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Divalent ions attenuate DNA synthesis by human DNA polymerase α by changing the structure of the template/primer or by perturbing the polymerase reaction

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ABSTRACT

DNA polymerases (pols) are sophisticated protein machines operating in the replication, repair and recombination of genetic material in the complex environment of the cell. DNA pol reactions require at least two divalent metal ions for the phosphodiester bond formation. We explore two understudied roles of metals in pol transactions with emphasis on polα, a crucial enzyme in the initiation of DNA synthesis. We present evidence that the combination of many factors, including the structure of the template/primer, the identity of the metal, the metal turnover in the pol active site, and the influence of the concentration of nucleoside triphosphates, affect DNA pol synthesis. On the poly-dT_{70} template, the increase of Mg^{2+} concentration within the range typically used for pol reactions led to the severe loss of the ability of pol to extend DNA primers and led to a decline in DNA product sizes when extending RNA primers, simulating the effect of "counting" of the number of nucleotides in nascent primers by polα. We suggest that a high Mg^{2+} concentration promotes the dynamic formation of unconventional DNA structure(s), thus limiting the apparent processivity of the enzyme. Next, we found that Zn^{2+} supported robust polα reactions when the concentration of nucleotides was above the concentration of ions; however, there was only one nucleotide incorporation by the Klenow fragment of DNA pol I. Zn^{2+} drastically inhibited polα, but had no effect on Klenow, when Mg^{2+} was also present. It is possible that Zn^{2+} perturbs metal-mediated transactions in pol active site, for example affecting the step of pyrophosphate removal at the end of each pol cycle necessary for continuation of polymerization.

1. Introduction

Eukaryotic polα-primase (polα-prim) complex synthesizes *de novo* millions of chimeric RNA-DNA primers at replication initiation sites, which are subsequently extended by polε and polδ [1–4]. The fragments are short and typically excised during Okazaki fragment maturation; but they are so numerous that polα-prim significantly contributes to genome stability [5–9]. Primase synthesizes 8–10 nucleotides of RNA and then the complex switches intra-molecularly to synthesis by polα [10,11]. The X-ray crystal structure of the catalytic domain of yeast polα with RNA/DNA substrates revealed that the RNA/DNA duplex forms an A-form structure that favors the binding of polα [12]. The extension pattern of RNA primer showed a prominent stop as the size of synthesized DNA reaches 20 nt. This led to the hypothesis of the intrinsic mechanism of counting of the number of nucleotides in a primer by polα due to a switch from DNA-RNA to DNA-DNA duplex [12]. Experimental evidence was consistent with the idea, as yeast polα extended RNA primers much more efficiently in comparison to DNA primers. Unfortunately, the reactions were done on the poly-dT template and all effects were caused by the unique properties of this template. In a recent study we confirmed that the pattern of

Abbreviations: pol, DNA polymerase; polα-prim, four subunit human polα-primase; polδ DNA, polymerase δ; polε, DNA polymerase ε; Me^{2+}, divalent metal cation; polα-core, catalytic domain of human polα.

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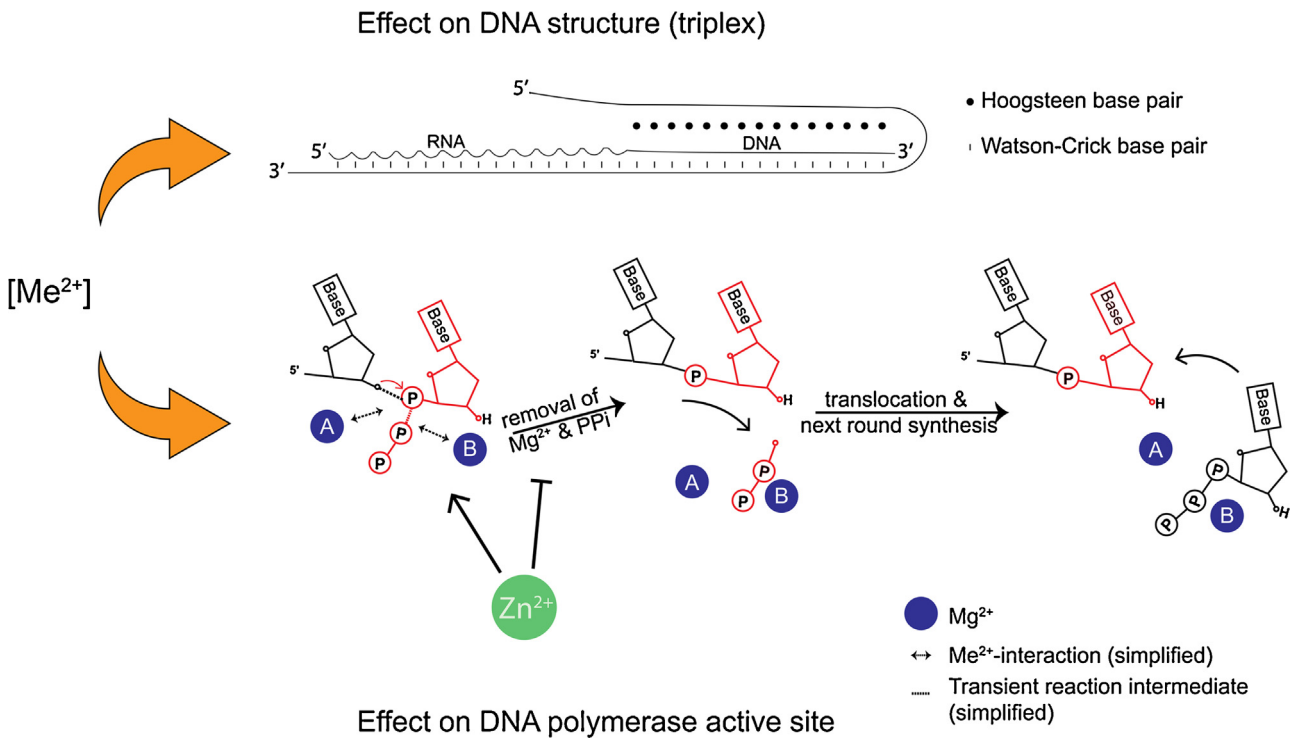

