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# The mammalian primase is part of a high molecular weight DNA polymerase α polypeptide

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**The recently discovered eukaryotic primases have been found in tight association with certain DNA polymerase α forms. Here I present evidence that the high mol. wt. catalytic polypeptide (125 000) of an apparently homogeneous DNA polymerase α from freshly harvested calf thymus contains both polymerase and primase activity. This conclusion derives from the following three facts: (1) the two enzyme activities cannot be separated upon velocity sedimentation in 1.7 M urea, (2) both activities elute at a pI of 5.25 upon chromatofocussing and (3) after SDS-electrophoresis, renaturation of the enzymes *in situ* and measurement of DNA polymerase and primase activities in the gels, both enzymes have identical mobilities and coincide with the high mol. wt. catalytic subunit of DNA polymerase α.**

*Key words:* calf thymus DNA polymerase α/calf thymus primase/catalytic subunit/enzyme renaturation after SDS-gel electrophoresis/identity

## Introduction

DNA replication in prokaryotes and eukaryotes is carried out continuously on the leading- and discontinuously on the lagging-strand (Kornberg, 1980). The nascent DNA pieces (Okazaki fragments) on the lagging-strand are initiated on RNA primers (Kornberg, 1980). Enzymes, called primases, that synthesize these RNA primers, have been described in prokaryotes (Scherzinger *et al.*, 1977; Rowen and Kornberg, 1978), during semi-discontinuous replication of polyoma DNA (Eliasson and Reichard, 1978), and in a variety of eukaryotic organisms such as *Drosophila melanogaster* (Conway and Lehman, 1982), *Xenopus laevis* and cultured mammalian cells (Kaufman and Hoffman, 1982; Yagura *et al.*, 1982a, 1982b; Tseng and Ahlen, 1982). All eukaryotic primases so far tested have been found in close association with DNA polymerase α (Conway and Lehman, 1982; Riedel *et al.*, 1982; Kaufman and Hoffman, 1982; Yagura *et al.*, 1982a, 1982b; Kozu *et al.*, 1982).

The calf thymus DNA polymerase α has been purified to apparent homogeneity by several authors (Albert *et al.*, 1982; Holmes *et al.*, 1976; Grummt *et al.*, 1979; Grosse and Krauss, 1980, 1981; Masahi *et al.*, 1982). From all these reports it appeared as if several forms of calf thymus DNA polymerase α existed *in vivo*. This complexity is further accentuated by artefactual proteolysis during isolation. Avoiding the latter complication we have isolated an apparently homogeneous form of calf thymus DNA polymerase α, defined as the core enzyme (Hübscher *et al.*, 1982). This consists of a high mol. wt. (125 000) catalytic subunit and several smaller mol. wt. (54 000 – 64 000) non-catalytic polypeptides. Proteolytic peptide mapping shows that the small mol. wt. polypeptides share structural features with the high mol. wt. polypeptide suggesting that they may derive from a single precursor molecule of >140 000 dalton (Albert *et al.*, 1982). When strict precautions are taken to prevent proteolysis during isolation, a holoenzyme form of calf thymus DNA polymerase α can be purified that is able to elongate *in vivo*-like DNA primer-templates (Hübscher *et al.*, 1982). In the course of these studies, a primase activity was found in certain DNA polymerase α preparations, and this primase activity could never be separated from the polymerase by the usual chromatographic and sedimentation techniques. Even our apparently homogeneous DNA polymerase α (Albert *et al.*, 1982) contained the primase activity. This raised the possibility that primase and polymerase activities might reside on the same polypeptide.

Here I present evidence that the high mol. wt. polypeptide of an apparently homogeneous calf thymus DNA polymerase α preparation (core enzyme; Hübscher *et al.*, 1982) contains primase activity. This conclusion derives from the facts that: (1) these two enzyme activities cannot be separated upon velocity sedimentation in 1.7 M urea, (2) both activities elute at a pI of 5.25 upon chromatofocussing and (3) after SDS-electrophoresis, renaturation of the enzymes *in situ* and measurement of DNA polymerase and primase activities in the gels (Spanos *et al.*, 1981; Spanos and Hübscher, 1982) both enzymes have identical mobilities and coincide with the high mol. wt. catalytic subunit of DNA polymerase α (Albert *et al.*, 1982; Hübscher *et al.*, 1981).

