrome *c* were from Koch–Light Laboratories Ltd., Colnbrook, Bucks., U.K.; bovine serum albumin, calf thymus DNA (type V), salmon sperm DNA (type III) were from Sigma (London) Chemical Co. Ltd., Kingston-upon-Thames, Surrey KT2 7BH, U.K.; dATP, dCTP, dGTP, dTTP, lactate dehydrogenase and alcohol dehydrogenase were from Boehringer (London) Ltd., London W5 2TZ, U.K.; ovalbumin was from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A.; poly(A)·oligo[d(pT)$_{10}$], poly[d(A)]·oligo-[d(pT)$_{10}$] and poly[d(A-T)] were from Miles Seravac (Pty.) Ltd., Maidenhead, Berks., U.K.; Whatman phosphocellulose P-11 and Whatman glass-fibre GF/C filter discs were from Reeve Angel Scientific Ltd., London SE1 6BD, U.K.; Blue Dextran, Sephadex G-100 and Sepharose 6B were from Pharmacia (G.B.) Ltd., London W5 5SS, U.K.; liquid-scintillation fluors were from Nuclear Enterprises Ltd., Edinburgh, U.K.; and [*Me*-^{3}H]dTTP (15–30Ci/mmol), [5-^{3}H]dCTP (5Ci/mmol) and [8-^{3}H]dGTP (5–15Ci/mmol) were from The Radiochemical Centre, Amersham, Bucks., U.K.

Native BHK-21/C13 cell DNA and human serum albumin were gifts respectively from Dr. R. J. Cooper and Dr. J. E. Fothergill, both of this department.

All reagent chemicals were A. R. grade.

Methods

Cell culture. Baby-hamster kidney cells (BHK-21/C13 cells; Macpherson & Stoker, 1962) were grown in monolayer culture at 37°C in 2.24litre roller-bottles (House & Wildy, 1965) with the use of the Glasgow modification of Eagle's medium containing 10% (v/v) tryptose phosphate broth and 10% (v/v) calf serum (Flow Laboratories Ltd.). During cell growth the bottles were rotated at 1rev./3min. The cells were checked regularly for contamination by mycoplasmas by the method of Fogh & Fogh (1964).

Cell harvesting and preparation of cell extracts. Cells in the late exponential phase of growth were harvested by rinsing the cell sheet (about 2.5×10^8cells/bottle) with 0.5mM-EDTA in phosphate-buffered saline (Dulbecco & Vogt, 1954) at 37°C, and were collected by centrifugation ($200g_{av.}$, 5min, 4°C). All subsequent operations were carried out at 4°C. The cells were washed by resuspension in TD buffer (5ml/bottle of cells), and collected by centrifugation as before. The sedimented cells were finally resuspended in the hypo-osmotic buffer A (5ml/bottle) and allowed to swell for 5min. They were then disrupted in a glass homogenizer tube with four strokes of a Teflon pestle (clearance 0.075mm) by using a Tri-R-Stir-R homogenizer operating at 1000rev./min. The resulting homogenate (100% cell breakage) was centrifuged at $800g_{av.}$ for 10min and the supernatant fluid retained as a 'cytoplasmic' fraction. The sedimented material consisting predominantly of nuclei was resuspended in buffer B (2.5ml/bottle of cells) and left for 60min. All insoluble material was then removed from both the 'cytoplasmic' and the 'nuclear' fractions by centrifugation in an MSE Superspeed 65 ultracentrifuge ($100000g_{av.}$, 90min, 4°C) in the 8×25ml fixed-angle rotor. The resulting supernatants were concentrated by the addition of solid $(NH_4)_2SO_4$ added with gentle stirring to 80% saturation at 4°C (Dawson *et al.*, 1969). After 25min the precipitated proteins were collected by centrifugation in an MSE Highspeed 18 centrifuge ($18000g_{av.}$, 20min, 4°C) in the 8×50ml fixed-angle rotor. The sedimented material was then dissolved in a minimal volume of buffer C, and dialysed against three changes (100vol./change) of buffer C with constant stirring for a minimum overall period of 3h. The dialysed supernatants were then clarified, if necessary, by centrifugation as before at $18000g_{av.}$ for 10min, and finally pooled and used as a source of enzyme for ion-exchange chromatography on columns of phosphocellulose or for fractionation by gel filtration. This preparative approach ensured removal of insoluble cellular debris and mitochondria before exposure of the 'soluble cytoplasmic' and 'nuclear' fractions to the hyperosmotic buffer B and high concentrations of $(NH_4)_2SO_4$ (see the Discussion section).

Column chromatography. (1) Phosphocellulose. The total cell extract obtained from 20 bottles (about 4×10^9cells) was loaded at 10ml/h on to a column (1.7cm×9cm) of phosphocellulose, equilibrated with buffer C. After extensive washing to remove unbound protein, stepwise elution was carried out with 0.4M- and 0.6M-potassium phosphate buffers, pH7.5, both containing 5mM-2-mercaptoethanol. Fractions (2ml) were collected and those containing DNA polymerase activity were pooled and dialysed at 4°C against buffer C.

