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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 pol α , 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-dT₇₀ template, the increase of Mg²⁺ 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 pol α . We suggest that a high Mg²⁺ concentration promotes the dynamic formation of unconventional DNA structure(s), thus limiting the apparent processivity of the enzyme. Next, we found that Zn²⁺ supported robust pol α 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. Zn²⁺ drastically inhibited pol α , but had no effect on Klenow, when Mg²⁺ was also present. It is possible that Zn²⁺ 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.

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Keywords

DNA polymerases; primase; DNA polymerase alpha; polymerase active site; magnesium; manganese; zinc; homopolymeric runs; non-B DNA

1. Introduction

Eukaryotic pol α -primase (pol α -prim) complex synthesizes *de novo* millions of chimeric RNA-DNA primers at replication initiation sites, which are subsequently extended by pol ϵ and pol δ [1–4]. The fragments are short and typically excised during Okazaki fragment maturation; but they are so numerous that pol α -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 pol α [10, 11]. The X-ray crystal structure of the catalytic domain of yeast pol α with RNA/DNA substrates revealed that the RNA/DNA duplex forms an A-form structure that favors the binding of pol α [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 pol α due to a switch from DNA-RNA to DNA-DNA duplex [12]. Experimental evidence was consistent with the idea, as yeast pol α 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 products synthesized on poly-dT by human pol α is similar to yeast pol α , 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 pol α on poly-dT was inversely correlated with concentration of Mg²⁺. We undertook a systematic investigation of the effects of divalent metal ions on pol α activity in comparison to other well-studied DNA pols. Results indicate that the pattern of synthesis on the poly-dT₇₀ template is determined by the effect of Mg²⁺ or Mn²⁺ on the formation of an unusual structure of DNA substrates/primers (Fig. 1, upper part). Another divalent ion, Zn²⁺, affected the pol reaction itself with peculiarities specific to each pol studied, pol α 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'-TYE₆₆₅ (Cy5-equivalent) labeled primers poly-dA₁₅, poly-rA₁₅, and hetero-DNA (5'-CTTGAAAACATAGCGA); templates poly-dT₇₀, 73a (5'-(T)₃₅-AGCGTCTTAATCTAAGCACTCGCTATGTTTCAAGTTT), and 73b (5'-GTCTGGAATGATGAAGATTACTAGTGAAGATTCTGAGCGTCTTAATCTAAGCACTCGCTATGTTTCAAGTTT). All oligonucleotides were from IDT Inc., Coralville, Iowa. The purification of human proteins, including the pol α catalytic core (further referred to as pol α -core), p70-p180 N (pol α), and p49-p58-p70-p180 N (pol α -prim), are described in [10, 11, 14]. The three-subunit wild-type yeast pol δ 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; 5,000 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 μ l) with solution B (7.5 μ l). Solution A is 30 mM HEPES-KOH pH 7.9, 1 mM DTT, 10 mM KCl, 0.15 μ M fluorescent primers pre-annealed with 0.2 μ M templates, and 10–15 nM pol α (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 μ M dNTPs (or dATP only for reactions with poly-dT₇₀ templates unless indicated otherwise) and Me²⁺ ions (MgCl₂, MnCl₂, and ZnCl₂ were used to provide Mg²⁺, Mn²⁺, and Zn²⁺, 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 minutes, and resolved by 17% urea-PAGE (UreaGel System (19:1 acrylamide/bisacrylamide); National Diagnostics) for 5 hours at 2000 V. The products were visualized using the Typhoon 9410 imager (emission of fluorescence at 670 nm). ImageQuant software (version 5.2, Molecular Dynamics) was used for the quantification of reaction products in gel images. The efficiency of each reaction (number under each lane in Figures) was calculated as the percentage of extended primers:

$$\frac{\text{sum of the intensity of all bands} - \text{the intensity of the unextended primer}}{\text{sum of the intensity of all bands}} \cdot 100\%$$

3. Results

3.1. Mg²⁺ and Mn²⁺ -induced inhibition of pol α progression creates the effect of “counting” of the number of nucleotides in the product on the poly-dT₇₀