## Results

Three types of experiment were conducted with the apparently homogeneous DNA polymerase α from calf thymus (core enzyme, Hübscher *et al.*, 1982). In the first, this preparation was centrifuged in a 10 – 30% glucose gradient containing 1.7 M urea (Figure 1). No separation of DNA polymerase α from the primase activity was evident. Under identical conditions the separation of the adenovirus DNA polymerase from its tightly associated terminal protein precursor (a special form of a priming protein, Lichy *et al.*, 1982) has been achieved. In addition, a similar treatment of *D. melanogaster* DNA polymerase α has resulted in the dissociation of the high mol. wt. catalytic subunit from the three small mol. wt. subunits (Villani *et al.*, 1980). Therefore, if the primase and the DNA polymerase α from calf thymus were separate entities the urea treatment should have brought about their dissociation.

In a second experiment, the same DNA polymerase α preparation was subjected to chromatofocussing (Figure 2). Again, no separation ensued as evidenced by the exact coincidence of polymerase and primase activity at an isoelectric point of 5.25.

Third, the DNA polymerase α was electrophoresed in SDS-polyacrylamide gels containing either gapped DNA or single-stranded M13 DNA. *Escherichia coli* DNA polymerase I and its Klenow fragment were used as size markers and controls. After electrophoresis, the enzymes were renatured *in situ* and their activities determined in the gels as described (Spanos *et al.*, 1981; Spanos and Hübscher, 1982). The results are shown

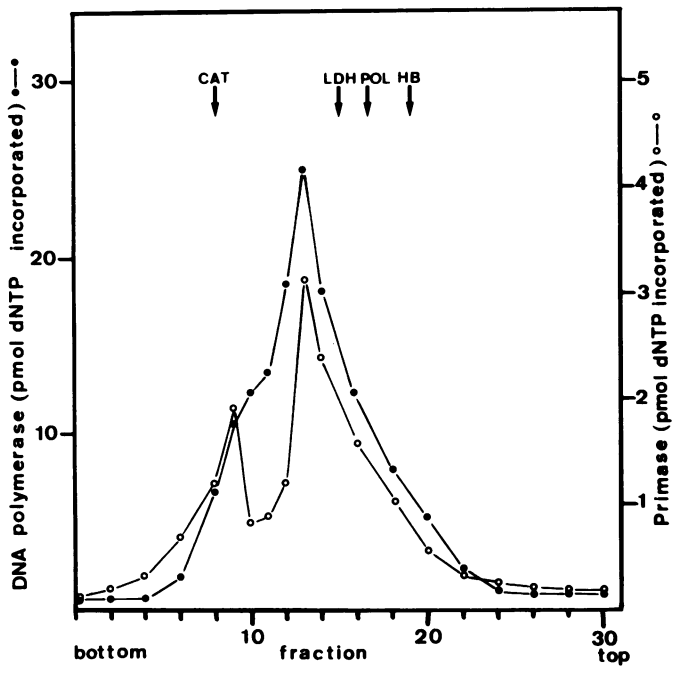


**Fig. 1.** Glucose gradient centrifugation of calf thymus DNA polymerase α in the presence of 1.7 M urea. 100 units of apparently homogeneous DNA polymerase α were dialysed against buffer A [20 mM Tris-HCl, pH 7.5; 100 mM ammonium sulfate; 1 mM EDTA, 2.5 mM DDT; 10% (v/v) glycerol; and 1.7 M urea] and applied to a 4.8 ml linear gradient of 10–30% (w/v) glucose in buffer A containing the same concentration of urea. Centrifugation, collection of fractions and determination of DNA polymerase α or primase activities were carried out as described in Materials and methods.

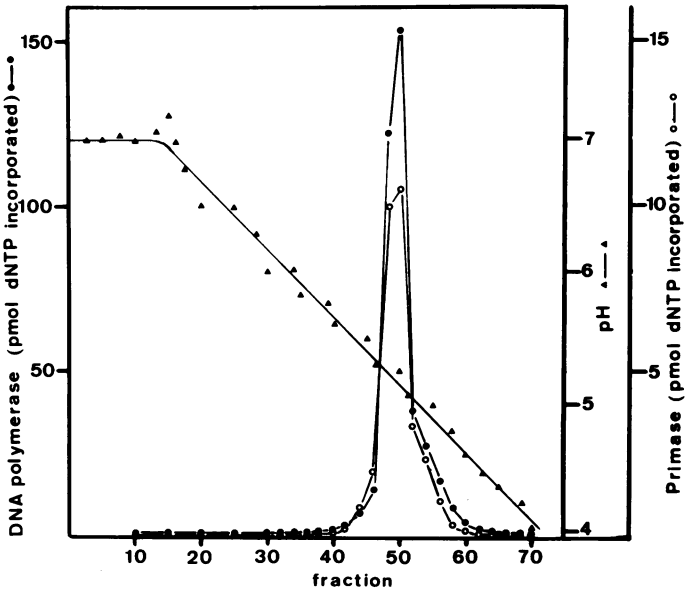


**Fig. 2.** Chromatofocussing of DNA polymerase α. 250 units of apparently homogeneous DNA polymerase α were dialysed against buffer B (25 mM imidazole HCl, pH 7.4; 20% (w/v) glycerol; 5 mM DTT and 1 μM pepstatin). Chromatofocussing and determination of DNA polymerase α or primase activities were performed as described in Materials and methods.

