Multiple Forms of Nuclear Deoxyribonucleic Acid Polymerases and their Relationship with the Soluble Enzyme

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1. DNA polymerase from nuclear and supernatant fractions of cultured mouse L929 cells was fractionated on columns of Sephadex G-200, Sepharose 4B and of DEAE-cellulose. Several peaks of activity are found on Sephadex chromatography and the distribution of activity between these depends on: (a) the source of the enzyme, i.e. nuclear or supernatant fraction; (b) the mode of extraction of the enzyme from the nucleus; (c) the amount of enzyme applied to the column. 2. The DNA polymerase activity in the lower-molecular-weight peaks (approximate molecular weights are 35000, 70000 and 140000) is firmly bound within the cell nucleus and shows a preference for native DNA as template, whereas the high-molecular-weight peak (peak I, molecular weight 250000 or greater) is found in supernatant fractions and shows greater activity with a denatured DNA template. 3. During periods of DNA synthesis the high-molecular-weight enzyme becomes more firmly bound within the nucleus. 4. Peak I enzymic activity is relatively unstable and is inhibited by thiol-blocking reagents and deoxycholate, but it is stimulated by univalent cations. 5. Very little endonuclease is present in the polymerase preparations, but a very active exonuclease and nucleoside diphosphokinase are present. On Sephadex chromatography, however, it was shown that the immediate precursors for DNA synthesis, at least by peak I enzyme, are the deoxyribonucleoside triphosphates. 6. Attempts to decrease the molecular weight of the peak I enzyme while still retaining activity failed.

Several groups (Wallace *et al.*, 1971; Haines *et al.*, 1971; Weissbach *et al.*, 1971) have now reported that the DNA polymerase activity found in supernatant fractions from animal cells is of higher molecular weight than the nuclear enzyme. However, no evidence has appeared as to the function and complexity of these enzymes nor as to their relationship with each other. It is, however, known that the supernatant enzyme, which shows greater activity with a denatured DNA template, is more active in growing tissues, whereas the nuclear enzyme, which normally prefers a native DNA template, shows considerable activity in cells and tissues not actively growing (Iwamura *et al.*, 1968; Lindsay *et al.*, 1970; Ove *et al.*, 1970).

We have confirmed that the supernatant enzyme is larger than the nuclear enzyme and have shown that the latter contains at least three species of DNA polymerase of approximate molecular weights 35000, 70000 and 140000.

Nuclei also contain DNA polymerase activity indistinguishable from the supernatant enzyme. There is increased association of the high-molecular-weight enzyme with the nucleus during S-phase, and this would account for the increased activity of S-phase nuclei with a denatured DNA template (Lindsay *et al.*, 1970).

We have been unable to degrade the high-molecular-weight enzyme to smaller active fragments, but have shown several important differences between the high-molecular-weight and the lower-molecular-weight enzymes, in particular with regard to their relative stability, sensitivity to thiol-blocking reagents and affinity for the DNA template.

Materials and Methods

Preparation of enzyme fractions

Mouse fibroblast (L929) cell cultures were subcultured in minimum essential medium (Eagle) supplemented with 10% (v/v) of calf serum (medium EC10). Cultures and media were obtained from Flow Laboratories Ltd., Irvine, Ayrshire, U.K., or from BioCult Laboratories, Glasgow, U.K. Cells, which as a routine were tested for mycoplasma, were harvested (a) while growing exponentially or (b) after they had entered the stationary phase or (c) 2h after reversal of a 16h aminopterin blockade, i.e. in S-phase (Adams, 1969). Nuclear and supernatant fractions were prepared as previously described (Lindsay *et al.*, 1970) by homogenizing the washed cells in 0.25M-sucrose buffered with 20mM-Tris-HCl buffer, pH7.5.

Table 1. *Purification of nuclear enzymes*

The values in parentheses represent the yields of enzymic activity.