(2) Sephadex G-100. A 3ml portion of the dialysed preparation from chromatography on phosphocellulose was applied to a column (1.2cm×86cm; 130ml bed volume) of Sephadex G-100 equilibrated with buffer C. Elution with buffer C was carried out at 5ml/h. Fractions (2ml) were collected and assayed for DNA polymerase activity at both pH7.6 and 8.9.

Sucrose-density-gradient centrifugation. Sucrose density gradients [4.8ml; 5–20% (w/v)] were prepared in buffer C by the freeze–thaw method of Baxter-

Gabbard (1972). The polymerase preparation (200 μl) and a suitable enzyme marker were applied to the top of the gradient, and centrifugation was carried out (15h, 130600$g_{av.}$, 4°C) in a Beckman–Spinco L2 ultracentrifuge in the SW50L rotor. Fractions (nine drops each) were collected by upward displacement of the gradient with 40% (w/v) sucrose, and were assayed for enzyme activity.

Determination of molecular weights. Molecular weights of DNA polymerase species were determined by both gel filtration through Sephadex G-100 (Andrews, 1965) and centrifugation on sucrose gradients (Martin & Ames, 1961).

The Sephadex G-100 column (see above) was calibrated by using Blue Dextran and the four standard proteins lactate dehydrogenase [pig heart; mol.wt. 140000 (Goldberg, 1972)], alcohol dehydrogenase [horse liver; mol.wt. 83000 (Theorell, 1964)], serum albumin [bovine; mol.wt. 67000 (Phelps & Putnam, 1960)], ovalbumin [hen; mol.wt. 45000 (Cunningham *et al.*, 1963)] and cytochrome *c* [horse heart; mol.wt. 12400 (Margoliash, 1962)].

The sucrose-density-gradient centrifugation was performed in the presence of lactate dehydrogenase which served as a marker protein. The marker enzymes used in these calibration experiments were assayed by standard techniques: namely lactate dehydrogenase by the method of Kornberg (1955) and alcohol dehydrogenase by the method of Bonnischen & Brink (1955). Blue Dextran, serum albumin, ovalbumin and cytochrome *c* were monitored in column eluates spectrophotometrically at 650, 280, 280 and 412nm respectively.

Assay of DNA polymerase. The assay measured the incorporation of mononucleotidyl residues from [^{3}H]deoxyribonucleoside 5′-triphosphate into an acid-insoluble product. Unless otherwise stated [*Me*-^{3}H]dTTP was used as the radioactive precursor. The standard assay mixture (100 μl) for DNA polymerase I contained 12.5 μg of 'activated' salmon sperm DNA as template; KCl (1.5mM); $MgCl_2$ (8mM); dATP, dCTP, dGTP (0.2mM each); [^{3}H]-dTTP (0.2mM; 10mCi/mmol), 2-mercaptoethanol (5mM), potassium phosphate buffer (pH7.5; 20mM), Tris–HCl buffer (pH7.6 at 37°C; 50mM) and enzyme. DNA polymerase II was assayed in an identical manner except that the Tris–HCl buffer was replaced by ammediol (pH8.7 at 37°C, 50mM). After 30min at 37°C, the samples were cooled on ice, and 100 μl of 10% (w/v) trichloroacetic acid was added. After 20min at 4°C, the precipitated material was collected under suction on glass-fibre discs (Whatman GF/C; 2.5cm diam.). Each disc was then washed individually under suction with four 5ml portions of 5% (w/v) trichloroacetic acid, followed (very briefly) by one 2ml portion of water and two 2ml portions of ethanol. The discs were dried, placed in glass vials (scintillation-grade) each containing 10ml of scintillation fluid and measured for radioactivity in an Intertechnique model SL30 liquid-scintillation spectrometer. The scintillation fluid contained 0.5% (w/v) 2,5-diphenyloxazole and 0.03% (w/v) 1,4-bis-(4-methyl-5-phenyloxazol-2-yl)benzene in toluene.

Modifications of the standard assay, appropriate to the properties of each type of DNA polymerase, are described in the Results section. All assays were conducted under conditions in which polymerase activity was linear with respect to time and to protein concentration.

Assay of DNAase activity.* (*a*) Acid DNAase. The assay mixture (0.25ml) contained 3 μg of ^{3}H-labelled (10^3c.p.m.) DNA from *Escherichia coli*, Mg^{2+} (4mM), sodium acetate buffer, pH5.3 (40mM), bovine serum albumin (0.02%), 2-mercaptoethanol (8mM) and enzyme. After 30min at 37°C the samples were chilled on ice to 0°C and 0.1ml of calf thymus DNA (2.5mg/ml) was added followed by 0.25ml of 7% (v/v) $HClO_4$. After 20min at 0°C the samples were centrifuged (1000$g_{av.}$, 45min, 2°C). The acid-soluble radioactivity in the resulting supernatant fluid was measured for radioactivity (liquid-scintillation spectrometry) by transfer of 0.1ml portions to vials containing 10ml of the above toluene-based liquid-scintillation fluid made 33% (v/v) with Triton X-100.

