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Hypothesis

Do DNA polymerases δ and α act coordinately as leading and lagging strand replicases?

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The activity ratio of DNA polymerases δ and α in calf thymus was found to be invariably 1:1, irrespective of extraction procedure (8 types) and subcellular localization (cytoplasm, nucleus and microsomes). This was established by separation of the two forms by hydroxyapatite chromatography and by their response to specific inhibitors and monoclonal antibodies. This finding supports the dimeric DNA polymerase model [(1980) J. Biol. Chem. 255, 4290–4303], which proposes that DNA polymerases δ and α act coordinately as leading and lagging strand enzymes, respectively, at the replication fork.

DNA polymerase δ; DNA polymerase α; Leading strand; Lagging strand; Dimeric model; Quantitation

1. INTRODUCTION

During DNA replication both DNA strands have to be copied with the same speed and accuracy. Due to the universal 5′ to 3′ directionality of any DNA polymerase known, one strand is synthesized continuously (the leading strand) and the other discontinuously (the lagging strand). A dimeric DNA polymerase has been proposed [1,2] and biochemically characterized in bacteria [3] which combines functions required for efficient polymerization of both strands. In higher eukaryotes, pol α has been known for a long time to play a major role in DNA replication [4]. Recently, data were presented suggesting that pol δ, a fourth cellular DNA polymerase [5], participates in DNA replication [6]. In addition, cell-free replication of SV40 DNA indicated that both pol δ and α have functional roles [7].

The establishment of a quantitative assay for pol δ and α allowed us to perform exact measurements in crude extracts. A pol δ to pol α activity ratio of invariably 1:1 was obtained in calf thymus. The results are based on (i) a variety of extraction conditions, (ii) different subcellular localizations, (iii) ammonium sulfate precipitation with subsequent standardized solubilization and (iv) separation of pol δ from pol α by hydroxyapatite chromatography. Our data support the model proposed by Sinha et al. [1] that pol δ and α might act coordinately as leading and lagging strand replicases.

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Abbreviations: pol, DNA polymerase; BuPdGTP, N^2-(*p*-*n*-butylphenyl) dGTP; BuAdATP, 2-(*p*-*n*-butylanilino) dATP

2. MATERIALS AND METHODS

Assays for pol δ and pol α contained the following components in a final volume of 25 μl: 75 mM Hepes-KOH (pH

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7.5), 1.25 mM dithiothreitol, 20% (v/v) glycerol, 10 mM $MgCl_2$, 0.24 mg/ml bovine serum albumin (DNase-free), 0.5 μg poly(dA)/oligo(dT)$_{12-18}$ (base ratio 10:1), 10 μM [^{3}H]dTTP (1000–1500 cpm/pmol) and enzyme fraction to be assayed. The pol α specific inhibitors BuPdGTP [8] and BuAdATP [9] were used at concentrations that inhibited >98% of pol α (5 μM) and the pol α and δ specific inhibitor aphidicolin at 50 μg/ml, resulting in inhibition of >95%. The neutralizing monoclonal antibody 132-20 [10] specific for pol α [11] was used in excess in an amount of 2.85 μg per assay. All inhibitors and the antibody were added to the reaction mixtures before the enzyme fractions. For determination of pol α only, the final volume of 25 μl contained: 20 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 4 mM dithiothreitol, 0.25 mg/μl bovine serum albumin, 10 mM $MgCl_2$, dATP, dGTP and dCTP each at 48 μM, 18 μM [^{3}H]dTTP (250–500 cpm/pmol), 3 μg activated DNA and enzyme fraction to be assayed. Incubations were at 37°C for 15 min. Trichloroacetic acid-insoluble material was determined as described by Hübscher and Kornberg [12]. One unit of enzyme activity is defined as 1 nmol dNTP incorporated into acid-precipitable material in 60 min at 37°C.