Fraction	Sample	Total protein (mg)	Sp. activity of DNA polymerase (units/mg)	
			Native template	Denatured template
I	Whole nuclei	110	0.5 (100)	0.2 (100)
II	0.4M-KCl extract	36	2.5 (160)	1.0 (167)
III	(NH ₄) ₂ SO ₄ precipitate	19.4	5.8 (204)	3.9 (367)

The homogenate was centrifuged at 800g for 10min and the supernatant fraction, after being centrifuged at 105000g for 60min (Spinco model L ultracentrifuge, rotor no. 40), was used as a source of soluble enzyme. The nuclear pellet was washed twice in buffered sucrose and was then extracted with medium containing (final concentrations) 0.4M-KCl, 20mM-Tris-HCl buffer, pH7.5, and 5mM-2-mercaptoethanol to yield the nuclear enzyme.

Enzymic preparations were concentrated by adding (NH₄)₂SO₄ to 80% saturation over 30min at 0°C with constant stirring. The suspension was centrifuged at 10000g for 20min in the MSE High Speed 18 centrifuge and the pellet was dissolved in and dialysed against column buffer [containing (final concentrations) 0.15M-KCl, 20mM-Tris-HCl buffer, pH7.5, and 5mM-2-mercaptoethanol]. When the sample was to be applied to a DEAE-cellulose column it was further dialysed against 20mM-Tris-HCl buffer, pH7.5, containing 5mM-2-mercaptoethanol. Table 1 gives details of the purification of the nuclear enzyme.

Column chromatography

Sephadex G-200 and Sepharose 4B were obtained from Pharmacia (G.B.) Ltd., London W5 5SS, U.K. Column chromatography was carried out at 4°C with a column (60cm×0.9cm diam.) that had been calibrated with molecular-weight markers of cytochrome *c*, bovine serum albumin, haemoglobin, human γ -globulin and urease. The flow rate was 5ml/h and 1ml fractions were collected.

DEAE-cellulose chromatography was carried out in a column (6.8cm×1.5cm diam.) with Whatman DE52 DEAE-cellulose (H. Reeve Angel and Co. Ltd., London EC4V 6AY, U.K.). The sample was applied in and washed with 20mM-Tris-HCl buffer, pH7.5, containing 5mM-2-mercaptoethanol, and then a 50ml linear gradient of 0–0.4M-KCl in the same buffer was applied. The column was run at 6.5ml/h and 1.3ml fractions were collected.

DNA polymerase assay

DNA polymerase was assayed basically as described by Shepherd & Keir (1966). Samples (0.1 ml) in column buffer were incubated at 37°C for 60min in a total volume of 0.25ml with 4 μ mol of Tris-HCl buffer, pH7.5, 1.5 μ mol of MgCl₂, 15 μ mol of KCl, 0.1 μ mol of EDTA, 3 μ mol of 2-mercaptoethanol, 100 μ g of DNA and 50nmol each of dATP, dGTP, dCTP and [Me-³H]dTTP (20nCi/nmol) (The Radiochemical Centre, Amersham, Bucks., U.K.). The reaction was terminated by the addition of 0.05ml of 2M-NaOH and the samples were reincubated for at least 1h at 37°C. Portions (100 μ l) were spotted on to Whatman 3MM paper disks (2.5cm diam.), and these were washed six times in 5% (w/v) trichloroacetic acid in 50mM-Na₄P₂O₇ and then dried with ethanol and ether. The DNA was dissolved by heating with 0.5ml of 1M-Hyammine hydroxide [Nuclear Enterprises (G.B.) Ltd., Edinburgh, U.K.] for 20min at 60°C in a counting vial. Radioactivity was determined after the addition of toluene-based scintillator [0.5% (w/v) 2,5-diphenyloxazole].

When diphosphates were used in the assay they replaced the triphosphates in equimolar amounts. When present, ATP was at 1.5mM.

A unit of DNA polymerase activity is defined as the amount required to catalyse incorporation of 1nmol of [Me-³H]dTTP into acid-insoluble material in 1h at 37°C.

Nucleoside diphosphokinase assay

To assay the interconversion of di- and tri-phosphates 100 μ l portions of the polymerase incubation mixtures were pipetted into 0.4ml of 5% trichloroacetic acid, and 50 μ l portions of the supernatant were chromatographed on Whatman no. 1 paper with isobutyric acid-aq. NH₃ soln. (sp.gr. 0.88)-EDTA (0.1M)-water (100:4.2:1.6:55.8, by vol.) (Krebs & Hems, 1952). Spots corresponding to

dTMP, dTDP and dTTP were cut out and radioactivity was counted by using toluene-based scintillator.