(*b*) Neutral and alkaline DNAases. The assays were similar to the above except that the pH was changed to 7.0 and 8.5 respectively by using appropriate buffers.

(*c*) General. All of the above assays for DNAase were performed by using a variety of native and denatured [^{3}H]DNA substrates, from both bacterial and mammalian sources. No definitive endodeoxyribonuclease assays were performed.

Assays for related enzymes. RNAase H was assayed by the method of Cooper *et al.* (1974). DNA ligase was assayed by the method of Lindahl & Edelman (1968) with the following modifications: (*a*) the [5′-^{32}P]phosphoryl DNA substrate was prepared with polynucleotide kinase extracted from rat liver nuclei as described by Ichimura & Tsukada (1971) and further purified by column chromatography on DEAE-cellulose and phosphocellulose (D. G. Evans, B. A. Milman, A. V. Muir & H. M. Keir, unpublished work); (*b*) 2-mercaptoethanol was used in the assay at 6mM; (*c*) the incubation was for 75min at 20°C; and (*d*) the acid-insoluble product was collected on a glass-fibre filter. Polynucleotide kinase was assayed by the method of Ichimura & Tsukada (1971) with the difference that K_2HPO_4 was present at 3mM to inhibit phosphatase action. The ligase and kinase assays were performed respectively by

* Abbreviations: DNAase, deoxyribonuclease; RNAase, ribonuclease.

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Preparation of templates for the polymerase reaction. Native DNA was dissolved with gentle stirring (16h; 4°C) in 10mM-Tris–HCl buffer (pH7.5 at 4°C), 10mM-KCl to a final concentration of 2mg/ml, and stored at −20°C. Denatured DNA was prepared by heating the native DNA (2mg/ml) at 100°C for 15min followed by rapid cooling in an ice–water bath.

'Activated' DNA was prepared by the method of Loeb (1969) or by the method of Momparler *et al.* (1973).

The synthetic templates poly(A)·oligo[d(pT)$_{10}$], poly[d(A)]·oligo[d(pT)$_{10}$] and poly[d(A-T)] were dissolved in 10mM-Tris–HCl buffer (pH7.5 at 4°C)–10mM-KCl to give a final concentration of 1mg/ml, and were stored at −20°C.

Hybrid molecules of DNA–RNA that were used as primer-templates, were prepared by the method of Cooper *et al.* (1974).

Protein determination. Protein was determined by the method of Miller (1959). Where protein concentrations were low, samples were dried under vacuum to remove 2-mercaptoethanol, redissolved in 0.5M-NaOH and assayed by the Miller (1959) method scaled down fivefold.

Results

Purification of DNA polymerases

BHK-21/C13 cell DNA polymerases were separated into two major species by a purification scheme involving subcellular fractionation to remove mitochondria and insoluble cellular debris, column chromatography on phosphocellulose and gel filtration on Sephadex G-100. Stepwise elution of the enzyme in the total cell extract from phosphocellulose resulted in the recovery of over 90% of the total DNA polymerase activity in the 0.4M buffer step (Fig. 1). Subsequent washing with 0.6M buffer did not elute any further DNA polymerase activity. The ensuing gel filtration of the most active fractions on Sephadex G-100 resolved this activity into two distinct fractions (Fig. 2). The first and major activity (DNA polymerase I) was eluted in the void volume as a peak with a slightly skewed profile, but the second activity (DNA polymerase II) was retarded giving an estimated molecular weight of 48000±2000.

Final purification of the enzymes with respect to the homogenate, calculated from polymerase assays at pH7.6 by the use of an activated DNA primer-template, was 114-fold for DNA polymerase I, and 154-fold for DNA polymerase II (Table 1). The most active enzyme fractions were made 20% (v/v) with ethylene glycol and stored at −70°C. Under these conditions there was no apparent loss of activity during storage for 3 months.

General properties

DNA polymerases I and II. Experiments to determine the optimal conditions of assay for each enzyme revealed pronounced differences in behaviour when an 'activated' DNA primer-template was used. These are described below.

Cation requirements. Both enzymes showed similar bivalent cation requirements, Mg^{2+} being preferred

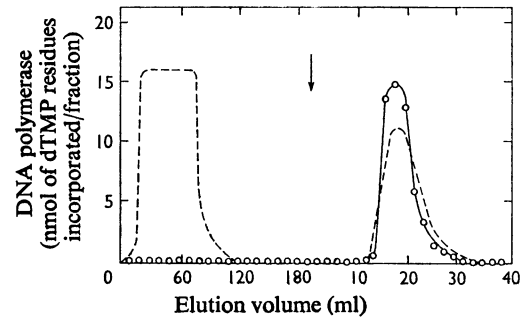

Fig. 1. *Column chromatography of DNA polymerase on phosphocellulose*

See the Experimental section. DNA polymerase was assayed under conditions optimal for DNA polymerase I. ----, E_{280}; ○, DNA polymerase; the arrow indicates the point at which washing of the column with buffer C was terminated and elution with 0.4M-potassium phosphate buffer (pH7.5)–5mM-2-mercaptoethanol commenced.