Fig. 1. Two mechanisms of Me^{2+} induced attenuation of DNA polymerase synthesis. The *upper arrow* points to a mechanism of Mg^{2+} induced inhibition by triplex formation the on poly-dT template. Our results indicate that this structure is most readily formed with the DNA•DNA duplex and ssDNA tail, but not the RNA•DNA duplex. Therefore, the rA_{15} primer can be extended by polα at high [Mg^{2+}], but polα is stalled when a sufficiently long DNA duplex appears and the template DNA is folded back on it. The *lower arrow* points to a mechanism of Zn^{2+}-dependent catalysis and inhibition. In comparison to the Mg^{2+} (blue) –dependent catalysis, the replacement of Mg^{2+} in both A and B sites by Zn^{2+} (green) inhibits the pyrophosphate removal from the active site in Klenow fragment but not in polα. The replacement of Mg^{2+} by Zn^{2+} in site A inhibits polα activity (shown as a structure-based model in Fig. 5).

products synthesized on poly-dT by human polα is similar to yeast polα, however, the pol synthesizes much longer products and has the same activity with either DNA or RNA primers on a template with a more natural random sequence [13]. Our current study was ignited by the observation that the length of DNA fragments synthesized by human polα on poly-dT was inversely correlated with concentration of Mg^{2+}. We undertook a systematic investigation of the effects of divalent metal ions on polα activity in comparison to other well-studied DNA pols. Results indicate that the pattern of synthesis on the poly-dT_{70} template is determined by the effect of Mg^{2+} or Mn^{2+} on the formation of an unusual structure of DNA substrates/primers (Fig. 1, upper part). Another divalent ion, Zn^{2+}, affected the pol reaction itself with peculiarities specific to each pol studied, polα and the Klenow fragment (Fig. 1, lower part).

2. Material and methods

2.1. Materials

We have used the following primers and templates for the DNA polymerase reactions: 5′-TYE_{665} (Cy5-equivalent) labeled primers poly-dA_{15}, poly-rA_{15}, and hetero-DNA (5′-CTTGAAAACATAGCGA); templates poly-dT_{70}, 73a (5′-$(T)_{35}$-AGCGTCTTAATCTAAGCACTCGCTATGTTTTCAAGTTT), and 73b (5 –GTCTGGAATGATGAAGATTACTAGTGAAGATTCTGAGCGTCTT-AATCTAAGCACTCGCTATGTTTTCAAGTTT). All oligonucleotides were from IDT Inc., Coralville, Iowa. The purification of human proteins, including the polα catalytic core (further referred to as polα-core), p70·p180ΔN (polα), and p49·p58·p70·p180ΔN (polα-prim), are described in [10,11,14]. The three-subunit wild-type yeast polδ and its exonuclease-defective variant, Pol3-5DV, were generous gifts from Dr. Polina Shcherbakova's laboratory (University of Nebraska Medical Center) and Dr. Peter Burgers' laboratory (Washington University Medical School), respectively. A large (Klenow) fragment of *E. coli* DNA Polymerase I (#M0210S; 5000 units/ml) was purchased from New England Biolabs Inc., Ipswich, Massachusetts. The 7-Deaza-dATP was purchased from TriLink BioTechnologies, Inc., San Diego, California. Mg and Zn chlorides were from Sigma (U.S.A.).

2.2. Methods

All experiments were repeated at least two times. Typical gels are shown to illustrate the reproducible results

2.2.1. Primer extension assay

Polymerase reactions were done as described in [13], with some modifications. Briefly, all reactions were assembled by mixing of solution A (7.5 μl) with solution B (7.5 μl). Solution A is 30 mM HEPES-KOH pH 7.9, 1 mM DTT, 10 mM KCl, 0.15 μM fluorescent primers pre-annealed with 0.2 μM templates, and 10–15 nM polα (or other polymerases) to achieve a 1:15-1:10 enzyme to template ratio; and solution B is 30 mM HEPES-KOH pH 7.9, 1 mM DTT, 10 mM KCl, 100 μM dNTPs (or dATP only for reactions with poly-dT_{70} templates unless indicated otherwise) and Me^{2+} ions ($MgCl_2$, $MnCl_2$, and $ZnCl_2$ were used to provide Mg^{2+}, Mn^{2+}, and Zn^{2+}, respectively) to achieve the concentrations indicated in the figures. All reactions were carried out at 35 °C for the time intervals indicated in the figures. Reactions were stopped by mixing with an equal volume of formamide loading buffer (95% formamide, 0.025% Orange G, 5 mM EDTA, and 0.025% SDS), heated at 65 °C for 10 min, and resolved by 17% urea-PAGE (UreaGel System (19:1 acrylamide/bisacrylamide); National Diagnostics) for 5 h at 2000 V. The products were visualized using the Typhoon 9410 imager