in Figure 3. Lanes 1 and 2 are positive controls. They show that in the presence of gapped DNA, representative of a multiply primed template, deoxynucleotide polymerisation occurred with *E. coli* DNA polymerase I (109 000 dalton) and

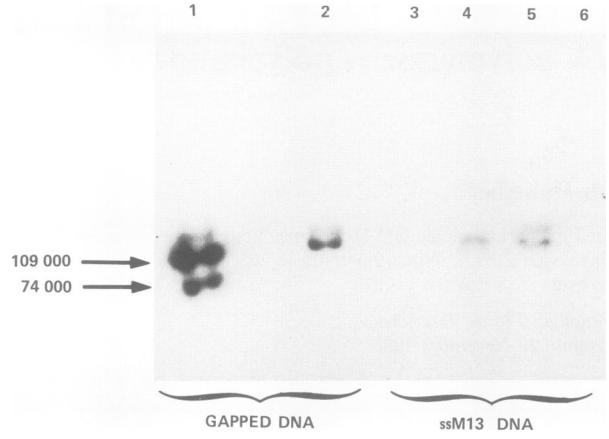


**Fig. 3.** Autoradiogram of the activities of DNA polymerases and primase following SDS-polyacrylamide gel electrophoresis and *in situ* renaturation of the enzymes. Preparation of the enzymes for electrophoresis, electrophoretic separation and renaturation of the enzymes in the gel were performed as described in Materials and methods. The gel containing gapped DNA was incubated in 3 ml of 70 mM Tris-HCl, pH 7.5; 7 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 14 μM each of dATP, dGTP and dTTP and 5 μCi [α-$^{32}$P]dCTP (3000 Ci/mmol) for 14 h at 37°C. Slices of the gel containing single-stranded (ss) M13 DNA were incubated in 3 ml of 70 mM Tris-HCl, pH 8.5; 10 mM $MgCl_2$; 10 mM 2-mercaptoethanol; 14 μM each of dATP, dTTP and dGTP (plus dCTP in **lane 4**); 5 μCi of [α-$^{32}$P]dCTP (omitted in **lane 4**) and 200 μM each of ATP and GTP (replaced by 5 μCi [α-$^{32}$P]GTP 3000 Ci/mmol, in **lane 4**), CTP and UTP (all rNTPs omitted in **lane 6**) for 14 h at 37°C. The unincorporated radioactive nucleoside triphosphates were washed out with 5% (w/v) TCA containing 1% (w/v) sodium pyrophosphate for 26 h. The gels were finally dried and autoradiographed on a Kodak X-5 film using an intensifying screen. The experiment was carried out as follows: two 7.5% polyacrylamide miniature gels of identical size were prepared, one containing gapped DNA and the other ss M13 DNA. After loading the enzymes, both gels were electrophoresed together under identical conditions in a slab gel apparatus and the enzymes were renatured as outlined (see Materials and methods and Spanos *et al.*, 1981). The gel containing ss M13 DNA was sliced into four pieces with sterile razor blade and were incubated as described above. After incubations and wash-out of the unincorporated nucleoside triphosphates, the sliced gel pieces containing ss M13 DNA were realigned and placed side by side with the gel containing gapped DNA. The gels were dried and autoradiographed. The lanes contained the following enzymes: **lanes 1** and **3**, a mixture of *E. coli* DNA polymerase I (0.1 unit) and its Klenow fragment (0.1 unit; both enzymes were a kind gift of A. Spanos, MRC, Mill Hill); **lane 2, 4, 5** and **6**, apparently homogeneous calf thymus DNA polymerase α (10 units).