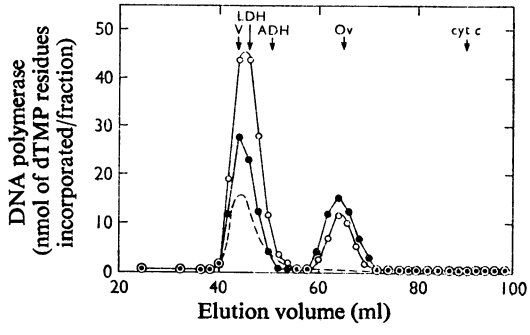

Fig. 2. *Gel filtration of DNA polymerase on Sephadex G-100*

The most active fractions from column chromatography on phosphocellulose were used (see the Experimental section). ----, E_{280}; ○, DNA polymerase assayed at pH7.6; ●, DNA polymerase activity assayed at pH8.9. The arrows indicate the positions of elution of the marker proteins. V, void volume; LDH, lactate dehydrogenase; ADH, alcohol dehydrogenase; Ov, ovalbumin; cyt *c*, cytochrome *c*.

Table 1. *Partial purification of DNA polymerases*

For details see the text. The specific activity is expressed as nmol of dTMP residues incorporated/30min per mg of protein at 37°C measured by the standard assay at pH7.6. The total activity is expressed as nmol of dTMP residues incorporated/30min per fraction at 37°C.

Fraction	Protein (mg/ml)	Total protein (mg)	Specific activity	Total activity	Yield (%)	Purification (fold)
Homogenate	11.0	1200	0.65	778.0	100	1
Pooled fractions from phosphocellulose	1.13	8.61	77.24	665.0	85.4	118.8
DNA polymerase I from Sephadex G-100	0.20	5.08	74.20	368.0	47.3	114.1
DNA polymerase II from Sephadex G-100	0.027	0.68	100.12	68.4	8.8	154.0

Table 2. *Activities of DNA polymerases I and II on various DNA templates*

100% activity is 61.23 and 186.2nmol of dTMP residues incorporated/mg of protein respectively for polymerases I and II. All assays contained 25μg of DNA in 200μl final volume, but were otherwise standard.

DNA template	Polymerase I (%)	Polymerase II (%)
'Activated' salmon sperm*	100	100
'Activated' salmon sperm†	42.0	16.0
Native salmon sperm	7.0	11.7
Denatured salmon sperm	9.7	1.7
Native BHK	0	<1.0
Denatured BHK	<1.0	<1.0
Native calf thymus	6.6	3.3
Denatured calf thymus	4.2	2.0
None	0	0

* 'Activated' by the method of Loeb (1969).
† 'Activated' by the method of Momparler *et al.* (1973).

to Mn^{2+} over a broad optimum range of concentration (4–12mM). However, whereas DNA polymerase II required the presence of a univalent cation (K^+), showing maximum activity at 60mM, DNA polymerase I was optimally active in the absence of K^+. If the KCl concentration in the assay was increased to 120mM, DNA polymerase I was inhibited by 40% and DNA polymerase II by only 10%, relative to the activities measured under optimum conditions.

pH optimum. Assays carried out over the pH range 6.5–10.0, with Tris–HCl or ammediol–HCl buffer at 50mM, revealed distinct and sharp pH optima for the separated polymerases. DNA polymerase I showed maximal activity at pH7.65; the activities at pH7.4 and pH7.9 were 65 and 68% respectively of maximum. DNA polymerase II was optimally active at pH8.5–8.9; the activities at pH8.3 and pH9.1 were 69 and 66% respectively of maximum. These assays were conducted with 'activated' DNA as primer-template, and the difference in pH optimum shown by the two enzymes was exploited to emphasize the presence or absence of either enzyme by criteria other than simply physical separation (see Fig. 2).

Template requirements. DNA polymerases I and II displayed an absolute requirement for a DNA primer-template, maximum activity occurring on a native DNA template submitted to prior treatment ('activated') with pancreatic deoxyribonuclease (Loeb, 1969; Momparler *et al.*, 1973). Native or denatured DNA preparations supported deoxyribonucleotide incorporation rather poorly, polymerases I and II showing no significant preference for either template (Table 2). When high-molecular-weight DNA (mol.wt. 1×10^8–3×10^8) purified from BHK-21/C13 cells was used as template in either the native or denatured form, no significant activity was shown by either enzyme. DNA 'activated' by the method of Momparler *et al.* (1973) (prepared by mixing equal amounts of denatured DNA, and denatured DNA made 100% acid-soluble by the action of DNAase I) was a distinctly less efficient primer-template than DNA 'activated' by the method of Loeb (1969), especially when used as a primer-template for DNA polymerase II.

The activities of DNA polymerases I and II on a denatured template were less than 10% (Table 2) of those shown on an 'activated' primer-template. When the enzymes were compared in this respect but over a longer incubation period (2h), then the activity of DNA polymerase I on a denatured template increased to 30% of the activity given on the 'activated' primer-template; in contrast, the activity of DNA polymerase II remained low.

