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Effect of divalent and monovalent cations on calf thymus PCNA-independent DNA polymerase δ and its $3' \rightarrow 5'$ exonuclease

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Recent data suggest that DNA polymerases α and δ might have a coordinate functional role at the replication fork. In this communication we show that Mg^{2+} is likely the natural metal activator for both enzymes. Mn^{2+}, a known mutagenic agent, is a competitive inhibitor of Mg^{2+} for DNA polymerase δ and acompetitive for DNA polymerase α. The $3' \rightarrow 5'$ exonuclease activity associated with DNA polymerase δ is not affected upon addition of Mn^{2+}. Be^{2+}, another mutagenic agent, on the other hand, has an inhibitory effect on the $3' \rightarrow 5'$ exonuclease, but not on the DNA polymerase δ. The data presented might explain the mutagenic and carcinogenic potential of these two divalent cations.

DNA polymerase δ; Exonuclease, $3' \rightarrow 5'$; Cofactor; Mutagenesis

1. INTRODUCTION

Mammalian cells contain 4 different DNA polymerases termed α, β, γ and δ [1]. Recent data suggested that two of them, DNA polymerases α and δ might play a coordinated role at the replication fork [2-4]. Two forms of DNA polymerase δ have been described: a PCNA-dependent enzyme [5] whose activity on natural DNA and processivity are apparently influenced by the cell cycle-regulated protein named proliferating cell nuclear antigen (PCNA) [6], originally identified as a DNA polymerase δ auxiliary protein [7]; and a PCNA-independent enzyme with similar catalytic properties in the absence of PCNA [8,9]. In our laboratory the overall DNA polymerase δ activity, present in calf thymus, was essentially PCNA-independent [9,10]. However, the purified DNA polymerase δ is very processive and can efficiently copy natural DNA such as primed M13 in the absence of PCNA. These properties, together with the lack of an associated DNA primase make it a candidate for leading strand synthesis, as proposed, by other authors, for the PCNA-dependent DNA polymerase δ. Whether there are two separate enzymes in calf thymus as in yeast [11] or whether these are two forms of the same enzyme is still a matter of investigation that at least awaits the purification of both forms of the enzyme from the same tissue.

On the contrary, DNA polymerase α, purified from the same source, being less processive and tightly associated with DNA primase, possesses properties supporting frequent reinitiations at the lagging strand of DNA [2-4]. In general DNA polymerase α does not have an associated $3' \rightarrow 5'$ exonuclease. Exceptions so far are DNA polymerase α of *Drosophila melanogaster* [12], a DNA polymerase α holoenzyme from calf thymus [13] and immunopurified DNA polymerase α from an human lymphoblast cell line [14]. DNA polymerase δ, on the other hand, was always found with an associated $3' \rightarrow 5'$ exonuclease [15].

All DNA polymerases require a divalent cation for catalysis. Its main function in DNA synthesis appears to be the coordination of the incoming deoxyribonucleoside triphosphate on the active site of the DNA polymerase molecule bound to DNA [1]. Published data suggested that Mg^{2+} is the physiological activator of DNA polymerase α [1].

In this communication we present data which indicate that: (i) Mg^{2+} is apparently also the physiological activator of PCNA-independent DNA polymerase δ; (ii) Mn^{2+}, a mutagen, inhibits DNA polymerase δ by competing with Mg^{2+} for the active site, but can support $3' \rightarrow 5'$ exonuclease proofreading of DNA polymerase δ; (iii) Be^{2+} did not act as an activator cation for DNA polymerase δ and $3' \rightarrow 5'$ exonuclease, but preferentially inhibited the $3' \rightarrow 5'$ exonuclease, when tested under optimal Mg^{2+} concentration; and finally (iv) under optimal Mg^{2+} concentration, DNA polymerase δ was stimulated by monovalent cations (Na^+, K^+) both on artificial and natural DNA templates.

2. MATERIALS AND METHODS

2.1. *Materials*

$MgCl_2$, $MnCl_2$, NaCl and KCl were purchased from Merck. $BeCl_2$ was purchased from Fluka.

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2.2. *Purification of DNA polymerase α and PCNA-independent DNA polymerase δ*

The enzymes were purified from calf thymus as described [6,9,10].