2.1. *Extractions*

All extraction methods were standardized. The thymus was finely ground at −20°C and immediately resuspended in the appropriate buffer. All buffers contained the two protease inhibitors sodium metabisulfide (10 mM) and pepstatin (1 μM). Extractions were performed at 0–2°C as fast as possible, and aliquots of 20–30 μl were frozen immediately and stored in liquid nitrogen. A given aliquot was thawed and assayed only once. Extracts from 3 g calf thymus were prepared as follows (see table 1 for lettering): (A) Cytoplasm as originally devised for pol α according to [13]. (B) Cytoplasm as originally devised for pol δ according to [14]. (C) Cytoplasm as originally devised for pol α and precipitated with 33% ammonium sulfate as described below. (D,E) To prepare nuclear extracts, 3 g calf thymus powder were dissolved in 9 ml of 10 mM Hepes-KOH (pH 7.5), 1.5 mM $MgCl_2$, 10 mM KCl, 0.5 mM dithiothreitol and homogenized with 10 strokes in a glass-teflon homogenizer at 500 rpm. After centrifugation in a Sorvall HB4 rotor at 2500 rpm for 10 min the pellet was washed with the same buffer, recentrifuged as above and resuspended in 2.5 ml of 20 mM Hepes-KOH (pH 7.5), 20% (v/v) glycerol, 0.1 M NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 0.5 mM dithiothreitol. This solution was rehomogenized as above, left on ice for 30 min and centrifuged in an HB4 rotor at 2500 rpm for 10 min. The supernatant was centrifuged in an SS34 rotor at 15000 rpm for 30 min. The final supernatant was designated as the 0.1 M NaCl nuclear extract (D). The pellet was then treated as above but with buffer containing 0.42 M NaCl, to yield the 0.42 M NaCl nuclear extract (E). Both extracts were concentrated in Centricon 10 tubes and finally frozen in aliquots in liquid nitrogen. (F) Microsomal extracts were prepared according to [14]. (G) An extraction optimal for in vitro replication of SV40 was carried out as described by Wobbe et al. [15]. (H,L) Whole cell extracts were prepared with modifications of a published procedure [16]. 3 g pulverized calf thymus were resuspended in 15 ml of 50 mM Tris-HCl (pH 7.5), 4 mM Mg acetate, 2 mM $CaCl_2$, 25 mM KCl, 10% (w/v) sucrose, 2 mM dithiothreitol, and homogenized with 20 strokes in a glass-teflon homogenizer at 500 rpm. The homogenate was brought to 1 M KCl and the viscous solution divided into halves. One half (9 ml) was dialyzed for 12 h against 4 × 250 ml of a solution containing 20 mM Hepes-KOH (pH 7.5), 1 mM EDTA, 20% (v/v) glycerol, 1 mM dithiothreitol and centrifuged at 100000 × g for 30 min. This fraction was designated as the dialyzed whole cell extract (L). The other half was left on ice for 30 min and then

Table 1

DNA polymerases α and δ in crude and partially purified enzyme fractions from calf thymus

Extract/enzyme fraction	Method[a]	Pol α (U/ml)	Pol δ (U/ml)	Pol α/pol($\alpha+\delta$)
A. Cytoplasm, originally devised for pol α	13	71	78	0.48
B. Cytoplasm, originally devised for pol δ	14	84	62	0.58
C. Cytoplasm, 33% ammonium sulfate precipitate	M	38	34	0.53
D. Nuclear extract, 0.1 M NaCl	M	86	38	0.69
E. Nuclear extract, 0.42 M NaCl	M	79	61	0.56
F. Microsomes	14	7[b]	8[b]	0.46
G. Extract optimized for SV40 replication	15	101	108	0.48
H. Whole cell extract, 1 M KCl, not dialyzed	M	23[b]	18[b]	0.56
I. As H, 80% ammonium sulfate precipitate, redissolved for 60 min	M	77	101	0.43
J. As I, but redissolved for 90 min	M	78	113	0.41
K. As I, but redissolved for 120 min	M	32	19	0.62
L. Whole cell extract, 1 M KCl, dialyzed	M	90	81	0.53
M. As L, 80% ammonium sulfate precipitate, redissolved for 90 min	M	55	44	0.55
N. Partially purified pol α/δ	M, 14	28	22	0.56
Arithmetical mean of the ratio pol α:pol$\alpha+\delta$ as calculated from A-N				0.53
O. Pol α after hydroxyapatite	fig.2	<1	24	<0.04
P. Pol δ after hydroxyapatite	fig.2	20	<1	>20