The main barrier for DNA pols on the poly-dT template is the formation of triplex DNA [15]. To study why pol α is virtually inactive on the poly-dT₇₀ template with DNA primers but active with RNA primers [12–13], we used several different assay conditions, varying enzyme/template ratio, temperature, reaction time and dATP concentrations. The DNA pol activity of all pol α variants tested, single polypeptide catalytic core (pol α -core), two-subunit pol α , and the full four-subunit pol α -prim [13], was invariably low at all of those different conditions (data not shown). There was one striking exception. When Mg²⁺ concentration was reduced from typically used 10 mM to 0.2 mM, the activity of the pol α -core with DNA primers was recovered and became identical to its activity with RNA primers (Fig. 2, lanes 2 and 3, 8 and 9). When the concentration of Mg²⁺ was increased to 0.5 mM, the activity of

the pol α -core was sharply reduced in the case of DNA primers (Fig. 2, lane 4), in comparison to RNA primers (Fig. 2, lane 10). However, the product size with RNA primers was shortened as [Mg²⁺] increased above 0.5 mM, to less than 20 nt long at 16 mM (Fig. 2, lanes 10–15). A similar pattern of synthesis by pol α -core was observed with Mn²⁺ (Fig. S1). The high Mg²⁺ and Mn²⁺ concentrations did not inhibit the overall activity of the pol α -core with RNA primers (right panel of Figs. 2 with quantification below and Fig.S1). The results of these experiments suggested a possibility that the formation of inhibitory triplex at high Mg²⁺ occurs right after annealing of the DNA template with the DNA primer and thus there is no synthesis by pol α . Apparently, the RNA primer annealed to the DNA template does not promote triplex formation, and the extension of RNA primer by a certain length of DNA is necessary before the triplex can form (Fig. 1, upper part).

The inability of pol α to perform efficient DNA primer extension is specific to the poly-dT template. Pol α extends DNA and RNA primers annealed to heterogeneous sequence equally well [13, 16]. To examine the effects of [Mg²⁺] on pol stalling on the poly-dT run, we used the hybrid template with 5' 35-nt poly-dT and heterogeneous sequence at 3' as the annealing site of the DNA primer. Pol α -core stalling was only observed within the 35-nt poly-dT zone (Fig. S2). Increasing concentrations of Mg²⁺ moderately increased the usage of the primer and led to a progressive shortening of the length of products synthesized on the poly-dT template, simulating the decrease of pol processivity. Apparently, the formation of a barrier for elongation, triplex DNA, occurred within shorter stretches of T/A duplexes with an increase of concentration of Mg²⁺.

3.2 Mg²⁺-induced restrain of pol activity is relieved when synthesis occurs in the presence of 7-Deaza-dATP

DNA pols can use 7-Deaza-dATP (7DdATP) instead of dATP [17]. Due to the lack of a N7 atom (substituted by a CH group), 7DdATP prevents Hoogsteen base pairing but forms normal Watson-Crick base pairs. This property can be used in DNA pol reactions to relieve the negative effects of triplex DNA formation on pol synthesis [15]. Human pol α can utilize 7DdATP, albeit 2.5-fold less efficiently compared to dATP [18]. The presence of 7DdATP did not affect the primer usage mediated by Mg²⁺ ions in comparison to dATP, indicating that the Hoogsteen base pair-dependent triplexes were formed before the reaction (Fig. 3A, and quantification in Fig. 3C). The reactions with 7DdATP led to the recovery of longer products of extension on dA₁₅ primers and the longer products can be easily observed at 2 mM Mg²⁺. This suggested that the incorporation of 7DdATP readily disrupted the triplexes. Similar to the extension of dA₁₅ primers, 7DdATP did not significantly affect the overall rA₁₅ primer usage, but strongly relieved the premature synthesis termination at high concentrations of Mg²⁺ (Fig. 3B, it is noticeable that the products with 7DdATP migrate faster than the dATP products). The products at comparable concentrations of Mg²⁺ were always longer with the deaza nucleotide, indicating that the formation of Hoogsteen base pairs in triplexes was impeded in a substantial proportion of DNA substrates. However, at 16 mM Mg²⁺ in reactions with 7DdATP, 50% of the extension products were still terminated around 10 nt. This suggested that high [Mg²⁺] might stabilize the Hoogsteen base pairing even in the presence of 7DdATP without the N7 atom. Alternatively, high concentrations of

Mg²⁺ may affect the template DNA *per se* in a way attenuating pol synthesis, as discussed in 4.1, thus contributing to the overall inhibition of pol movement.