Preparation of ^{14}C -labelled DNA from L929 cells

L929 cells were grown in medium EC10 containing $[2\text{-}^{14}\text{C}]\text{thymidine}$ $[50\text{nCi}$ ($0.86\text{nmol}/\text{ml}$)] for 3 days before they were harvested. The washed cells were suspended in balanced salt solution and poured into an equal volume of 2% (w/v) sodium dodecyl sulphate containing 4mM-EDTA and 6% (w/v) of sodium *p*-aminosalicylate. The sample was stirred at 70°C for 30min and cooled to room temperature, and an equal volume of phenol-*m*-cresol (22:3, v/v) containing 0.1% (w/v) 8-hydroxyquinoline was added. After being stirred for 40min the aqueous layer was overlaid with 2vol. of cold ethanol and the DNA spooled out. The DNA was redissolved and respoiled before it was finally dissolved in 50mM-KCl and dialysed against 50mM-KCl (Kirby, 1964).

Non-radioactive DNA was prepared similarly.

Activation of DNA. This was carried out as described by Aposhian & Kornberg (1962).

DNA endonuclease assays

(1) A portion ($20\mu\text{g}$) of ^{14}C -labelled L929-cell DNA ($7000\text{d.p.m.}/\mu\text{g}$) was incubated at 37°C for 1h with $2\mu\text{mol}$ of Tris-HCl buffer, pH7.5, $0.5\mu\text{mol}$ of 2-mercaptoethanol, $2\mu\text{mol}$ of NaCl, $0.6\mu\text{mol}$ of KCl, $0.5\mu\text{mol}$ of MgCl_2 and the sample in a total volume of 0.2ml. The reaction was terminated by cooling the samples to 0°C , and $50\mu\text{l}$ portions were chromatographed on Whatman DE81 chromatography paper (H. Reeve Angel and Co. Ltd.) with $0.75\text{M-NH}_4\text{HCO}_3$, pH8.6, as solvent (Furlong, 1966). Oligonucleotides of chain length less than 30 nucleotides migrate from the origin, and this was monitored by cutting the chromatogram into strips and assaying their radioactivity with the aid of toluene-based scintillator.

(2) A portion ($1\mu\text{g}$) of simian virus 40 DNA (predominantly form I) was incubated at 37°C for 1h with $0.2\mu\text{mol}$ of NaCl, $0.5\mu\text{mol}$ of MgCl_2 and the sample in a total volume of $40\mu\text{l}$. The reaction was terminated by cooling to 0°C and the DNA was analysed by band centrifugation through CsCl in a Spinco model E analytical ultracentrifuge. We are indebted to Dr. R. Eason for the gift of the simian virus 40 DNA and for the ultracentrifugational analyses.

DNA exonuclease assay

A portion ($5\mu\text{g}$) of ^{14}C -labelled L929-cell DNA ($7000\text{d.p.m.}/\mu\text{g}$) was incubated at 37°C for 1h with the sample, $0.15\mu\text{mol}$ of KCl, $2\mu\text{mol}$ of MgCl_2 ,

$0.5\mu\text{mol}$ of 2-mercaptoethanol and $20\mu\text{mol}$ of either Tris-HCl buffer, pH7.5, or glycine-NaOH buffer, pH9.2, in a total volume of 0.3ml. The reaction was terminated by cooling the samples to 0°C and 0.4mg of unlabelled salmon testis DNA was added as carrier. Acid-insoluble radioactivity was assayed with the aid of toluene-based scintillator. Protein was determined by the method of Lowry *et al.* (1951). Phospholipase C and trypsin were obtained from Sigma Chemical Co., St. Louis, Mo., U.S.A. Salmon testis and calf thymus DNA were obtained from Worthington Biochemical Corp., Freehold, N.J., U.S.A.