The response of both enzymes to synthetic homopolymer templates was compared with the response to 'activated' DNA under a variety of conditions (Table 3). Irrespective of the primer-template used or the conditions of the assay, DNA polymerase I always showed a marked preference for an 'activated' DNA primer-template. No significant incorporation of deoxyribonucleotides was detected on the poly(A)·oligo[d(pT)$_{10}$] primer-template, and only limited incorporation was observed when poly-[d(A-T)] or poly[d(A)]·oligo[d(pT)$_{10}$] primer-template was used. In contrast, DNA polymerase II

Table 3. *Activities of DNA polymerases I and II on various synthetic templates*

All assays contained 5 μg of template and the appropriate deoxyribonucleoside 5'-triphosphates in a final assay volume of 100 μl. The conditions of assay were: A, 37°C, 8 mM-Mg^{2+}; B, 37°C, 0.5 mM-Mn^{2+}; C, 30°C, 8 mM-Mg^{2+}; D, 30°C, 0.5 mM-Mn^{2+}. Incubation time was 30 min. Activities are expressed as percentage of activity at 8 mM-Mg^{2+} at 37°C; polymerase I was assayed at pH 7.6 and polymerase II at pH 8.9; 100% activity was 17.3 and 73.6 nmol of dTMP residues incorporated/mg of protein respectively for polymerases I and II. DNA was activated by the method of Loeb (1969).

Enzyme ...	DNA polymerase I				DNA polymerase II			
Temperature of incubation...	37°C		30°C		37°C		30°C	
Cation ...	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn^{2+}	Mg^{2+}	Mn_2^{+}	Mg^{2+}	Mn^{2+}
Assay condition	A	B	C	D	A	B	C	D
Poly[d(A-T)]	36.6	19.0	12.4	8.5	175.0	216.0	109.0	81.1
Poly[d(A)]·oligo[d(pT)$_{10}$]	84.8	27.9	69.5	44.0	2.7	5.1	14.6	6.3
Poly(A)·oligo[d(pT)$_{10}$]	0	4.0	0.5	16.6	0	2.8	2.6	1.7
'Activated' DNA from salmon sperm	100	96.6	98.7	90.7	100	56.4	65.8	50.4

Table 4. *Activities of DNA polymerases I and II with respect to the deoxyribonucleoside triphosphate composition of the reaction mixture*

All triphosphates were individually present at 0.2 mM. Specific activity is expressed as nmol of dTMP residues incorporated/mg of protein under standard assay conditions

	dCTP	dGTP	dATP	dTTP	Specific activity	Activity (% of maximum)
DNA polymerase I	−	−	−	+	4.75	11.4
	−	−	+	+	6.20	14.9
	−	+	+	+	14.5	34.7
	+	+	+	+	41.8	100
DNA polymerase II	−	−	−	+	48.0	33.0
	−	−	+	+	62.2	42.7
	−	+	+	+	78.4	53.8
	+	+	+	+	145.5	100

showed maximal activity with either poly[d(A-T)] or 'activated' DNA but no significant activity with either poly(A)·oligo[d(pT)$_{10}$] or poly[d(A)]·oligo-[d(pT)$_{10}$] primer-templates, under any of the assay conditions used. It is to be stressed that all enzyme activities in Table 3 are expressed as nmol of dTMP residues incorporated per mg of protein; had the same results been expressed as total deoxyribonucleotide residues incorporated (the non-radioactive dAMP, dCMP and dGMP as well as the radioactive dTMP), then the overall preference of DNA polymerase I for an 'activated' DNA primer-template and that of DNA polymerase II for both the 'activated' DNA and the poly[d(A-T)] primer-template would have been even more emphatic.

When the DNA–RNA hybrid [prepared by the action of DNA-dependent RNA polymerase from *E. coli* on denatured DNA from salmon sperm as template (see under 'Methods')] was tested for primer-template activity for the DNA polymerases, it was used only by DNA polymerase I giving a response equal to that of the 'activated' DNA primer-template from salmon sperm.

In the presence of an 'activated' salmon sperm DNA primer-template, both enzymes showed maximal activity in the presence of all four deoxyribonucleoside 5'-triphosphates (Table 4). Omission of one, two or three of the triphosphates from the assay did not completely abolish incorporation. In the absence of three deoxyribonucleoside triphosphates DNA polymerase II still retained 33% of maximum activity, whereas DNA polymerase I retained only 11%. This was not, however, simply an indication of the inability of either enzyme to copy the template by complementary addition of deoxyribonucleotide residues, as both enzymes when assayed with the poly[d(A-T)] primer-template, could polymerize only the monomers complementary to the template. Thus in the presence of dATP, but with [^{3}H]dGTP or [^{3}H]dCTP replacing [^{3}H]dTTP, less than 1% of maximum activity was observed. Similarly, uridine 5'-triphosphate could not

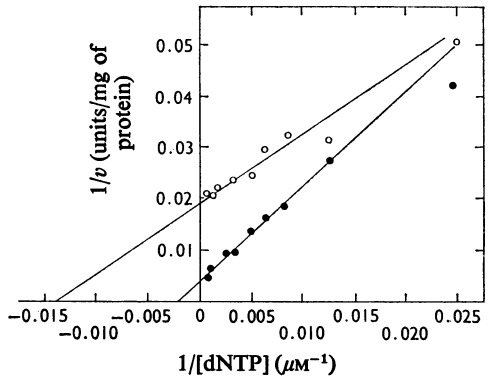

Fig. 3. *Activities of DNA polymerases I and II as a function of substrate concentration*

The four deoxyribonucleoside triphosphates were present in equimolar amounts for each point. ○, DNA polymerase I; ●, DNA polymerase II. One unit is 1 nmol of dTMP residues incorporated in the standard assay. dNTP signifies the equimolar mixture of dATP, dCTP, dGTP and dTTP.