[a] The extracts were prepared from 3 g of fresh calf thymus as described in the literature (number) or as described in section 2 (M)

[b] These low values are due to salt inhibition

centrifuged at 100000 × g for 30 min, resulting in the non-dialyzed whole cell extract (H).

2.2. *Ammonium sulfate precipitation (C,I–K,M)*

Extracts from cytoplasm (C), whole cell dialyzed (L) and whole cell non-dialyzed (H) were brought to the ammonium sulfate concentration of choice (see table 1) by adding solid salt during 30 min to the solution while stirring with a magnetic stirrer. After stirring for a further 30 min the precipitates were collected in an SS34 rotor at 15000 rpm for 30 min. The pellets were resuspended for exactly 60 min in 0.2 vol. (compared to the precipitated extract) of the following buffer: 50 mM Tris-HCl (pH 7.5), 1 mM EDTA, 10 mM ammonium sulfate, 1 mM dithiothreitol, 20% (v/v) glycerol, 20% (w/v) glucose (C,I). The undissolved material was centrifuged as above. The supernatants were saved (C,I) and the pellets resuspended in the same buffer for another 30 min. After centrifugation the supernatants were saved again (J,M). In the case of the non-dialyzed whole cell extract, the procedure was repeated a third time for 30 min and the final supernatant (K) saved. All fractions (C,I–K,M) were dialyzed against the above buffer, frozen in aliquots and stored in liquid nitrogen.

2.3. *Isolation of pol α and pol δ*

Calf thymus pol α and pol δ were isolated according to published procedures ([13,14] respectively). For the separation of pol α from pol δ 107 units of α/δ (phosphocellulose fraction according to [14]; pol α/δ defines a given fraction that contains pol α and pol δ) were dialyzed against buffer A [20 mM potassium phosphate (pH 7.5), 20% (v/v) glycerol, 1 mM dithiothreitol, 5 mM sodium metabisulfite, 0.1 mM phenylmethylsulfonyl fluoride] and loaded on a 1.5 ml hydroxyapatite column previously equilibrated in buffer A. The column was washed with three column volumes of buffer A, and a 15 ml gradient from 0.02 to 0.4 M potassium phosphate in buffer A was developed at a rate of 2.7 ml/h. Fractions of 0.2 ml were collected and aliquots assayed for activity on poly(dA)/oligo(dT)$_{12-18}$ or on activated DNA.

3. RESULTS AND DISCUSSION

We first established an assay that permitted the accurate determination of pol δ and α in crude and partially purified enzyme fractions. It exploits the facts that the two enzymes can be discriminated with a monoclonal antibody and a variety of specific inhibitors: First, the pol α specific monoclonal antibody 132-20 [10] neutralizes pol α completely but not pol δ [11]; second, aphidicolin inhibits both pol δ and pol α and therefore permits detection of interfering pol β and/or pol γ, if present, both completely resistant to this compound; third, the two deoxyribonucleoside triphosphate analogues BuPdGTP [8] and BuAdATP [9] are specific inhibitors of pol α and are more than 1000-fold less effective against pol δ [17]. Poly(dA)/oligo(dT)$_{12-18}$ was used as the template, which is efficient for pol α [18] as well as for pol δ. In the latter case, however, the presence of an auxiliary protein, called proliferating cell nuclear antigen/cyclin, is necessary [19,20]. Fig.1A,B demonstrates the effects of these compounds on purified calf thymus pol δ [14] and pol α [13], respectively. The assay can also be used for determination in crude extracts, as exemplified for the cytoplasm in fig.1C. Maximal inhibition by the monoclonal antibody 132-20 and the two analogues BuPdGTP and BuAdTP is exactly the same. Total inhibition by aphidicolin suggests the absence of pol β and pol γ. Fig.1C shows furthermore that in a crude cytoplasmic extract the difference between the baseline of antibody and BuPdGTP/BuAdATP on the one hand and the baseline of aphidicolin on the other can be attributed to pol δ.