Results

Fractionation of polymerase activities

Nuclear extract and high-speed supernatant fractions of mouse L929 cells were prepared as described in the Materials and Methods section and fractionated on columns of Sephadex G-200, Sepharose 4B or DE52 DEAE-cellulose. Results are shown in Figs. 1 and 2.

The supernatant fraction contains most of the DNA polymerase activity in a single fraction, which is eluted just after the excluded peak on Sephadex G-200 (and hence has a molecular weight greater than 250000) and from the DE52 DEAE-cellulose column at a KCl concentration of 0.21M. Greater activity is shown with a denatured DNA template.

The nuclear fraction (either fraction II or fraction III) shows a more complex picture, DNA polymerase activity being found in three or four distinct regions. On both Sephadex G-200 and DE52 DEAE-cellulose there is some activity in the region where the supernatant enzyme is eluted, and this activity is usually greater with a denatured DNA template. However, there is activity eluted from Sephadex G-200 in regions shown by comparison with markers of known molecular weight to correspond to enzyme of molecular weights approx. 35000, 70000 and 140000. Enzyme eluted in this region shows greater activity with a native DNA template, as does the activity eluted from DE52 DEAE-cellulose columns at KCl concentrations of zero, 0.09M and 0.15M.

Sepharose 4B chromatography suggested that the molecular weight of peak I enzyme may be about 10^6 , although there is slight aggregation to produce a small peak of activity in the excluded fraction.

When the eluate from the Sephadex G-200 column was assayed with a native DNA template but only one triphosphate (dTTP), residual activity (20–40%) was found associated with each fraction. Fractions 18–24 from Sephadex fractionation of an extract of stationary nuclei were pooled and reprecipitated with $(\text{NH}_4)_2\text{SO}_4$. On rechromatography on Sephadex G-200 maximum activity is found in fraction 22 and

Fig. 1. *Sephadex G-200 chromatography of DNA polymerase preparations*

Four preparations were subjected to fractionation on Sephadex G-200 as described in the Materials and Methods section. They were: (a) 14mg of a high-speed supernatant fraction [concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation] from exponentially growing cells; (b) 4mg of fraction II from stationary-phase nuclei; (c) 4mg of fraction II from S-phase nuclei prepared in parallel with (b); (d) 4mg of an extract obtained from nuclei of exponentially growing cells. This last extract was prepared from nuclei that had already been extracted with 0.4M-KCl. The extracted nuclei were solubilized in 1.0M-KCl in 20mM-Tris-HCl buffer, pH7.5, and the viscous solution was dialysed against 100vol. of 0.15M-KCl in 20mM-Tris-HCl buffer, pH7.5, for two periods of 1h before centrifugation at 10000g for 20min. The supernatant fluid was concentrated by $(\text{NH}_4)_2\text{SO}_4$ precipitation before application to the column. ●, DNA polymerase activity with a native DNA template; ○, DNA polymerase activity with a denatured DNA template.

peak I activity is negligible, even with a denatured DNA template.

When a smaller sample of nuclear extract containing only 15% of the normal amount of protein

was applied to the same Sephadex G-200 column the pattern of elution was changed, most activity now being eluted in fraction 26 (i.e. molecular weight approx. 35000).

Fig. 2. DEAE-cellulose chromatography of DNA polymerase preparations

Two preparations from exponentially growing cells were subjected to fractionation on Whatman DE52 DEAE-cellulose after precipitation with $(\text{NH}_4)_2\text{SO}_4$. They were: (a) 34mg of a high-speed supernatant fraction; (b) 30mg of a 0.4M-KCl nuclear extract. ●, DNA polymerase activity with a native DNA template; ○, DNA polymerase activity with a denatured DNA template.

Binding of enzyme to nuclei and DNA

It was considered possible that the high-molecular-weight DNA polymerase activity present in nuclear extracts was caused by contamination with supernatant enzyme, and we investigated this by extracting nuclei with Tris-HCl buffer, pH 7.5, containing increasing concentrations of KCl. The results are shown in Fig. 3. Enzyme extracted at 50mM-KCl shows only a slight preference for a native DNA template (native DNA/denatured DNA activity ratio 1.5), whereas the enzyme extracted between 0.15M- and 0.30M-KCl shows a native DNA/denatured DNA activity ratio of 2.7. Fig. 1(d) shows the result of Sephadex G-200 chromatography of the fraction extracted from nuclei between 0.4M- and 1.0M-KCl. There is no high-molecular-weight activity and peak IV is again enhanced. Conversely peak I is proportionately larger in 0.10M-KCl extracts, and dialysis of 0.4M-KCl extracts, in the presence of DNA, against 0.15M-KCl causes preferential precipitation of the lower-molecular-weight activity.