3.3. Mg²⁺-induced poly-dT template-specific stalling is characteristic for different DNA pols

Pol α -prim possesses RNA pol and DNA pol activities [13]. The inhibitory effects of Mg²⁺ on synthesis on the poly-dT₇₀ template with both DNA and RNA primers by the whole complex of pol α -prim were similar to the single polypeptide pol α -core possessing DNA pol active site only (Fig. S3A). Experiments with the Klenow fragment (Fig. S3B) and yeast pol δ (WT and exonuclease-deficient variant, Pol3-5DV, 3-subunit complexes; Figs. S3 C and D, respectively) showed that the Mg²⁺ induced polymerase stalling on the poly-dT₇₀ template was a general phenomenon. Yeast pol δ was most resistant to the inhibitory effect of high concentrations of Mg²⁺ than the Klenow fragment and human pol α , but still the effect was very clear. Thus, the high Mg²⁺ induced triplex DNA and disturbed pol reactions for various DNA pols.

Next, to get more information on the effects of Mg²⁺, we directly compared the pol α -prim and Klenow fragment on the template with heterogeneous random sequences (73b, Materials and Methods). Pol α extended the primer to the full length with an increasing efficiency when the concentration of Mg²⁺ elevated from 0.2 to 4 mM (Fig. 4, lanes 2–5), then mildly slowed down at 8 mM (Fig. 4, lane 6), and stalled more severely at 16 mM (Fig. 4, lane 7). The Klenow fragment, on the other hand, reached its activity plateau at 0.2 mM Mg²⁺ (Fig. 4, right panel), and produced almost the same pattern of elongated product over a range of concentrations of Mg²⁺. Its activity slightly declined only when [Mg²⁺] was 16 mM (Fig. 4, lanes 9–14). It is noticeable that the early sites of termination of synthesis by the Klenow fragment were more pronounced at a low [Mg²⁺] concentration, a mirror-like effect in comparison to pol α -prim (Fig. 4, lanes 9 and 10). Overall, the experiments in this section confirmed that high concentrations of Mg²⁺ were harmful for pol reaction only when abnormal DNA structures could form. This discovery prompted our further study of Mg²⁺ effects on other templates that can potentially form unusual structures.

3.4. Zn²⁺-catalyzed polymerase reactions

To explore whether the mechanism of Mg²⁺ effects seen in our experiments is a general feature of divalent metal ions, we studied the effects of Zn²⁺. Zn²⁺ is not a traditional catalytic metal ion in polymerase reactions, but it could be tightly associated with polymerases. Two zincs are seen in the crystal structure of the C-terminal domain of the catalytic subunit of yeast pol α [19]. In the structure of the ternary complex of human pol α with RNA-primed DNA template and incoming dCTP (Fig. 5), the metal site B in the catalytic center is occupied by Mg²⁺ whereas the site A is occupied by Zn²⁺. The Zn²⁺ at the A site shows octahedral coordination when the 3'-OH group of the primer is modeled. The modeling was performed because in the crystal structure the primer contained a dideoxy cytosine at the 3'-end to prevent catalysis. The binding of Zn²⁺ in site A indicated that this site is not specific to Mg²⁺, and Zn²⁺ has a potential to influence (support or inhibit) the polymerase reaction. It is not well known how Zn²⁺ behaves in the DNA pol cycle. DNA synthesis by *E.coli* DNA pol I proceeds with Zn²⁺, but at lower rate than with Mg²⁺ [20–

22]. In these earlier studies, only bulk incorporation was measured, which did not provide information on the sizes of reaction products. Zn^{2+} in fact forms a more stable complex with carboxylate residues as well as the triphosphate group of nucleotides than Mn^{2+} and Mg^{2+} [23, 24].

On the heterogeneous 73b template with DNA primer, 10 to 50 μM Zn^{2+} supported DNA synthesis by both the pol α (tetramer) and the Klenow fragment (Fig. 6). The increase of Zn^{2+} concentration led to longer products, but the total amount of extended primers was increased insignificantly (Fig. 6A, lanes 4–6 and 12–14; quantification is below the gel). The activity of the pol α with 50 μM Zn^{2+} was, on average, 2.5-fold less than with 8 mM Mg^{2+} (Fig. 6A, compare lanes 4–6 to lane 3, lanes 12–14 to lane 11 and quantification below the gel image). The Klenow fragment extended more than 80% of primers to the end of the template within a 1 min reaction with 8 mM Mg^{2+} (Fig. 6B, lane 19). Strikingly, when 10–50 μM Zn^{2+} was used alone, an average of 60% of the primers were extended, but the reaction primarily stopped after incorporation of the first nucleotide (Fig. 6B, lanes 20–22).