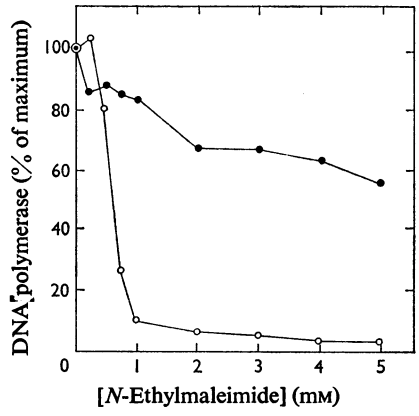

Fig. 4. *Inhibition of the activities of DNA polymerases I and II by N-ethylmaleimide*

Assay conditions for the two enzymes were standard except that 2-mercaptoethanol was omitted. ○, DNA polymerase I; 100% activity was 38.8 nmol of dTMP residues incorporated/mg of protein without the inhibitor added. ●, DNA polymerase II; 100% activity was 164 nmol of dTMP residues incorporated/mg of protein without the inhibitor added.

replace dTTP in the reaction under standard assay conditions.

There was a marked difference in the affinities of the two enzymes for the triphosphates, the K_m values given by DNA polymerases I and II for equimolar amounts of all four triphosphates being 7.28×10^{-5} M and 5.00×10^{-4} M triphosphate respectively (Fig. 3).

Inhibition by N-ethylmaleimide. When assayed in in the absence of 2-mercaptoethanol, but in the presence of increasing amounts of *N*-ethylmaleimide (Fig. 4), DNA polymerase I activity was almost totally abolished at concentrations of *N*-ethylmaleimide greater than 1 mM, whereas DNA polymerase II activity was decreased by only 12 and 44% by the inhibitor at 1 and 5 mM respectively.

Associated enzyme activities. Both enzyme preparations were assayed for enzyme activities likely to be associated with the replication mechanism. No exodeoxyribonuclease activity could be detected by a variety of assay conditions all involving the release of acid-soluble fragments from a [^{3}H]DNA substrate tested in either the native or the denatured condition at pH values of 5.3, 7.0 and 8.5. Both enzyme preparations contained a RNAase H activity (Cooper *et al.*, 1974). When assayed for 60 min at 37°C under conditions of pH and ionic composition optimal for the respective DNA polymerase but in the absence of deoxyribonucleoside 5′-triphosphates and 'activated' DNA, and in the presence of a [^{3}H]RNA–DNA hybrid, polymerase I converted 20% and polymerase II 79% of the [^{3}H]RNA of the hybrid into an acid-soluble form. The DNA polymerase I preparation contained some DNA ligase activity (45 pmol of [5′-^{32}P]phosphoryl termini in the DNA substrate rendered resistant to alkaline phosphatase per mg of protein), and polynucleotide kinase activity (29 pmol of ^{32}P from [γ-^{32}P]ATP rendered acid-insoluble per mg of protein). Neither ligase nor kinase activity was detectable in the DNA polymerase II preparation.

Heterogeneity of DNA polymerase I

In order to investigate possible heterogeneity and estimate molecular weight(s) of the DNA polymerase I preparation, a portion of the pooled fractions after gel filtration on Sephadex G-100 (Fig. 2; Table 1) was analysed by sucrose-gradient centrifugation, sedimentation coefficients being determined with respect to the position of a marker enzyme, lactate dehydrogenase (7.3 S; Martin & Ames, 1961). The density-gradient profile (Fig. 5) revealed the heterogeneous nature of DNA polymerase I; there were two major overlapping but distinct peaks of activity corresponding to sedimentation coefficients of 7.6 S and 8.8 S. A minor but distinct shoulder of activity was also apparent at 5.5 S.