2.3. *Assay for DNA polymerase on activated DNA*

A final volume of 25 μl contained: 20 mM potassium phosphate (pH 7.2), 0.1 mM EDTA, 4 mM dithiothreitol, 10 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, dATP, dGTP, dCTP, 48 μM each, 18 μM [^{3}H]dTTP (500 cpm/pmol), 1.5 μg of activated DNA and enzyme to be tested. When DNA polymerase δ was assayed the reaction mixture included 100 mM KCl. After incubation at 37°C for 15 min, 20 μl of the mixture were spotted onto GF/C filter. The filters were washed three times in 5% (w/v) trichloroacetic acid for 5–10 min, twice in ethanol and the acid-insoluble radioactivity was determined in Omnifluor-based scintillation fluid. One unit is defined as 1 nmol of dNTPs incorporated in 60 min at 37°C.

2.4. *Assay for DNA polymerase on poly(dA)/oligo(dT)$_{12-18}$*

A final volume of 25 μl contained: 75 mM Hepes-KOH (pH 7.5), 1.25 mM dithiothreitol, 20% glycerol (v/v), 10 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, 10 mM KCl, 0.5 μg poly(dA)/oligo(dT)$_{12-18}$ (base ratio 10:1), 10 μM [^{3}H]dTTP (250 cpm/pmol) and enzyme to be tested. After incubation at 37°C for 15 min, 20 ml of the mixture were spotted onto GF/C filters and processed as described above. One unit is defined as 1 nmol of dTTPs incorporated in 60 min at 37°C.

2.5. *Assay for 3′→5′ exonuclease*

A final volume of 25 μl contained: 75 mM Hepes-KOH (pH 7.5), 1.25 mM dithiothreitol, 20% glycerol (v/v), 10 mM $MgCl_2$, 0.25 mg/ml bovine serum albumin, 10 mM KCl, 1 μg poly(dA)/oligo(dT)$_{12-18}$-[^{3}H]dC (1500 cpm/pmol 3′-OH termini) and enzyme to be tested. Incubation was at 37°C for 15 min. Samples were then adsorbed onto 2 cm^2 DEAE-81 paper, washed in 0.3 M ammonium formate (pH 7.8), dehydrated twice in 95% ethanol and counted in Omnifluor-based scintillation fluid. One unit is defined at 1 nmol dCMP removed in 60 min at 37°C.

3. RESULTS

3.1. *Magnesium appears to be the natural divalent activator cation for PCNA-independent DNA polymerase δ*

DNA polymerases α and δ require Mg^{2+} at 10 mM for optimal catalytic activity on activated DNA (fig.1A and B). If $MnCl_2$ replaces $MgCl_2$ the DNA polymerase α activity is reduced more than 50% and DNA polymerase δ more than 70%. With poly(dA)/oligo(dT)$_{12-18}$ as template (fig.2) the activity of DNA polymerase δ was optimal with 10 mM Mg^{2+} concentration. With this template Mn^{2+} was virtually ineffective

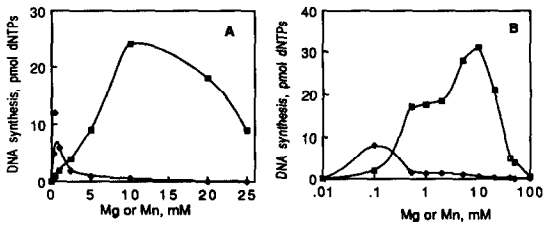

Fig.1. Effect of divalent cations on DNA polymerases α and δ. 0.12 units of DNA polymerases were tested as described in section 2 by using activated DNA as template. (A) DNA polymerase α; (B) DNA polymerase δ; Mg^{2+} (□—□) and Mn^{2+} (◆—◆).

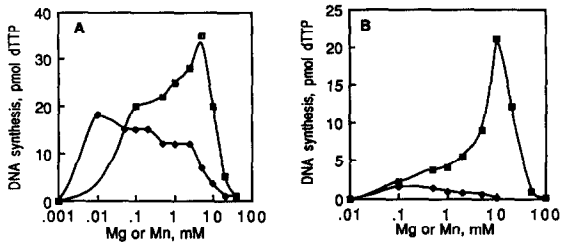

Fig.2. Effect of divalent cations on DNA polymerases α and δ. 0.06 units of DNA polymerases were tested as described in section 2 by using poly(dA)/oligo (dT)$_{12-18}$ as template. (A) DNA polymerase α; (B) DNA polymerase δ; Mg^{2+} (□—□) and Mn^{2+} (◆—◆).

as activator. DNA polymerase α again could use Mn^{2+} as a cofactor at about 50% compared to optimal Mg^{2+} concentration (fig.2A).