its Klenow fragment (74 000 dalton) on the one hand (lane 1) and with calf thymus DNA polymerase α on the other (lane 2). With the latter, a single high mol. wt. (125 000 dalton) activity band was observed. This was slightly distorted as often noticed with purified enzymes in such renaturation gels (Spanos *et al.*, 1981; Spanos and Hübscher, 1982) (compare also the shape of the band of the 109 000 dalton *E. coli* DNA polymerase I in lane 1). When these enzymes were tested on unprimed, single-stranded M13 DNA (lanes 3–6) in the presence of all four ribo- and [α-$^{32}$P]deoxynucleoside triphosphates, the *E. coli* enzymes no longer were capable of deoxynucleotide polymerisation (lane 3). This was in agreement with their lack of primase activity (Kornberg, 1980) and, consequently, their absolute dependency on an exogenous primer (Kornberg, 1980). In contrast, the DNA polymerising activity of calf thymus DNA polymerase α was not affected by the absence of preformed primer as long as the four ribonucleoside triphosphates were available (lane 5) but was suppressed when the latter were omitted from the reaction mixture (lane 6). This indicated that the high mol. wt. activity band produc-

ed its own primer for DNA synthesis. That this was the case was shown by directly assaying the DNA polymerase α for primase activity by incubating the polymerase with [α-$^{32}$P]-ribonucleoside triphosphates in the presence of cold deoxynucleoside triphosphates. This produced a single band of RNA polymerase activity (lane 4), which exactly corresponded to the band of DNA polymerase activity visible in lane 5.

## Discussion

All reports on eukaryotic primases documented that this enzyme is tightly associated with DNA polymerase α (Conway and Lehman, 1982; Riedel *et al.*, 1982; Kaufman and Hoffman, 1982; Yagura *et al.*, 1982b). Separation cannot be effected by chromatography on DEAE-cellulose, phosphocellulose, hydroxylapatite and single-stranded or double-stranded DNA cellulose, by sedimentation under non-denaturing conditions and by ammonium sulfate fractionation. Here I have added three further items to this list. Though the number of vain separation attempts is strongly indicative of a common peptide for primase and polymerase activity, convincing proof so far has been lacking. The problem is exemplified by two of the techniques used in the present study. While sedimentation in 1.7 M urea has proven ability to achieve dissociation of non-covalently associated proteins (Lichy *et al.*, 1982) but is of low resolution, chromatofocussing has high resolution power but lacks denaturing capacity. Clearly, what is needed is a method that combines strong denaturion with high resolution. SDS-polyacrylamide gel electrophoresis followed by renaturation of the separated peptides and enzyme assay in the gels fulfills these stringent criteria (Spanos *et al.*, 1981; Spanos and Hübscher, 1982). Using this method I have demonstrated that the high mol.wt. (125 000 dalton) polypeptide of calf thymus DNA polymerase α contains both primase and polymerase activity.

The term DNA replicase has been used for a form of DNA polymerase α tighly associated with primase activity (Kozu *et al.*, 1982; Yagura *et al.*, 1982a). It has been suggested that this form is a good candidate for performing discontinuous DNA replication. Thus the enzyme that combines DNA polymerase α and primase activity may be responsible for synthesis of Okazaki fragments at the DNA replication fork.

## Materials and methods

*Chemicals and preparation of DNAs*

Unlabelled ribonucleoside and deoxynucleoside triphosphates were from P-L Biochemicals. [$^3$H]dTTP, [α-$^{32}$P]dCTP and [α-$^{32}$P]GTP were from Amersham. Urea, from Schwarz/Mann, was dissolved immediately before use as outlined by Villani *et al.*, 1980. Gapped calf thymus DNA was prepared as described for *in vitro* assays (Hübscher *et al.*, 1977) and for polymerisation into the SDS-polyacrylamide gels (Spanos *et al.*, 1981). M13 virus production and isolation of the single-stranded M13 DNA was performed as reported by Sadowski and Hurwitz, 1969.

*In vitro assays for DNA polymerase α and primase*

The DNA polymerase α activity was determined in an assay mixture (final volume 25 μl) containing 20 mM potassium phosphate, pH 7.2; 0.1 mM EDTA; 4 mM dithiothreitol (DTT); 0.25 mg/ml bovine serum albumin (BSA); 10 mM $MgCl_2$; dATP, dGTP and dCTP each at 48 μM; 18 μM [$^3$H]dTTP (100–200 c.p.m./pmol) and 16.5 nmol gapped DNA (DNA concentration expressed as nucleotides). Incubation was at 37°C for 10 min. The primase activity was determined in an assay (total volume 25 μl) containing 20 mM Tris HCl, pH 8.5; 4% (w/v) sucrose; 8 mM DTT; 80 μg/ml BSA; 10 mM $MgCl_2$; 5 mM ATP; 200 μM each of GTP, CTP and UTP; 40 μM each of dGTP, dATP and dCTP; 18 μM [$^3$H]dTTP (400 c.p.m./pmol) and 800 pmol unprimed, single-stranded M13 DNA (DNA concentration expressed as nucleotides). Incubation was at 37°C for 60 min. Under these conditions, incorporation of [$^3$H]dTTP into acid-precipitable material occurs only in the presence of a primase (Conway and Lehman, 1982). The reactions were stopped, the DNA precipitated, the precipitates collected on Whatman GF/C filters and the radioactivity determined as described (Hübscher and Kornberg, 1979). One unit of activity is defined as the incorporation of 1 nmol dNTPs into acid-insoluble DNA in 60 min at 37°C.