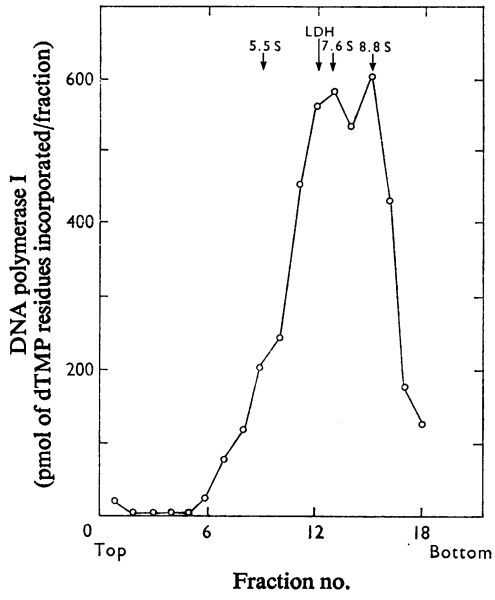

Fig. 5. *Sedimentation analysis of DNA polymerase I by sucrose-density-gradient centrifugation*

The enzyme was extracted from exponentially growing cells and purified by ion-exchange chromatography on phosphocellulose then by gel filtration on Sephadex G-100. The fractions were assayed for DNA polymerase I and lactate dehydrogenase (LDH). Sedimentation was from left to right.

Discussion

The experimental scheme adopted for the fractionation and partial purification of BHK-21/C13 cell DNA polymerases, gave a high yield and also an acceptable degree of purity of the enzymes, without contamination by mitochondrial DNA polymerase (Meyer & Simpson, 1968), or by material from microsomal fractions, ribosomes and cell debris that would have been solubilized by buffer B. The results clearly demonstrate the presence of two major species of DNA polymerase in these cells, DNA polymerases I and II, the enzymes corresponding respectively to the 6–8S and 3.3S species identified in a variety of other tissues and cell lines (Craig & Keir, 1974).

Apart from the molecular-weight estimations, several enzymic properties of the BHK-21/C13 DNA polymerases I and II are particularly characteristic of the 6–8S and 3.3S species (Table 5). The DNA polymerase II exhibits an alkaline pH optimum when used with an 'activated' primer-template and displays substantial resistance to thiol-group alkylating agents, properties generally characteristic of the 3.3S polymerases. In contrast, DNA polymerase I exhibits a pH optimum nearer to neutrality, but is strongly inhibited by thiol-group alkylating agents, properties generally characteristic of the 6–8S polymerases.

Template specificity and precursor requirements also closely resemble those of previously characterized 6–8S and 3.3S species. K_m values for deoxyribonucleoside 5′-triphosphates resemble those obtained from the KB cell DNA polymerases (Sedwick *et al.*, 1972). Both enzymes have a remarkably high activity on an 'activated' DNA primer-template in the absence of a full complement of deoxyribonucleoside triphosphates. Similar results have been reported for a variety of tissues (see Craig & Keir, 1974). Such activity cannot be attributed to ability of the enzymes only to copy the template in a non-specific manner, as both enzymes have the ability to produce a faithful complementary copy of the template using the alternating co-polymer poly[d(A-T)]. Only dATP and dTTP are polymerized, even when dCTP and dGTP are provided. Similarly such incorporation cannot be ascribed to a terminal deoxyribonucleotidyltransferase activity, since the lack of incorporation of a ribonucleoside 5′-triphosphate, the requirement for complementary base-pairing, and the substantial increase in activity observed in the presence of all four deoxyribonucleoside triphosphates, are all responses incompatible with activities reported for the terminal deoxyribonucleotidyltransferase (Yoneda & Bollum, 1965; Chang & Bollum, 1971). Thus incorporation must be due to complementary synthesis. Chang & Bollum (1972) using a 3.3S polymerase have explained a similar result on the basis of template restriction. According to them, the reaction apparently consists of very limited replication of many short regions on the 'activated' DNA primer-template, the replicative polymerization at each region representing what is possible with the provision of fewer than four species of deoxyribonucleoside 5′-triphosphate, but at the same time always being effected through complementary base-pairing of the incoming monomer(s) with the template strand. However, this overall concept of template restriction cannot be entirely true; if it were, the assay of both the 6–8S and 3.3S enzymes using the same template, without a full complement of deoxyribonucleoside 5′-triphosphates, should reveal a similar relaxed template requirement for both. This was not found to be so; the activity of DNA polymerase I was markedly less than that of DNA polymerase II in the presence of a single triphosphate. This observation seems not to be compatible with an explanation based purely on template restriction.

Sedwick *et al.* (1972) have questioned the ability of the 3.3S enzyme from KB cells to traverse single-stranded gaps of a DNA primer-template. Such a

Table 5. *Comparison of the properties of DNA polymerases I and II partially purified from exponentially growing BHK-21/C13 cells*

Property	Conditions of experiment	DNA polymerase I	DNA polymerase II
pH optimum	Standard	7.6–7.8	8.5–8.9
Mg^{2+}	Standard	4–12mM	4–12mM
Mn^{2+}	Standard	0.25–1.0mM	0.5–1.0mM
K^+	Standard	None	60mM
K_m for triphosphates	Standard	7.28×10^{-5}M	5.0×10^{-4}M
Maximum activity with only 1 triphosphate (%)	Standard	11	33
Heat inactivation (%)	1min at 45°C in absence of substrate before assay	82	36
Inhibition by *N*-ethylmaleimide (%) at	No 2-mercaptoethanol present		
(*a*) 1.0mM		(*a*) 90	(*a*) 12
(*b*) 5.0mM		(*b*) 96	(*b*) 44
Preferred template	Standard	'Activated' DNA	'Activated' DNA