Zn^{2+} became sharply inhibitory to pol α when Mg^{2+} (8 mM) was also present in the reactions. The activity of the pol α was unaffected at 10 μM Zn^{2+} (Fig. 6A, comparing lanes 7 and 15 to 3 and 11, respectively), inhibited by four-fold at 25 μM Zn^{2+} (Fig. 6A, lanes 8 and 16), then by six-fold at 50 μM Zn^{2+} . The template sequence-independent stimulatory and inhibitory effects of Zn^{2+} at micro-molar concentrations suggested that these effects could be related to binding of metals at the A and B sites in the catalytic center (see Introduction). The addition of Zn^{2+} to reactions containing 8 mM Mg^{2+} did not result in inhibition of the Klenow fragment to the same extent as the pol α (Fig. 6B, lanes 23–25). This suggests that the Klenow fragment strongly preferred Mg^{2+} to Zn^{2+} for catalysis, whereas the processes in the active site of the pol α was easily perturbed with Zn^{2+} .

On the poly-dT₇₀ template with RNA primers, Zn^{2+} also sharply inhibited pol α reaction (about two-fold at 25 μM and four-fold at 50 μM), compare to 1 mM Mg^{2+} alone and with 10 μM Zn^{2+} (Fig. S4A, compare lanes 6 and 7 with lane 4 and 5; quantification is below the gel). Similar effects were also observed with Mn^{2+} (Fig. S4A, lanes 8–11). The Klenow fragment was mildly inhibited by Zn^{2+} (at 10–50 μM) in the presence of 0.2 mM Mg^{2+} (Fig. S4B). Zn^{2+} did not cause the same premature termination on the poly-dT template as did the Mg^{2+} and Mn^{2+} , suggesting that the inhibition is not likely due to the formation of an unusual DNA structure. Note that the concentrations when Zn^{2+} supported and inhibited pol reaction were in the micromolar range. This may explained why we did not see its effects on the structure of the template, which was seen at millimolar concentrations of Mg^{2+} .

To further understand the inhibition of pol α activity in the presence of both Zn^{2+} and Mg^{2+} , we studied the usage of Zn^{2+} as catalytic Me^{2+} by pol α core in more depth. It was likely that this inhibition on pol α was mainly driven by the concentration of Zn^{2+} reaching a certain threshold, because the dramatic effects were caused by tiny changes in $[\text{Zn}^{2+}]$ (Figs 6 and S4). Pol α reactions shown in these figures proceeded with 10–50 μM Zn^{2+} in the presence of a standard concentration of nucleotide triphosphates (100 μM). Nucleotide triphosphates are able to chelate the positively charged Zn^{2+} . It was possible that the ratio of $[\text{dNTP}]/[\text{Zn}^{2+}]$ affects pol activity stimulation by Zn^{2+} . The range of $[\text{Zn}^{2+}]$ supporting the pol α shifted

from 5 to 80 μM when the concentration of dATP was increased from 10 to 160 μM , respectively (Fig. 7; lanes 3, 7, 11, and 15). As predicted, once $[\text{Zn}^{2+}]$ exceeded $[\text{dATP}]$, polymerase activity decreased (Fig. 7; lanes 4–5, 8–9, 12–13, and 16–17). Reactions with 160 μM of all dNTPs were generally similar to 160 μM dATP at the same $[\text{Zn}^{2+}]$ (Fig. 7, lanes 11–13, 15–17). We noticed that the overall product sizes at 80 μM Zn^{2+} were slightly smaller with dNTPs (Fig. 7, compare lane 15 to lane 11). This was probably due to the slowed polymerase activity in the presence of the non-cognate nucleotides and Zn^{2+} . It was noticeable that traces of Me^{2+} were present in the purified human pol α samples that supported the polymerase reactions (Fig. 7, lanes 2, 6, 10, and 14). This residual synthesis was more prominent at lower $[\text{dATP}]$ (10 and 40 μM ; Fig. 7, lanes 2 and 6). Since these reactions reflected unusual conditions with a limited quantity of trace metal ions, it was possible that higher $[\text{dATP}]$ chelated these trace metal ions that decreased the probability of Me^{2+} -binding at the active site. At 1 μM Zn^{2+} , pol α is slightly stimulated comparing to reactions supported by the trace metal ions but much less than at 5 μM Zn^{2+} (Figure S5). The chelation effect by dATP in the presence of 1 and 5 μM Zn^{2+} is more pronounced in comparison to 25 μM Zn^{2+} (Figure S5, compare lanes 5–7, 8–10 to 11–13).