The nuclear enzyme readily combines with native DNA at 0°C in 20mM-Tris-HCl buffer, pH 7.5, containing 5mM-2-mercaptoethanol, and the complex sediments on centrifugation at 105000g for 4h. Similarly the complex is eluted with the excluded fraction from Sepharose 4B. Binding is inhibited by 0.15M-KCl.

Variation of activity during the cell cycle

We have previously reported that nuclei from stationary-phase cells show greater DNA polymerase activity when native DNA is used as template, whereas nuclei from S-phase cells prefer a denatured DNA template (Lindsay *et al.*, 1970). Although there was no consistent difference in the elution pattern of enzyme extracted from stationary or exponentially growing cells, nuclear extracts from S-phase cells show a massive increase in DNA polymerase activity in peak I, but little change in the activity of lower-molecular-weight fractions, when compared with extracts of stationary-phase cells (Fig. 1c).

Fig. 3. *Extraction of nuclei with potassium chloride*

Nuclei, prepared in the standard manner, were extracted with successively higher concentrations of KCl in 0.25M-sucrose in 20mM-Tris-HCl buffer, pH7.5. The extractions were carried out at 0°C for 10min, after which the nuclei were sedimented at 800g for 10min. The extracts were dialysed against 100vol. of 0.15M-KCl in 20mM-Tris-HCl buffer, pH7.5, for two periods of 1h each before being assayed. The results are expressed in a cumulative fashion. ●, DNA polymerase activity with a native DNA template; ○, DNA polymerase activity with a denatured DNA template; X, DNA polymerase activity in the absence of added template; —, concentration of protein.

These observations lead to the possibility that peak I activity becomes bound in the nucleus during S-phase when certain binding sites on the DNA are exposed.

Membrane-bound activity

In view of the suggestion of Baril *et al.* (1970) that the supernatant DNA polymerase is associated with a post-mitochondrial membrane fraction, we treated preparations with reagents such as phospholipase C, sodium deoxycholate and ribonuclease in an attempt to decrease its molecular weight while still retaining activity. Treatment of a supernatant preparation with ribonuclease (50 µg/ml for 1h at 37°C) before application to a Sephadex G-200 column removes the RNA that is normally co-eluted with peak I, but causes no change in the elution profile of the DNA polymerase.

Treatment of supernatant preparations with deoxycholate caused inhibition of activity with both native and denatured DNA, but the latter was somewhat more sensitive (Fig. 4a). When 0.03% deoxycholate was included in the buffer used to elute the enzyme from a Sephadex G-200 column the DNA polymerase activity recovered in peak I showed greater activity with a native DNA template, but no increased activity in the lower-molecular-weight regions was discernible (Fig. 4b). Higher concentrations of deoxycholate (0.1%) decreased peak I activity drastically, but still no activity appeared in latter fractions.

Preincubation of supernatant preparations with phospholipase C (up to 50 µg/ml for 1h at 37°C or for 2h at 25°C), amylase (2 µg/ml for 1h at 37°C) or neuraminidase (4 µg/ml for 1h at 37°C) produced no inhibition of DNA polymerase activity, nor did these treatments alter the profile of elution of the enzyme from Sephadex G-200. Treatment with trypsin produced results similar to treatment with deoxycholate, in that inhibition of the activity by 50% with a native DNA template required four times the concentration of trypsin (1.6 µg/ml for 20min at 37°C) as was required to bring about a similar inhibition when a denatured DNA template was used.