*Purification of DNA polymerase α*

DNA polymerase α (designated as the core enzyme, see Hübscher *et al.*, 1982) from freshly harvested calf thymus (frozen in liquid nitrogen <5 min after killing the 4 month-old animal) was purified to apparent homogeneity as described (Albert *et al.*, 1982; Grummt *et al.*, 1979) in the presence of pepstatin (1 μM) and sodium bisulfite (10 mM) to prevent proteolytic degradation of the high mol. wt. catalytic subunit (Hübscher *et al.*, 1981).

*Glucose gradient centrifugation in the presence of urea*

100 units of the pure DNA polymerase α were dialysed against buffer A [20 mM Tris.HCl, pH 7.5; 100 mM ammonium sulfate; 1 mM EDTA, 2.5 mM DTT; 10% (v/v) glycerol; and 1.7 M urea] and applied to a 4.8 ml linear gradient of 10–30% (w/v) glucose in buffer A containing the same concentration of urea. The centrifugation was carried out in a Beckman SW 50.1 rotor at 45 000 r.p.m. for 20 h at 0–2°C. Fractions (150 μl) were collected from the bottom of the tube and assayed for DNA polymerase α and primase activity. The sedimentation coefficient markers were run in a parallel gradient. These were: horse hemoglobin (HB, 4.13S), *E. coli* DNA polymerase I (POL, 5.5S), rabbit muscle lactate dehydrogenase (LDH, 7.2S) and bovine catalase (CAT, 11.3S).

*Chromatofocussing*

250 units of apparently homogeneous DNA polymerase α were dialysed against buffer B [25 mM imidazole.HCl, pH 7.4; 20% (w/v) glycerol; 5 mM DTT and 1 μM pepstatin]. A 10 ml (15 x 0.9 cm) chromatofocussing column (Polybuffer Exchange 94 from Pharmacia) was poured and equilibrated with 30 column volumes of buffer B at a rate of 63 ml/h. The dialysed DNA polymerase α was applied to the column at 25 ml/h. The column was then washed with 10 volumes of buffer B and the DNA polymerase α eluted with 110 ml polybuffer 74.HCl (Pharmacia) diluted 1:8 with buffer B at the same rate. Fractions of 1.5 ml were collected and assayed for DNA polymerase α and for primase activity. The pH was determined with a standard pH electrode.

*Activities of DNA polymerases and primase following SDS-polyacrylamide gel electrophoresis and in situ renaturation of the enzymes*

Details of this technique have been described (Spanos *et al.*, 1981; Spanos and Hübscher, 1982). This method has been adapted to a miniature slab gel system with a gel volume of <1 ml per slab gel (47 x 30 x 0.8 mm). Briefly, the enzymes in 65 mM Tris-HCl, pH 6.8, 1 mM EDTA; 125 mM 2-mercaptoethanol; 10% (v/v) glycerol and 1% (w/v) SDS were heated at 37°C for 3 min and electrophoresed in a 7.5% (w/v) polyacrylamide gel containing 0.1% SDS; 2 mM EDTA and either gapped DNA or single-stranded M13 DNA at a final concentration of 120 μg/ml. Electrophoresis was at 2.5 mA constant current for 2 h. SDS was removed (30 min in 50 mM Tris.HCl, pH 7.5; 10 mM 2-mercaptoethanol and 1 mM EDTA at 37°C) and the enzymes were renatured (50 mM Tris.HCl, pH 7.5; 3 mM 2-mercaptoethanol and 1 mM EDTA, first for 30 min at 37°C and then for a further 28 h at 4°C). DNA polymerase and primase activities were then determined *in situ* as described in detail in the Legend to FIgure 3.

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**Note added in proof**

The faster sedimenting primase peak co-sedimenting with a shoulder of DNA polymerase $\alpha$ in Figure 1 appears to be an aggregated form of DNA polymerase $\alpha$.