In order to test whether Mn^{2+} is unable to activate DNA polymerase δ activity or if it exerts an inhibitory effect on DNA synthesis, increasing amounts of Mn^{2+} were added to optimal concentration of Mg^{2+} (10 mM). Fig.3A indicates that Mn^{2+} inhibited DNA polymerase δ. In fact at 5 mM Mn^{2+} the extent of polymerization was reduced to 10% and it nearly disappeared at 30 mM.

Competitive studies, using different Mg^{2+} and Mn^{2+} concentrations demonstrated that Mn^{2+} inhibited DNA polymerase δ activity by competing for the metal cation active site (fig.3B). The Lineweaver-Burk plot in fig.3B shows the competitive curves with a K_m for Mg^{2+} of 5 mM. The relative K_i of Mn^{2+} was 0.26 mM. The low K_i indicates that the enzyme has a very strong affinity for the inhibiting ion. Analogous experiments with DNA polymerase α on activated DNA resulted also in an inhibition by Mn^{2+} (fig.4A), but apparently in an acompetitive way (fig.4B).

3.2. *Effect of magnesium and manganese on the 3′→5′ exonuclease activity of PCNA-independent DNA polymerase δ*

DNA polymerase δ has an intrinsic 3′→5′ exonuclease activity [8–10] that is stimulated by Mg^{2+} with an

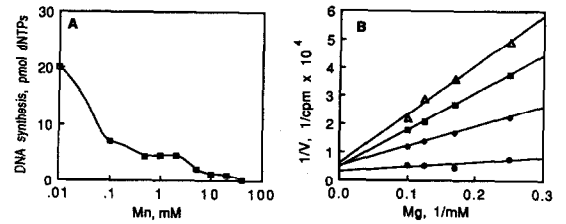

Fig.3. (A) Effect of increasing Mn^{2+} on DNA polymerase δ in presence of optimal Mg^{2+} concentration (10 mM). DNA polymerase δ (0.12 units) was tested by using poly(dA)/oligo(dT)$_{12-18}$ as template as described in section 2. (B) Lineweaver-Burk plot of the effect of Mn^{2+} on the activity of DNA polymerase δ. The enzyme (0.03 units) was assayed as described in section 2 at the Mg^{2+} concentration indicated in abscissa. No Mn^{2+} (●); 0.05 mM Mn^{2+} (◆); 0.1 mM Mn^{2+} (■); 0.2 mM Mn^{2+} (△).

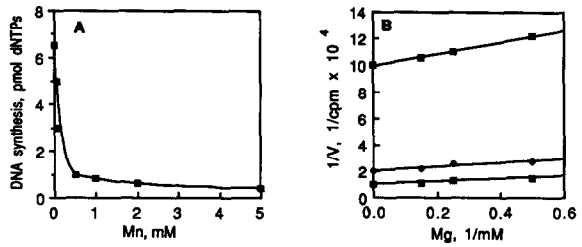

Fig.4. (A) Effect of increasing Mn^{2+} on DNA polymerase α in presence of optimal Mg^{2+} concentration (10 mM). DNA polymerase α (0.12 units) was tested by using activated DNA as template as described in section 2. (B) Lineweaver-Burk plot of the effect of Mn^{2+} on the activity of DNA polymerase α. The enzyme (0.03 units) was assayed as described in section 2 at the Mg^{2+} concentration indicated in abscissa. No Mn^{2+} (●); 0.05 mM Mn^{2+} (♦); 0.1 mM Mn^{2+} (■); 0.2 mM Mn^{2+} (△).

optimum at 2 mM (fig.5). However the 3′→5′ exonuclease activity is inhibited by Mn^{2+} to a lower extent than the polymerizing activity (compare fig.2 and fig.5). The exonuclease activity is approximately reduced by 50% only. Optimal concentration for Mn^{2+} was 0.5 mM.