Differences between high- and low-molecular-weight enzymes

When nuclei are prepared from stationary-phase cells most of the DNA polymerase activity that uses a native DNA template is recovered in the lower-molecular-weight fractions after fractionation on Sephadex. Conversely, nearly all of the activity measured with a denatured DNA template is found in peak I. In the following experiments the responses of the denatured-DNA- and native-DNA-primed reactions of nuclear extracts have been compared and these are equated with the responses of peak I enzyme and the lower-molecular-weight enzymes respectively.

Univalent cation effects. The activity with a denatured DNA template, i.e. peak I activity, is stimulated over 40% by K⁺ and NH₄⁺, showing maxima at 40 and 5mM respectively. NH₄⁺ produces no stimulation in the presence of 60mM-KCl. Spermine and spermidine produce a similar stimulation, but no stimulation is produced by Na⁺. With a native DNA template (i.e. lower-molecular-weight activity) all these ions produce inhibition of activity.

Effects of thiol-blocking reagents. The DNA polymerase activity that uses a denatured DNA template is much less stable than the activity that uses a native DNA template, especially in dilute solutions. It can, however, be stabilized by the addition of 2-mercaptoethanol. In agreement with this finding is the observation that the activity with a denatured DNA template is much more sensitive to thiol-blocking

Fig. 4. Effect of sodium deoxycholate on supernatant DNA polymerase

(a) Effect of increasing concentrations of deoxycholate present in the assay of $260\mu\text{g}$ of supernatant enzyme. (b) Supernatant enzyme (5.2mg) in 0.03% (w/v) deoxycholate was subjected to Sephadex G-200 chromatography in buffer containing 0.03% (w/v) deoxycholate. ●, DNA polymerase activity with a native DNA template; ○, DNA polymerase activity with a denatured DNA template.

reagents (e.g. *p*-chloromercuribenzoate and *N*-ethylmaleimide) than is the native-DNA-requiring enzyme.

Thus $19\mu\text{g}$ of fraction III, when preincubated for 1 h at 0°C in the presence of 2.5mm -*N*-ethylmaleimide, lost 83% of its activity when assayed with a denatured DNA template but only 36% of its activity when assayed with a native DNA template.

In all these experiments the nuclear activity measured with a denatured DNA template (i.e. peak I activity) resembles the activity found in supernatant fractions, and the results are similar to those of Shepherd & Keir (1966), who used a soluble enzyme from Landschütz ascites-tumour cells.

Template requirement. Although we refer to enzymes showing greater activity with a native DNA or denatured DNA template, this is only of relevance under our defined conditions, as it depends on the source and pretreatment of the DNA. Thus native salmon testis DNA (Worthington) is of relatively low molecular weight and is a very good template, but even this can be activated by treatment with pancreatic deoxyribonuclease. Native DNA prepared from L929 cells in our laboratory is a very poor

template, but a high degree of activation is possible (Fig. 5).

Contaminating activities

Exonuclease. Negligible exonuclease activity (i.e. less than 3% degradation in 1 h at 32°C) was found with a native DNA substrate whether the assay was carried out at pH 7.5 or 9.2 with either $30\mu\text{g}$ of fraction II or $116\mu\text{g}$ of fraction III. However, both fractions showed marked activity with denatured DNA as substrate, over 85% of the DNA being rendered acid-soluble at pH 9.2 after incubation with $116\mu\text{g}$ of fraction III.

Endonuclease. Fig. 5 shows that native L929-cell DNA is a very poor template in a polymerase assay, yet as little as 0.5ng of pancreatic deoxyribonuclease can bring about a doubling of activity when measured with $70\mu\text{g}$ of fraction III enzyme, i.e. fraction III is almost completely devoid of endonuclease activity on native DNA. This is further borne out by the results of the endonuclease assay carried out by the first method described in the Materials and Methods

Fig. 5. Activation of DNA by treatment with pancreatic deoxyribonuclease

Samples (25 μ g) of L929-cell DNA in 0.1 ml were activated by treatment with pancreatic deoxyribonuclease I for 15 min at 37°C. After inactivation of the endonuclease by heating at 77°C for 5 min they were used as a template (with or without denaturation) for 70 μ g of fraction III. ●, DNA polymerase activity with a native DNA template; ○, DNA polymerase activity with a denatured DNA template.

section. Table 2 shows the presence of significant activity on denatured DNA, but very little degradation of native DNA. To get an unequivocal answer, however, the second endonuclease assay method was used. A 1 μ g portion of simian virus 40 form I DNA, on incubation with 27 μ g of fraction III enzyme for 60 min, was degraded to form II and other products. In contrast, 10 μ g of peak III enzyme from a Sephadex fractionation under similar conditions nicked less

than 30% of the simian virus 40 form I DNA, yielding simian virus 40 form II DNA.