The concentration-dependent effect of nucleotides on the pol synthesis in the presence of Zn^{2+} was further explored by titration of $[\text{Zn}^{2+}]$ with 160 μM dATP at three time points. At 40 μM Zn^{2+} , pol α extended more and more primer as reaction time increased from 0.5 to 8 minutes (Fig. S6; lanes 4–6) similar to reactions with Mg^{2+} (lanes 16–18). At 80 μM Zn^{2+} , pol α extended the primer faster at 0.5 minutes (Fig. S6; lane 7) than at 40 μM Zn^{2+} , and reached the maximum primer usage at 2 minutes (lane 8). There was almost no increase in primer usage when time was 8 minutes (compare lane 9 to lane 8). At 160 and 320 μM Zn^{2+} , the activity of pol α decreased 2- and 4-fold, respectively, in comparison to at 80 μM Zn^{2+} (lanes 10–12 and 13–15). Moreover, the extent of primer usage was unchanged throughout all three reaction time points. The reaction with 160 μM Mg^{2+} shows a slower synthesis at 0.5 minutes in comparison to all reactions with Zn^{2+} at this time point (lane 16 in comparison o lanes 4, 7, 10, and 13). Thus, the concentration of nucleotides differently affected pol synthesis in the presence of the two metals.

4. Discussion

4. 1. Structure of DNA polymerase substrates, metal ions and pol activity

The formation of unusual structures of DNA has a tremendous impact on DNA and RNA transactions in living organisms [25–26]. Interactions of divalent metal ions with nucleic acids play a significant role in changing nucleic acid geometry [27]. The severe effects of repeats in template DNA on pol transactions are ubiquitous [28]. For example, the Klenow fragment and pol α from calf thymus paused on repeats of $\text{d}(\text{CT})_{27}$ [29–30]; *Xenopus* DNA pol γ and human pol α stalled on templates containing poly-dT tracts [13–15]. The inhibition was likely due to the formation of triplexes by a folded back repeated template DNA strand ahead of synthesized repeated duplex (illustrated in upper part of Fig. 1), because it was alleviated when the 7-Deaza derivative of dATP, unable to form stable Hoogsteen pairs, was used for synthesis [15–31] and current Fig. 3. High concentrations of Mg^{2+} increase the stability of triplexes and concomitantly impede DNA synthesis by thermostable pols [32].

Pol α is one of the few DNA pols that can efficiently extend from both DNA and RNA primers [13–16]. Our novel finding is that, with our experimental setup, the formation of triplexes impeding the advancement of DNA polymerases is much more efficient with DNA primers, than with RNA primers. This is supported by the observation that the pattern of usage of the dA₁₅ primers was similar with dATP or 7DdATP, therefore, DNA primer was unavailable for pol α at a higher [Mg²⁺] prior to the start of DNA synthesis (Fig. 3A). It is interesting that the threshold when severe inhibition occurred was shifted to 4 mM Mg²⁺ for reactions with 7DdATP, suggesting that the triplexes are dynamic and start to melt when destabilizing nucleotides are incorporated. When pol α extends the RNA (rA₁₅) primer, the formation of triplex with the growing DNA part causes pausing and dissociation of the polymerase, which is also dependent on the concentration of Mg²⁺.

We observed some peculiarities in how different DNA pols handle poly-dT templates (Fig. S3). Yeast pol δ was more robust than DNA pol α and *E.coli* pol I, even with functional proofreading exonuclease (panels C and D). The roles of the DNA pol α and *E.coli* pol I in replication are limited to the synthesis of relatively short starts of Okazaki fragments (pol α) and short patches of DNA after removal of RNA primer during Okazaki fragment maturation (pol I) [33]. Pol δ , on the other hand, is one of the major replicative pols in eukaryotes that synthesize the majority of the genome [2–4, 34–35]. It looks rational that it evolved to tolerate the inhibitory effects of certain templates/conditions better than pol α and the Klenow fragment.