3.3. *In presence of magnesium, berillium preferentially inhibits the 3′→5′ exonuclease activity of DNA polymerase δ*

Be^{2+} cannot substitute for Mg^{2+} as metal cofactor, since DNA polymerase δ and its 3′→5′ exonuclease activity are completely inactive in the presence of this bigger ion (data not shown). However, Be^{2-}, in presence of optimal Mg^{2+} concentration (10 mM), preferentially inhibited the 3′→5′ exonuclease activity, while DNA polymerase δ activity was not affected (fig.6).

3.4. *Sodium and potassium stimulate PCNA-independent DNA polymerase δ activity*

The effect of NaCl and KCl (Na^+ and K^+) was tested on the DNA polymerase δ activity both on poly(dA)/oligo(dT)$_{12-18}$ and activated DNA. Fig.7 shows that both ions stimulated DNA polymerase δ similarly, but their optimal concentration depends on the template added: using poly(dA)/oligo(dT)$_{12-18}$ the optimal salt concentration was 50 mM (fig.7A), whereas this was 100 mM with activated DNA (fig.7B). This is in contrast to the known inhibitory effect of monovalent ions on DNA polymerase α using activated DNA [1].

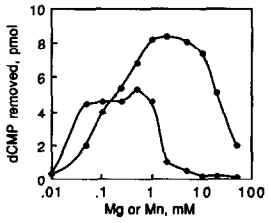

Fig.5. Effect of divalent cations on 3′ →5′ exonuclease activity of DNA polymerase δ. 0.12 units of DNA polymerase δ were tested as described in section 2. Mg^{2+} (●——●); Mg^{2+} (♦——♦).

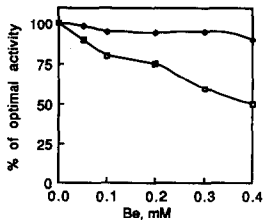

Fig.6. Effect of beryllium on DNA polymerase δ (♦——♦) or on its 3′ → 5′ exonuclease (●——●). The enzyme (0.12 units) was assayed under optimal Mg^{2+} concentration (10 mM) as described in section 2.

4. DISCUSSION

All prokaryotic and eukaryotic DNA polymerases require a divalent cation for catalysis. The major function of the metal activator appears to involve the coordination between the incoming nucleoside triphosphate substrates and the catalytic site of the DNA polymerase molecule. Additional roles of metal ions involve the interaction between the DNA polymerase molecule and nucleic acids such as the binding of the DNA template and the primer.

In this paper we presented evidence indicating that Mg^{2+} appears to be the natural divalent metal activator for PCNA-independent DNA polymerase δ. Substitution of Mg^{2+} by Mn^{2+} had a dramatic effect on DNA polymerization by DNA polymerase δ. Interestingly the intrinsic 3′→5′ exonuclease activity of DNA polymerase δ is less inhibited by Mn^{2+}. These observations might suggest that an adaptive answer to a stressing situation occurs in presence of the mutagenic ion Mn^{2+}. The cell would then slow down DNA replication while the proofreading by 3′→5′ exonuclease could still assure an acceptable degree of fidelity of DNA synthesis.

A metal that reduces the fidelity of DNA synthesis but does not serve as an activator of DNA polymerase is beryllium. Our data suggest that in the presence of optimal Mg^{2+} concentrations addition of Be^{2+} preferentially inhibits the proofreading 3′→5′ exonuclease activity of DNA polymerase δ. Thus the Be^{2+}-induced infidelity of DNA polymerases might probably be due not only to a diminished nucleotide discrimination capacity by the enzyme [1] but, at least

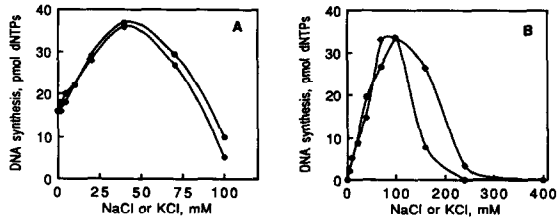

Fig.7. Effect of monovalent cations on DNA polymerase δ. 0.03 units of DNA polymerase were tested as described in section 2 with different concentrations of Na^+ (●——●) or K^+ (♦——♦). (A) poly(dA)/oligo(dT)$_{12-18}$; (B) activated DNA.

in the case of DNA polymerase δ, to the direct inhibition of its proofreading 3′ →5′ exonuclease activity.

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