Nucleoside diphosphokinase. Within 1 min of the start of a standard DNA polymerase incubation with fraction III enzyme, one-third of the dTTP is converted into dTDP (Table 3). This conversion is independent of the presence of DNA. Despite this the DNA polymerase reaction is linear for at least 2 h. In a similar manner an incubation where the triphosphates are replaced by diphosphates leads to rapid triphosphate synthesis in the presence of ATP or any deoxyribonucleoside triphosphate (50% of the [3 H]dTDP is converted into [3 H]dTTP within 2 min). The monophosphates, however, are not further phosphorylated, nor are they produced from di- or tri-phosphates. The presence of this nucleoside diphosphokinase, which allows rapid interconversion of di- and tri-phosphates (Mourad & Parks, 1966), makes it impossible to determine which are the precursors for synthesis of DNA when crude enzyme preparations are used. On Sephadex fractionation of a nuclear fraction III preparation the kinase separates from peak I, which cannot catalyse the synthesis of DNA from diphosphates alone, or from diphosphates in the presence of non-radioactive triphosphates. This shows that the triphosphates are the precursors of DNA synthesis at least for peak I polymerase.

Discussion

We have been interested in the relationship between the nuclear and supernatant DNA polymerases, since the possibility arose that the latter may act as a reservoir of enzyme that could be drawn upon by the nucleus at times of increased DNA synthesis (Lindsay *et al.*, 1970; Loeb & Fansler, 1970). However, chromatography on columns of Sephadex G-200 reveals that, although this may well be true for peak I (high-molecular-weight) enzyme, there are present other species of enzyme firmly associated with the nucleus whose activity is not related to the rate of synthesis of DNA.

Chang & Bollum (1971) and Baril *et al.* (1970) suggest that the supernatant enzyme may be present in the cytoplasm, and the latter group obtained evidence for its association with plasma membranes and free ribosomes. Although the supernatant enzyme is of very high molecular weight this is not caused by its association with ribosomes. This conclusion is reached from the effect of ribonuclease and from the molecular weight of the enzyme. We thus have no evidence to support a cytoplasmic location of this enzyme.

It does appear, however, that the lower-molecular-weight enzymes are firmly bound within the nucleus and do not appear in the supernatant fraction,

Table 2. *Endonuclease activity of DNA polymerase preparations*

^{14}C -labelled L929-cell DNA was incubated for 1 h with the DNA polymerase preparation and portions of the reaction mixture were then chromatographed on Whatman DE81 paper as described in the Materials and Methods section. Oligonucleotides produced by endonuclease action separate from DNA, which remains at the origin.

Enzyme	% of ^{14}C migrating more than 1.5 cm	
	Native DNA	Denatured DNA
Buffer alone	0.4	0
Fraction II (30 μg)	1.2	1.3
Fraction II (240 μg)	4.0	4.2
Fraction III (58 μg)	0.5	11.6
Fraction III (320 μg)	2.5	44.8
Deoxyribonuclease I (5 ng)	8.6	1.9

Table 3. *Precursors for DNA polymerase*

ATP was present at 1.5 mM, dATP (tube 9) at 0.8 mM and the four deoxyribonucleoside triphosphates (tube 12) were each present at 0.2 mM. The peak I enzyme was taken from the leading edge of the peak as kinase activity was present in the sample 14.