It is interesting that the threshold for pol stalling with both DNA and RNA primer is the same, 0.5 mM Mg²⁺ (Fig. 2). Both primers were extended equally well at concentrations below this threshold. Magnesium in mammalian tissue is present at a typical concentration of 30 mM, but most of it (more than 95%) is bound to proteins, NTPs, or phospholipids [36]. The concentration of free Mg²⁺ in nuclei, however, varies between cell cycle phases from 3 to 11 mM [37], and it was assumed that the physiological concentration of Mg²⁺ available for DNA binding is 10 mM [38]. The Mg²⁺ is capable of coordinating six water molecules forming its first octahedral solvation shell. The hydrated Mg²⁺ interact with DNA molecules via water-mediated hydrogen bonds. Primary DNA interaction sites include the phosphodiester backbone, the guanine base in the major groove and the A-T pair in the minor groove [39]. Moreover, two additional solvation shells can be built upon the first that helps interaction with different DNA molecules [40]. Therefore, the potential of Mg²⁺ mediated intra- and inter- strand interactions other than Watson-Crick base pairing is very high, and it is sequence-dependent. It is known that Mg²⁺ and other Me²⁺ stabilize H-DNA structures (reviewed in [41]). Mg²⁺ induces bending of nucleosomal linker DNA (up to 80 bp repetitive sequence [42]), which contributes to folding of nucleosomal arrays [43]. The RNA-Mg²⁺ interactions, on the other hand, are direct in the majority and are not mediated by water molecules [44]. This may explain the reason for the better extension of the poly-rA₁₅ primer on templates with polynucleotide runs. In conclusion, the poly-dT template is a very specific substrate for polymerases and should be used with caution to obtain general conclusions on the mechanisms of pol reactions.

Both pol α and the Klenow fragment utilized the hetero-DNA primer well throughout the wide concentration range of Mg²⁺. On the hybrid poly-dT₃₅-hetero template (73a), Mg²⁺

created a barrier for pol α between 10 to 20 nt within the poly-dT zone, without affecting the stimulation of the utilization of the primers (Fig. S2). On the template with a totally scrambled sequence (73b), pol α (pol α -core) showed a slow-down at a high 16 mM of Mg $^{2+}$ around 20 nt synthesis, whereas the Klenow fragment did not (Fig. 4). Secondary DNA structural prediction analysis revealed several potential hairpin sites in this template. Mg $^{2+}$ is known to increase the stability of the secondary structures of DNA and RNA [45]. It looks like pol α is more sensitive to irregularities in the template than the Klenow fragment. The latter is known to be able to synthesize over some hairpins [46].

4. 2. Dynamics of metals in the polymerase active site and the effect of Zn $^{2+}$

DNA and RNA polymerases (pols) use at least two divalent metal ions (Me $^{2+}$) (lower part of Fig. 1), at the specific positions of the active site, called A and B, during each cycle of polymerization [47]. The relative orientations of two catalytic Mg $^{2+}$, the 3'OH group of primer terminus, and the phosphates of incoming (deoxy)ribonucleoside triphosphate ((d)NTP) are invariant, despite the differences in pol domain structures and in the ways these Mg $^{2+}$ are coordinated [48, 49]. Both metals are required throughout the polymerase reaction: active positioning of the 3'-OH with α -phosphate by rearranging the side chains of carboxylate residues (Asp or Glu); stabilization of the pentacovalent intermediate during the transition state; and substrate translocation [50, 51]. Recent structural studies of DNA polymerases suggest there might be a third, transient, Me $^{2+}$ in the polymerase active site that participates in the reaction, which probably stabilizes the intermediate and prevents translocation of substrate DNA before the completion of the reaction cycle [50, 52].

Manganese (Mn $^{2+}$) is able to effectively substitute for Mg $^{2+}$ by DNA pols *in vitro*, because of the close chemical properties of these two ions [53–56]. Studies *in vitro* have shown that substitution with Mn $^{2+}$ is not benign and increases error rates, drastically increases pol activity or changes the specificity of many pols [57–60], including pol α [61]. Another divalent ion that has often been found in DNA pols is Zn $^{2+}$. Purified *E.coli* DNA pol I contains Zn atoms at 0.1 to 2 moles per mole of enzyme, depending on the strain and purification method [21, 62–64]. The effects of Zn $^{2+}$ on DNA pols are controversial. Both stimulation and inhibition of polymerase and exonuclease activities have been observed [20, 22, 65, 66]. A NMR study of pol β revealed that Zn $^{2+}$ not only occupies the active site, but also adopts an active conformation analogous to Mg $^{2+}$ [67].

