Purification and partial characterization of a DNA polymerase α species from calf thymus

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SUMMARY

We have purified a DNA polymerase α species from calf thymus to near homogeneity. The enzyme sediments at 5.7 S and contains two polypeptides of 123000 and 134000 daltons in about equimolar ratio. The enzyme is inhibited by aphidicolin and N-ethylmaleimide, and retains its activity in buffers containing moderate salt conditions. Activated DNA is a better substrate than poly-(dA) $\cdot$ (dT) $_{10}$.

INTRODUCTION

DNA polymerases in mammals have been classified according to their catalytic properties into α , β and γ species. It is now commonly assumed that the α species is involved in the replication of the cellular DNA.

In spite of considerable efforts spent in purifying DNA polymerase α from a variety of tissues, its subunit structure has remained unclear. Several laboratories have found a 150000 dalton catalytic core enzyme that can associate with other subunits having molecular weights in the range of 50000-70000 daltons (1, 2, 3). However, it has been also reported that DNA polymerase α consists of subunits of 50000-70000 daltons only (4, 5, 6). The difficulty in assessing the subunit structure of this enzyme may arise from several factors. The small amounts of enzyme that have been purified and the uncontrolled proteolysis encountered during purification may be factors contributing to the conflicting results concerning the subunit structure of this enzyme. However it is also possible that different forms of DNA polymerase α exist in different tissues. Such forms could arise from a common core polymerase by genetically programmed proteolysis.

Polymerase species created this way may perform distinct functions in the replication process. We wish here to report the purification of one species of DNA polymerase α from calf thymus to near homogeneity. The final enzyme preparation consists of a mixture of two polypeptides of 123000 and 134000 daltons. No smaller subunits are detectable in this preparation.

MATERIALS AND METHODS

Unlabeled deoxyribonucleotides and RNA polymerase were purchased from Boehringer-Mannheim (Germany). Deoxyribonucleoside triphosphates (^3H) were from Amersham and calf thymus DNA was from Sigma. Activated DNA was prepared by a slight modification of the procedure given in (7). Plasmid pBR 322 was a kind gift from J. Langowski of our laboratory. Poly (dA) was a product of Miles laboratories. Ampholine (pH 3.5 -10) was from LKB. DEAE-cellulose DE-52 and phosphocellulose P-11 were purchased from Whatman. Bio Rex 70 was from Bio Rad. Sepharose 4B Cl and BrCN activated Sepharose were obtained from Pharmacia. Heparin was a product of Serva. Coupling of heparin to BrCN activated Sepharose 4B Cl was performed according to the instructions of Pharmacia. The amount of heparin bound was determined to be 0.5 mg heparin / mg sepharose using the method given in (8). Cibacron blue 3GA was a gift of Ciba - Geigy (Basel). Coupling of this dye to Sepharose 4B Cl was done according to (9). Aphidicolin was a gift of ICI.

Buffers: Buffer A: 0.1 M K-phosphate pH 7.2, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonylfluoride; Buffer B: as buffer A, but with 0.3 M K-phosphate. Buffer C: as buffer A, but with 0.5 M K-phosphate. Buffer D: 30 mM K-phosphate pH 7.8, 1 mM EDTA, 5 mM 2-mercaptoethanol, 0.5 mM phenylmethanesulfonylfluoride, 20% (w/v) sucrose, 20% (v/v) ethylene glycol.

Assays: DNA polymerase was assayed at 37°C in a volume of 50 μl containing 60 mM Tris-HCl pH 7.8, 5mM MgCl_2 , 1 mM dithioerythritol, 0.3 mg/ml bovine serum albumin, 0.5 mg/ml activated DNA, 0.8% ampholine, 100 μM each of dATP, dGTP, dCTP, and ^3H -dTTP (5cpm/pmol). Assays using poly(dA)•(dT)₁₀ as substrate were performed as given in (10). The reaction was usually initiated with

the addition of 5 μ l of enzyme solution, and aliquots were pipetted on Whatman GFC filter disks. Incorporation of labeled nucleotides into acid insoluble product was as outlined in (11). RNA polymerase was assayed according to (12), terminal transferase was assayed according to Bollum et al. (13). Endonuclease activity was assayed by following the conversion of double-stranded superhelical plasmid pBR 322 to nicked or to linear forms in the presence of DNA polymerase. Analysis was performed in agarose gels with ethidium bromide staining. Nuclease activity was also measured by following the loss of acid precipitable radioactivity from ^3H -poly (dA) in the presence of DNA polymerase α .