Table 1 demonstrates that under a variety of extraction and fractionation conditions the activity ratio of pol δ to pol α is close to 1:1. Extracts from cytoplasm originally devised for pol α (table 1A) or pol δ (table 1B), from nuclei performed at moderate (0.1 M NaCl) or intermediate (0.42 M NaCl) salt concentrations (table 1D,E), from microsomes (table 1F), and from a procedure established for in vitro replication of SV40 DNA (table 1G) all resulted in a similar activity ratio of pol δ to pol α. The same activity ratio emerged when whole cell extracts were prepared with 1 M KCl (table 1H,L), a procedure that was described for the isolation of multienzyme complexes involved in DNA synthesis [16]. Results were indistinguishable whether the extracts were dialyzed before centrifugation (table 1L) – to remove the nucleohistones [16] – or not (table 1H). Ammonium sulfate fractionation of these whole cell extracts (table 1I–K,M) and of a cytoplasmic extract originally devised for pol α (table 1C) again resulted in a 1:1 ratio of both activities. The precipitated fractions were dissolved under standardized conditions for 60, 90 and 120 min (table 1I–K). The relative amounts of the two enzymes were again close to 1:1 suggesting that the solubilities of both were similar.

We then purified from a cytoplasmic extract [13] pol α/δ according to a published procedure [14] over phosphocellulose and separated the two activities on hydroxyapatite (fig.2). After phosphocellulose chromatography the pol α:δ

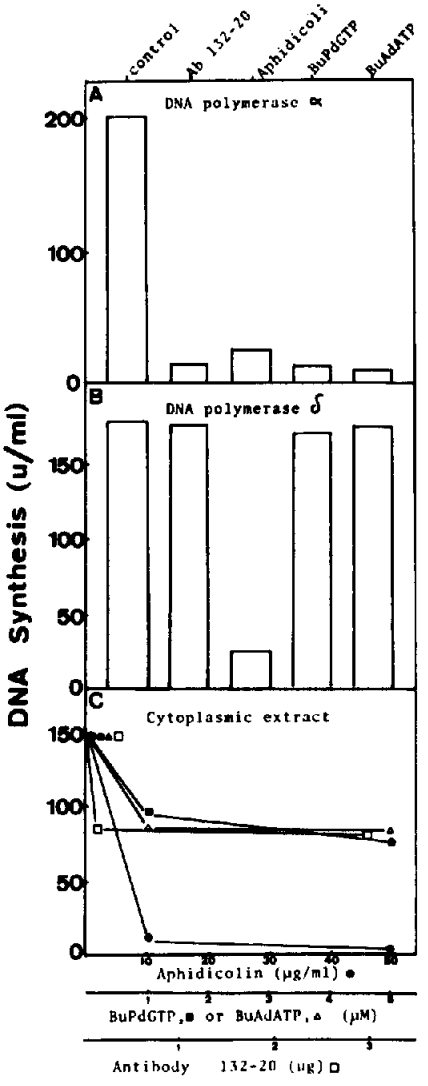

Fig.1. Assays specific for DNA polymerases α and δ. Assays were performed as described in section 2. (A) Purified pol α holoenzyme from calf thymus [13] (700 U/ml); (B) purified pol δ from calf thymus [14] (180 U/ml); (C) crude cytoplasmic extract prepared according to [13].