Zn $^{2+}$ differs from Mg $^{2+}$ in certain interactions with DNA components and proteins. It has a higher affinity toward tri-phosphate and the Asp/Glu residues [23, 24] and directly binds to the N7 position on the adenine ring [68]. The stop after the first incorporated nucleotide by the Klenow fragment in the presence of Zn $^{2+}$ suggests that the reaction mediated by Zn $^{2+}$ freezes at the translocation stage, which prevents binding of the next dNTP (Fig. 6B). The translocation is critical for the growth of the DNA chain as the step necessary for the release of pyrophosphate and bound Mg $^{2+}$ [51]. A recent single molecule-based kinetic study with bacteriophage Phi29 DNA polymerase showed that the translocation happens after the release of the pyrophosphate and before the binding of the incoming dNTP, along the DNA template, one nucleotide at a time [69]. DNA polymerase at the dNTP/pyrophosphate-free translocation state can thermally diffuse between the pre- and post-translocation state. The

binding of the correct dNTP stabilizes the posttranslocation stage with the finger domain closure. It is possible that because of the higher affinity of Zn^{2+} toward the nucleotides or catalytic carboxylates, the pyrophosphate bound to Zn^{2+} at the site B could not be easily released by the Klenow fragment, preventing the translocation (Fig. 6B). Alternatively, the open finger cannot switch back to the post-translocation conformation in the presence of Zn^{2+} . The human pol α behaves differently. Zn^{2+} supports full reaction cycles, but only when there is an excess of nucleotide triphosphates to help overcome the negative effects of Zn^{2+} . Our results show that the Zn^{2+} concentrations supportive for the robust polymerization are always less than the concentration of the nucleotides, except for 1 μM and 5 μM Zn^{2+} in the presence of 40 and 160 μM dATP likely because of chelation of all metal ions (Fig. 7, Figs S5 and S6). When $[\text{Zn}^{2+}]$ exceeded the concentration of nucleotides, the activity of pol α was low and the primer usage and product length remained constant over time (Fig. S6; lanes 13–15). This suggests that pol α at these conditions was not able to dissociate from the extended primer and/or re-bind it. The drastic inhibition of pol α by Zn^{2+} in the presence of Mg^{2+} could be explained by the idea that the configuration of the active site with the two different ions in sites A and B in pol α is improper for a polymerase reaction. In the absence of Mg^{2+} , both A and B sites are occupied by Zn^{2+} , which allows a reaction to proceed when dNTPs are in excess.

The effects of Zn^{2+} on the pol activities of the Klenow fragment and pol α might also be explained by the appearance of a third Mg^{2+} binding site after breaking the bond between the α - and β -phosphates of dNTP and before the dissociation of the pyrophosphate [50]. As was seen in Pol η , the third Mg^{2+} transiently stabilizes the leaving group. In case of the Klenow fragment, Zn^{2+} might have a much stronger affinity to this site in comparison to Mg^{2+} , which complicates the release of pyrophosphate. Therefore, the translocation is blocked. It will be interesting to directly compare the structural and kinetic basis of Zn^{2+} - and Mg^{2+} - catalyzed DNA polymerase reactions (i.e. how differ are these two ions in binding with catalytic residues, primer 3' end, and incoming nucleotide; metal ions position and hydrogen bonding at active site post reaction; or possible conformational changes).

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

pol	DNA polymerase
polα-prim	four subunit human pol α -primase
polδ	DNA polymerase δ
polϵ	DNA polymerase ϵ
Me²⁺	divalent metal cation; and pol α -core, catalytic domain of human pol α

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Highlights

- Mg^{2+} at suboptimal concentration inhibited DNA pol α and several other pols on templates with repetitive sequence by stabilizing unconventional DNA structure(s).
- Zn^{2+} supported DNA synthesis reactions by pol α with low efficiency, but inhibited synthesis when Mg^{2+} was present, suggesting the possibility that the step of removal of divalent metal ions after the formation of the phosphodiester bond is critical for polymerase reaction.
- The balance of the divalent metal ions is critical for the efficient DNA polymerase function.