SDS polyacrylamide gel electrophoresis was carried out in slab gels using the system of Lämmli (14). Samples were prepared for electrophoresis by precipitating the enzyme with trichloroacetic acid. The precipitate was dissolved in 50 μ l of Lämmli loading buffer. Prior to electrophoresis samples were heated to 95°C for 3 minutes. Preparative electrophoresis was carried out in cylindrical gels according to Fisher and Korn (4). After electrophoresis gels were frozen and cut into pieces. One of the gels was stained with coomassie blue. The gel pieces were incubated in 50 μ l of 10 mM Tris-HCl pH 7.8, 1mM EDTA, 5 mM 2-mercaptoethanol for 30 minutes at 5°C and aliquots of 25 μ l of the extraction buffer were assayed for DNA polymerase α activity. Gel pieces exhibiting activity were incubated overnight in 50 μ l of three-fold concentrated Lämmli loading buffer. The gel pieces and the incubation buffer were subjected to electrophoresis in the same slot of a denaturing slab gel. Preparative ultracentrifugation was carried out in the SB 283 rotor of an IEC B60 ultracentrifuge. Isokinetic sucrose gradients (15) in 50 mM Tris-HCl pH 7.8, 0.4 M KCl, 1 mM EDTA, 5 mM 2-mercaptoethanol and 0.5% ampholine were used. The sucrose concentration at the meniscus was 0.18 M. Reference proteins were: catalase (11.3 S), lactate dehydrogenase (7.3 S), and elongation factor TU (3.2 S). Protein concentration was determined according to Lowry (16) employing an acid precipitation step before the determination, and using bovine serum albumin as reference protein. In highly purified enzyme preparations the protein concentration was determined by assuming

$A_{280}^{0.1\%}$ to be equal to 1.

RESULTS

Purification of the enzyme

If not otherwise stated all operations are performed at 4°C. The purification scheme is outlined in Table 1. Calf thymus (1 kg) is thawed overnight and homogenized with 8 l of buffer A in a Waring blender (2 minutes at low speed). The homogenate is centrifuged for 45 minutes at 13000 g and the supernatant is poured through eight layers of cheesecloth. Adsorption of the filtrate to phosphocellulose is achieved by stirring the solution with 1 l of phosphocellulose for 30 minutes. After centrifugation at 1000 g for 4 minutes the supernatant is discarded. Subsequently, the phosphocellulose is washed in the centrifuge bottles by repeated (4-5 times) suspension and sedimentation with a total of about 20 l of buffer A. The phosphocellulose is then poured into a column and eluted with buffer B. Fractions containing polymerase activity are combined (=fraction I) and passed through a DE 52 column (10 x 10 cm, equilibrated with buffer B). The column is washed with buffer B until all polymerase α activity has passed through. This step removes any residual nucleic acid. The effluent is precipitated by addition of 410 g/l of

Table 1: Purification scheme for DNA polymerase α .
One unit corresponds to 1 nmol dTTP incorporated in 1 h at 37°C. The purification scheme refers to 1 kg of calf thymus.

Fraction	Total activity (units)	Specific activity (units/mg)
I. Crude extract	550000	5
II. Phosphocellulose	450000	250
III. Cibacron blue Sephadex	250000	1400
IV. Heparin-Sephadex	180000	10000
V. DEAE cellulose	60000	30000

solid ammonium sulfate. The resuspended precipitate is dialyzed overnight against buffer A and is applied to a Cibacron blue-Sepharose column (5 x 20 cm, equilibrated in buffer A). Elution is carried out with buffer C (=fraction III). Fraction III is diluted with 4 volumes of distilled water and is applied to a heparin-Sepharose column (5 x 20 cm). The column is developed with a linear gradient composed of 1500 ml each of buffer A and buffer B. Both buffers contained additionally 20% (v/v) glycerol and 20% (w/v) sucrose. DNA polymerase activity elutes at about 0.16 M K-phosphate. Fractions containing DNA polymerase are diluted with 1 volume of distilled water and are loaded on a small heparin-Sepharose column (1.5 x 2 cm). Elution with buffer B yields fraction IV. Fraction IV is diluted with eight volumes of distilled water and is applied to a DE 52 column (0.4 x 4 cm, equilibrated in buffer D). A linear phosphate gradient (30-250 mM) elutes enzyme of specific activity of 20000-40000 units/mg. The most active fractions were concentrated from a 1 ml heparin sepharose column (fraction V).