Enzyme	Precursor	Incubation time (min)	DNA synthesis		Acid-soluble material (% of radioactivity in dTTP)
			Native template	Denatured template	
1 —	$[^3\text{H}]\text{dTTP}$	0	—	—	95
2 —	$[^3\text{H}]\text{dTDP} + \text{ATP}$	0	—	—	5
3 Fraction III (53 μg)	$[^3\text{H}]\text{dTTP}$	60	16000	8400	42
4 Fraction III (53 μg)	$[^3\text{H}]\text{dTTP} + \text{ATP}$	1	300	200	69
5 Fraction III (53 μg)	$[^3\text{H}]\text{dTTP} + \text{ATP}$	60	18200	8600	85
6 Fraction III (53 μg)	$[^3\text{H}]\text{dTDP}$	60	2700	1300	5
7 Fraction III (53 μg)	$[^3\text{H}]\text{dTDP} + \text{ATP}$	2	200	100	49
8 Fraction III (53 μg)	$[^3\text{H}]\text{dTDP} + \text{ATP}$	60	11400	5800	72
9 Fraction III (53 μg)	$[^3\text{H}]\text{dTDP} + \text{dATP}$	60	10500	5300	45
10 Peak I (160 μg)	$[^3\text{H}]\text{dTTP}$	60	5000	5700	85
11 Peak I (160 μg)	$[^3\text{H}]\text{dTDP} + \text{ATP}$	60	100	100	5
12 Peak I (160 μg)	$[^3\text{H}]\text{dTDP} + \text{ATP} + 4\text{dNTP}$	60	100	100	5

whereas peak I activity is bound to a variable extent, there being far more bound in nuclei from S-phase cells. The factors that lead to this increased binding of peak I polymerase during DNA synthesis are not yet known.

The enzyme extracted from nuclei exists in several sizes, which may be related to each other as monomer, dimer and tetramer. This is supported by the increased proportion of monomer present in dilute solutions. That the monomer is not simply a terminal addition enzyme as isolated from calf thymus by Bollum *et al.* (1964) is supported by the fact that it is not associated with a peak of incorporation of $[^3\text{H}]\text{dTTP}$

when that triphosphate is the only one present in the reaction mixture. Secondly, all our assays were performed in the presence of 0.4 mM-EDTA, which has been shown to inhibit the terminal addition enzyme (Chang & Bollum, 1970).

The possibility exists that peak I enzyme is a complex that contains the lower-molecular-weight DNA polymerase, bound to other proteins that confer on it the ability to preferentially use a denatured DNA template. One such protein may resemble the gene-32 protein found by Alberts & Frey (1970). It would then be the action of this supplementary protein that was stimulated by univalent ions and

inhibited by thiol-blocking reagents. However, all our attempts to release an active lower-molecular-weight enzyme from peak I failed. Peak I activity was selectively inhibited by treatment with thiol-blocking agents, trypsin and deoxycholate, and the presence of membranes may be important for its function. That lower-molecular-weight activity was not recovered from breakdown of peak I enzyme may mean that its activity is greatly enhanced when membrane-associated or that the enzymes are unrelated. However, treatment with phospholipase C did not produce any significant effect.

Although RNA is associated with peak I from supernatant preparations, its removal does not cause any loss of activity, nor does this decrease the size of peak I enzyme. This appears to rule out the possibility that a ribosome-like structure is involved in the synthesis of DNA by the high-molecular-weight enzyme. Small traces of RNA did, however, remain after ribonuclease treatment, and we cannot rule these out as initiators of replication.

It is obvious from the results of Fig. 5 and from previous work (Lindsay *et al.*, 1970; Wallace *et al.*, 1971) that the activity of the native-DNA-primed enzyme in particular is dependent on the number of nicks present in the DNA. However, there is very little endonuclease activity present in fraction II and still less in fraction III. From Table 2 it would appear that 1 mg of fraction III contains less than 5 ng of deoxyribonuclease I. The experiments with simian virus 40 DNA do, however, confirm its presence. As all routine experiments were carried out with salmon testis DNA that shows evidence of a much greater extent of nicking (unactivated, it is seven times as good a template as unactivated L929-cell DNA, yet the two are comparable after activation), the trace of endonuclease is not going to affect the results.

There is present, however, an active exonuclease that will degrade denatured DNA. This brings about the loss of a small amount of template in some cases, but as the product is double-stranded DNA (Lindsay *et al.*, 1970) it does not seriously affect the results.

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