Figure 1. Two mechanisms of Me²⁺ induced attenuation of DNA polymerase synthesis

The *upper arrow* points to a mechanism of Mg²⁺ induced inhibition by triplex formation 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 rA₁₅ primer can be extended by polα at high [Mg²⁺], 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 Zn²⁺-dependent catalysis and inhibition. In comparison to the Mg²⁺ (blue) - dependent catalysis, the replacement of Mg²⁺ in both A and B sites by Zn²⁺ (green) inhibits the pyrophosphate removal from the active site in Klenow fragment but not in polα. The replacement of Mg²⁺ by Zn²⁺ in site A inhibits polα activity (shown as a structure-based model in Fig. 5).

Figure 2. High concentrations of Mg²⁺ attenuate DNA synthesis by the polα-core on the poly-dT₇₀ template

Extension of DNA (poly-dA₁₅) or RNA (poly-rA₁₅) primers was carried out by polα-core (enzyme to primer/template ratio = 1:15) in the absence of Mg²⁺ or in the presence of increased concentrations of Mg²⁺. Reactions were carried out at 35° C for 4 minutes. The efficiency of each reaction (number under each lane) was calculated as the percentage of extended primers (Materials and Methods).

Figure 3. Inclusion of 7-Deaza-dATP into reactions relieves polα pausing on the poly-dT template
(A) Extension of DNA (poly-dA₁₅) primers by human DNA polα core (enzyme to primer/template ratio = 1:5) using dATP (lanes 1–4) or 7DdATP (lanes 5–8) in the presence of 0.2–4 mM Mg²⁺, respectively. Reactions were carried out at 35 °C for 5 minutes. (B) Extension of RNA (poly-rA₁₅) primers by human DNA polα core (enzyme to primer/template ratio = 1:10) using dATP (lanes 1–4; 1 min reaction at 35 °C) or 7DdATP (lanes 5–8; 3 min reaction, due to the lower efficiency of 7DdATP incorporation by polα) in the presence of 0.2–4 mM Mg²⁺. Quantifications of the primer usage are below each panel. (C) Comparison of the efficiency of the pol reactions with dATP vs 7DdATP.

Figure 4. Comparison effects of Mg^{2+} concentration on the activity of pol α -prim and the Klenow fragment on the template with random sequence
Sequence of the 3' portion of the template 73b (excludes the 5' primer-binding region) that can potentially form two hairpins (marked by * and **) is shown. The extension of hetero-DNA primers annealed with the template contains a heterogeneous sequence (73b) by pol α -prim (enzyme to primer/template ratio = 1:15) and the Klenow fragment (enzyme to primer/template ratio = 1: 5) in the absence or presence of 0.2–16.0 mM Mg^{2+} . Reactions were carried out at 35 °C for three minutes (pol α -prim) and one minute (Klenow fragment).

Figure 5. The active site of the human polα in the ternary complex with RNA-primed DNA-template and incoming dCTP

Polα is represented as schematic and colored green. The DNA, RNA, and dCTP as well as polα residues involved in the coordination of divalent metal ions are shown as sticks and colored blue for nitrogen, red for oxygen, and grey, yellow and green for the carbons of DNA/RNA, dCTP and polα, respectively. The Mg^{2+} , Zn^{2+} and water molecules are shown as spheres and colored magenta, cyan and red, respectively. Metal coordination is depicted with blue dashed lines. The 3'-OH group of the RNA primer was modeled with the PyMOL Molecular Graphics System, Version 1.3 (Schrodinger, LLC) using the coordinates of the PDB structure 4QCL.

Figure 6. Comparison of the effects of Zn²⁺ alone and in combination with Mg²⁺ on DNA synthesis by pol α -prim and the Klenow fragment
(A) Extension of hetero-DNA primers annealed with heterogeneous 73b template by pol α -prim (enzyme to primer/template ratio = 1:10) and (B) the Klenow fragment. (enzyme to primer/template ratio = 1:5). Lanes 2–9, pol α -prim for one minute; Lanes 10–17, pol α -prim for eight minutes; Lanes 18–25, the Klenow fragment for one minute; all with 100 μ M dNTP at 35 °C. Reactions contained no enzyme (lane 1), no additional Me²⁺ (lanes 2, 10, 18), 8 mM Mg²⁺ (lanes 3, 11, 19), 10 to 50 μ M Zn²⁺ (lanes 4–6, 12–14, 20–22), and 8 mM Mg²⁺ with 10 to 50 μ M Zn²⁺ (lanes 7–9, 15–17, 23–25).