Comments on the purification scheme. Batch adsorption to phosphocellulose has proved to be the most efficient step in achieving a quick enrichment from the crude extract. It is superior to precipitation with ammonium sulfate or polyethylene glycol (17). Elution conditions from blue-Sepharose may depend on the batch of blue-Sepharose used. Final purification depends critically on the performance of the heparin-Sepharose chromatography. The heparin-Sepharose also separates all terminal transferase activity, that elutes before the polymerase α at 0.11-0.12M K-phosphate. With respect to the DEAE-cellulose step we could not reproduce a separation of distinct polymerase α species as described by Holmes et.al. (3). Rather we observed a wide distribution of polymerase activity over a rather broad range of salt.

Final purification and identification of subunits

Fraction V can be purified further using either preparative ultracentrifugation, preparative electrophoresis or Bio Rex 70 chromatography.

Preparative ultracentrifugation. Fraction V separates in isokinetic gradients into two activity peaks, one sedimenting at

8.0-9.0 S, the other sedimenting at 5.7 ± 0.25 S, the latter containing 70-80% of the total activity. In SDS electrophoresis the 5.7 S polymerase α yields two protein bands of approximately equal staining intensity (fig.1). The molecular weights of the polypeptide chains are 123000 and 134000 daltons, using the subunits of RNA polymerase as molecular weight standards. In preparative electrophoresis the 5.7 S polymerase migrates as a single band (fig.1). DNA polymerase α activity corresponds to this band.

Preparative electrophoresis. When fraction V is subject to preparative electrophoresis several protein bands show up (fig.2). Only one activity peak is detectable; it corresponds to the main protein band (fig.2). Analysis of this band in SDS gels again reveals two polypeptide chains of 123000 and 134000 daltons (gels not shown).

Bio Rex 70 chromatography. Fraction V is diluted with 1 volume of distilled water and is applied to a Bio Rex 70 column (0.4 x 3 cm, equilibrated in buffer A). A linear gradient from 0.1 to 0.3 M potassium phosphate is used for elution. Analysis of the fraction with the highest specific activity (100000 units/mg) in SDS gels indicates the presence of two main polypeptide bands of molecular weight 123000 and 134000 daltons (fig.3). There are still impurities of lower molecular weight present that could not be removed by ion exchange chromatography.

Fig 1: Analysis of the subunit structure of the 5.7 S DNA polymerase α

Enzyme of specific activity 40000 units/mg was separated in a isokinetic sucrose gradient. The activity peak sedimenting at 5.7 S was subjected to SDS electrophoresis (A) and to electrophoresis in native gels (B).

Fig.2: Preparative electrophoresis of enzyme of specific activity 40000 units/mg and distribution of DNA polymerase α activity along the gel.

Gels were prepared and assayed as described in "Materials and Methods."

Our results clearly indicate the presence of two polypeptides in the highly purified DNA polymerase α preparation. The DNA polymerase α migrates as a single band in preparative electrophoresis. We could not detect additional subunits of lower molecular weight, Due to the small amount of enzyme which we were able to prepare now it is not possible to show unequivocally whether or not the 5.7 S DNA polymerase α is a native dimer having a molecular weight of about 250000 daltons, or if it exists as a mixture of the two polypeptides. A preliminary molecular weight determination in the analytical ultracentrifuge supports the latter possibility. In accordance with this interpretation is also the sedimentation coefficient of 5.7 S that is

Fig.3: SDS electrophoresis of enzyme of specific activity 100000 units/mg (A). The subunits of RNA polymerase were used as molecular weight markers (B).

more compatible with a native molecular weight of about 130000 daltons than with a molecular weight of 250000 daltons. The co-purification of the two polypeptides in about equimolar ratio may be explained by a loose association between the two polypeptides and/or by a very similar amino acid composition, that does not allow separation during chromatography.