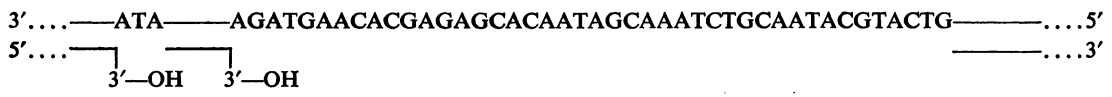

Fig. 6. *Replication of 'activated' DNA*

A stretch of DNA primer-template is shown. The upper strand is intact and serves as template. The lower strand contains two gaps, one of which is short and the other long. Replication catalysed by DNA polymerase proceeds in the 5′ → 3′ direction from the priming 3′-hydroxyl termini, filling in the gaps by complementary base-pairing. DNA polymerase I can fill in both gaps; DNA polymerase can fill the shorter gap but can proceed no farther than ten nucleotides along the larger one because of its postulated inability to traverse long gaps. If this primer-template were used in an experiment of the type described in Table 4 using [^{3}H]dTTP alone and with one or more of non-radioactive dATP, dCTP and dGTP, the percentage incorporation of [^{3}H]dTMP residues would be similar to the values shown in the Table.

proposal might explain the difference in apparent stringency of triphosphate requirement between the two BHK cell enzymes. Thus it would require that DNA polymerase II in the presence of a full triphosphate complement be capable of only very limited addition of triphosphates. However, the ability of DNA polymerase II to utilize a primer-template efficiently might depend on the length of single-stranded regions between successive oligodeoxyribonucleotide initiators, the greater the gap, the less efficient the polymerization, whereas the ability of DNA polymerase I might not be so restricted in utilizing primers adjacent to long single-stranded template regions. Thus the percentage values given in Table 4 for the activities of DNA polymerases I and II with respect to the deoxyribonucleoside 5′-triphosphate composition of the reaction mixture and with an 'activated' DNA primer-template, would be given by the hypothetical stretch of 'activated' primer-template shown in Fig. 6. Such a hypothesis is substantiated not only by the greater efficiency of DNA polymerase I in using a denatured template with increasing time of incubation, but also by the relative inability of DNA polymerase II to utilize an 'activated' primer-template prepared by the method of Momparler *et al.* (1973) (Table 2). The method involves the annealing of denatured DNA with an equimolar amount of denatured DNA hydrolysed to a 100% acid-soluble form by pancreatic DNAase before mixing. As the initiating 3′-hydroxyl terminus must be provided by an oligonucleotide at least seven nucleotides long (Chang & Bollum, 1972), it is likely that only a very small percentage of the oligonucleotides present would be capable of priming DNA synthesis [because of the established mode of action of pancreatic DNAase I (Laskowski, 1961)], by giving rise to a primer-template with extensive single-stranded regions. As expected, DNA polymerase I utilized such a template with appreciable efficiency in comparison with DNA polymerase II (Table 2).

The apparent inability of DNA polymerase II to utilize either a poly(A)·oligo[d(pT)$_{10}$] or a poly-[d(A)]·oligo[d(pT)$_{10}$] template under all assay conditions tested was unexpected, as 3.3S enzymes from a variety of sources (see Craig & Keir, 1974) have considerable activity on such primer-templates. However, until an exhaustive examination of bivalent metal cation and pH requirements for each synthetic primer-template has been carried out (Chang &

Bollum, 1972) it would be unwise to conclude that DNA polymerase II from BHK cells differs in its primer-template requirements from the 3.3S enzyme from other sources. Similar reservations of course also apply to the DNA polymerase I and other 6–8S DNA polymerases. Our results do, however, indicate the absence of contaminating RNA-dependent DNA polymerases from our DNA-dependent DNA polymerase preparations.

Our observations on multiple forms of DNA polymerase I by centrifugation on sucrose density gradients agree with the work of Lazarus & Kitron (1973) and clearly identify DNA polymerase I with the 6–8S DNA polymerases from other species (Craig & Keir, 1974). Similarly, the estimations of molecular weight of DNA polymerase II by gel filtration through Sephadex G-100, are close to the values obtained for the 3.3S enzyme from calf thymus (Chang, 1973) and KB cells (Sedwick *et al.*, 1972; Wang *et al.*, 1974). Thus it seems justifiable to conclude that proliferating BHK-21/C13 cells contain both of the DNA polymerase activities identified in a variety of tissues and cell lines, and that the BHK cell polymerases possess properties similar to these enzymes.

It is difficult to assign a definitive intracellular role(s) to either enzyme on the basis of the data presented here. However, the apparent ability of DNA polymerase I to traverse single-stranded regions (albeit inefficiently) and its ability to utilize a DNA–RNA hybrid as template (in spite of the presence of RNAase H activity in the polymerase preparation), suggest a possible role in the replication of DNA. Likewise, evidence for DNA polymerase II suggests a DNA-repair type of function which may or may not be required in the replication process. These putative roles are discussed in the light of evidence presented in subsequent papers (Craig & Keir, 1975; Craig *et al.*, 1975).

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