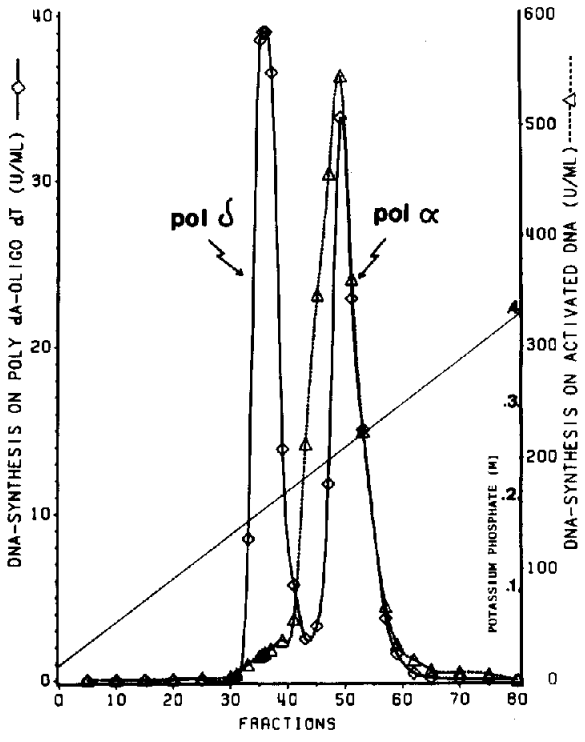

Fig.2. Separation of DNA polymerases α and δ by chromatography on hydroxyapatite. Pol α/δ from a phosphocellulose column [15] was chromatographed on hydroxyapatite as described in section 2. For the determination of pol α and pol δ the template was poly(dA)/oligo(dT)$_{12-18}$ (◇——◇), and for pol α alone the template was activated DNA (△——△). For details see section 2.

ratio remained unchanged (table 1N). Hydroxyapatite resulted in the resolution of pol δ and pol α (fig.2 and table 1O,P) as expected [14]. The total recovery of 38 units for pol δ and 32 units of pol α indicated again an activity ratio of 1:1 for the two DNA polymerases.

Our finding might fit a model proposed by Sinha et al. [1], which postulates a dimeric DNA polymerase working at the replication fork. Dimerization has so far been demonstrated for *E. coli* pol III holoenzyme [3]. The fact that pol δ and pol α appear to be present in equal activities are supportive of the idea that they could act together at the fork. We are aware of the fact that quantitation only measures active enzyme molecules and that a monoclonal antibody against pol δ in connection with quantitative immunoprecipitation would be needed to prove our hypothesis.

It is still not yet clear whether pol δ and pol α are two different enzymes or two different subassemblies of pol α forms [21]. Our consistent finding of a 1:1 activity ratio at least suggests that pol δ and pol α can be obtained in equal activities, and argues against pol δ as a simple isolation artefact. As suggested [22], the two enzymes might fulfill functional tasks at the leading and lagging strand of the replication fork. Pol δ is a candidate for leading strand synthesis [23,24] since in the

presence of proliferating cell nuclear antigen/cyclin it is an extremely processive enzyme, can perform strand displacement synthesis, lacks a primase and might even be regulated by proliferating cell nuclear antigen/cyclin [20,24]. Pol α, on the other hand, is a candidate for lagging strand synthesis [4] since it is only moderately processive satisfying the needs for discontinuous DNA synthesis and recycling at the lagging strand, cannot perform extensive strand displacement synthesis [4], contains a tightly associated primase and exists as a multipolypeptide complex which might be important for the ability to recycle frequently at the lagging strand [4,13].

In conclusion, it appears that pol δ and α exist in equal activities in calf thymus suggesting coordinated functions at the replication fork as leading and lagging strand replicases. Antibodies and specific inhibitors against pol δ will provide insight into the functional roles of both enzymes in DNA replication either separately or in a dimeric complex [1,2,25].

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