The specific activity of our 5.7 S DNA polymerase α is higher than 100000 units/mg. This value was determined with the Bio Rex 70 fraction that, as illustrated by fig.3, still contains contaminants. Due to the small amount of enzyme we could not determine the specific activity accurately for the fractions obtained after preparative electrophoresis or ultracentrifugation. The 5.7 S polymerase represents only part of the total polymerase α activity. We also detect a polymerase α sedimenting at about 8-9 S, which is much more difficult to purify. In highly enriched preparations of this enzyme a subunit of 150000 daltons and other subunits in the molecular weight range of 40000-70000 daltons can be demonstrated. This observation confirms earlier results of Hesslewood et al. (10). The preparation of the larger DNA polymerase α requires strict precautions for avoiding proteolysis (results to be published).

Catalytic properties

Our highly purified polymerase preparation shares most of the properties commonly assigned to DNA polymerase α . The enzyme is very sensitive to N-Ethylmaleimide (NEM). The influence of NEM was followed by incubating the enzyme with different concentrations of the inhibitor at 25°C for 10 minutes prior to the assay. 0.2 mM NEM inactivates the enzyme completely. At one fifth of this concentration (0.04 mM) the enzyme loses 50% of its activity within 10 minutes. Inhibition is also observed with aphidicolin. This compound has been shown to be a specific inhibitor of DNA polymerase α (19). 50% inhibition is observed at a concentration of 0.1 μ g/ml of aphidicolin.

Addition of ampholine has a marked effect on the stability of the enzyme. A low enzymatic activity is measured when small amounts of highly purified enzyme are assayed or when the enzyme

is prediluted from the stock solution, e.g. in buffer A, prior to the assay. Inclusion of 0.8% ampholine in the assay prevents this inactivation (fig.4). Thus a 5-fold higher activity may be measured in the presence as compared to the absence of ampholine. This protective effect of ampholine is very helpful in detecting small amounts of the enzyme, e.g. in sucrose gradients or preparative gels. Therefore we routinely included ampholine in the assay mixture. Addition of KCl also has a stimulating effect on the enzyme activity (fig.4), although the influence of KCl is much larger if ampholine is omitted (fig.4). In this case a maximal stimulation is achieved with 125 mM KCl. Both the stimulating effect of ampholine and KCl are not observed if larger enzyme concentrations ($>0.1 \mu\text{g}$) are used in the assay. Evidently the enzyme is inactivated when present at low concentrations, which is prevented by ampholine or salt. Determinations of specific activities have been performed at enzyme concentrations high enough to ensure full activity in the absence of ampholine. It has to be pointed out that the 5.7 S polymerase is rather resistant to salt, retaining full activity at 0.1 M KCl; at 0.2 M KCl, 25% of the enzyme activity is still detectable (table 2). The 5.7 S polymerase strongly prefers activated DNA over poly (dA)·(dT)₁₀ (table 2). It is not active when poly (rA)·(dT)₁₀ is used as substrate. The enzyme is free of RNA polymerase activity and terminal transferase activity. Digestion experiments with pBR 322 as substrate show that the

Fig.4: Influence of ampholine and KCl on the enzyme when assayed at low concentration. DNA polymerase α (100000 U/mg, 75 $\mu\text{g}/\text{ml}$) was diluted 5-fold in buffer D. 2 μl of this solution were assayed in 50 μl of the standard assay for 5 minutes. Activity in the presence (●) and absence (○) of 0.8% ampholine, at different KCl concentrations.

Table 2: Template utilization and salt dependence of the 5.7 S DNA polymerase α . 0.3 μ g of enzyme (100000 units/mg) were incubated at 37°C. An initial incorporation of 500 pmol/min was measured. Values are given relative to the standard assay.

Template	other conditions	activity
standard assay		
activated DNA	no ampholine	100 %
activated DNA	0.1 M KCl	100 %
activated DNA	0.2 M KCl	25 %
poly dA·(dT) ₁₀ (10:1)		10 %
poly rA·(dT) ₁₀ (10:1)		2 %

enzyme of a specific activity of 100000 units/mg contains trace amounts of a double strand specific nuclease activity. It can be calculated that the nuclease activity of this fraction causes fewer than 5×10^{-10} double strand breaks per hour and per mg of protein. No nuclease activity is detectable using ³H-poly (dA) as substrate.

DISCUSSION

We have purified to near homogeneity a DNA polymerase α species that consists of a mixture of two polypeptides of molecular weight 123000 and 134000 daltons. As shown by their equal uptake of coomassie blue in SDS gels they are present in about equimolar amounts. We do not at present have data which indicate which of these polypeptides (if not both) has catalytic activity. However our results clearly demonstrate the existence in calf thymus of catalytically active polypeptides in the molecular weight range 130000 daltons. Our findings are in contrast to a recent report of Grummt et al. (5), who have isolated from calf thymus catalytically active polypeptides having molecular weights in the range of 50000-70000 daltons. At present we cannot explain this discrepancy. Hesslewood et al. (10) have described a polymerase α species, named D-enzyme, of 135000 daltons

molecular weight. This D-enzyme apparently is not identical to our polymerase α , since the two enzymes have different activities with poly(dA)·(dT)₁₀ and exhibit different salt sensitivity. Holmes et al. (3) also report the occurrence of a subunit of 15000 daltons in calf thymus. Our data (not shown) support this observation. We have evidence that the 5.7 S polymerase described in the present paper is derived from a larger DNA polymerase by limited proteolysis (results to be published). It is interesting to note that the specific activity of the 5.7 S polymerase is considerable higher than that of other highly purified DNA polymerase α species (enzyme A₁, A₂, C in (10), (1)). Although it may be difficult to compare specific activities derived in different laboratories, the possibility remains that the proteolytic degradation of the larger enzyme to a smaller one leads to a substantial increase in the catalytic activity. A similar phenomenon has been described in the rat thymus system (20). It remains to be shown whether or not such a limited proteolysis is also active during replication. The marked heterogeneity of DNA polymerase α that confuses workers in this field may be due to the occurrence of different polymerase species in different stages of replication and perhaps in different compartments of the cell. In this context, it is remarkable that the polymerase described in the present paper exhibits only a moderate dependence on salt. This enzyme is still active at ionic conditions that are assumed to exist in the cell.

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References

- 1) Mechali, M., Abadiebat, J. and de Recondo, A.M., (1980) *J.Biol.Chem.* 255, 2114-2122
- 2) Banks, G.R., Boezi, J.A. and Lehman, J.R. (1976) *J.Biol.Chem.* 254, 9886-9892
- 3) Holmes, A.M., Hesslewood, I.P. and Johnston, I.R. (1976) *Eur.J.Biochem.* 62, 229-235
- 4) Fisher, P.A. and Korn, D. (1977) *J.Biol.Chem.* 252, 6528-6535
- 5) Grummt, F., Walzl, G., Jantzen, H.M., Hamprecht, K., Huebscher, U. and Kuenzle, C.C. (1979) *Proc.Natl.Acad.Sci.USA* 76, 6081-6085
- 6) Chen, Y.C., Boln, E.W., Planck, R. and Wilson, S.H. (1979) *J.Biol.Chem.* 254, 11678-11687
- 7) Aposhian, H.V. and Kornberg, A. (1962) *J.Biol.Chem.* 237, 519-525
- 8) Anatonopoulos, C.A. (1962) *Acta.Chem.Scand.* 16, 1521-1522
- 9) Böhme, H.J., Koppenschlaeger, G., Schulz, J. and Hofmann, E. (1972) *J.Chrom.* 69, 209-214
- 10) Hesslewood, I.P., Holmes, A.M., Wakeling, W.F. and Johnston, I.R. (1978) *Eur.J.Biochem.* 84, 123-131
- 11) Fasiolo, F., Befort, N., Boulanger, Y. and Ebel, J.P. (1970) *Biochem.Biophys.Acta* 217, 305-317
- 12) Burgess, R.R. (1969) *J.Biol.Chem.* 244, 6160-6167
- 13) Bollum, F.J., Chang, L.M.S., Tsiapilis, C.M. and Dorson, J.W. (1974) in *Methods in Enzymology*, Vol. XXIX, pp 70-81 Academic Press, New York
- 14) Laemmli, U.K. (1970) *Nature* 227, 680-685
- 15) Noll, H. (1967) *Nature* 215, 360-363
- 16) Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) *J.Biol.Chem.* 193, 265-275
- 17) Duguet, M., Mechali, M. and Rossignol, J.M. (1978) *Anal.Biochem.* 88, 399-405
- 18) Holmes, A.M., Hesslewood, I.P. and Johnston, I.R. (1974) *Eur.J.Biochem.* 43, 487-499
- 19) Pedrali-Noy, G. and Spadari, S. (1979) *Biochem.Biophys.Res. Commun.* 88, 1194-2002
- 20) Penner, P. (1979) *Canad.J.Biochem.* 57, 1026-1029