Human DNA Primase: Anion Inhibition, Manganese Stimulation, and Their Effects on In Vitro Start-Site Selection†

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ABSTRACT: We examined the effects of Mn^{2+} on eukaryotic DNA primase both in the presence and absence of 5 mM Mg^{2+}. In the absence of Mg^{2+}, Mn^{2+}-supported primase activity to a level 4-fold greater than that obtained with Mg^{2+} alone, and adding low levels of Mn^{2+} (100 μM) to assays containing 5 mM Mg^{2+} greatly stimulated primase. Increased activity was primarily due to more efficient utilization of NTPs, as reflected in a lower K_M for NTPs. Under conditions of saturating NTPs, Mn^{2+} had minimal effects on both the rate of initiation (i.e., dinucleotide synthesis) and processivity. The effects of Mn^{2+} involve multiple metal binding sites on primase and may involve both the catalytic p49 subunit as well as the p58 subunit. Physiological levels of salt can inhibit primase activity due to the presence of an anion binding site and low levels of Mn^{2+} significantly decreased this salt sensitivity. The implications of these results with respect to the biological role of primase are discussed.

Eukaryotic DNA primase is a two subunit RNA polymerase essential for DNA replication (*2*−*6*). In conjunction with DNA polymerase α (pol α),[1] primase is responsible for initiating the synthesis of each Okazaki fragment on the lagging strand and, presumably, on the leading strand. In addition, primase likely helps couple DNA replication and repair. Yeast containing mutations in primase are unable to delay entry into S phase and slow DNA synthesis upon treatment with DNA damaging agents (*7*, *8*).

Primase consists of two subunits of mass 49 and 58 kDa. The p49 subunit of primase contains phosphodiester bond formation activity and is a member of the class-X nucleotidyl transferase superfamily (*9*). The p49 subunit alone can catalyze complete primer synthesis, but only if it is purified in the presence of either Mn^{2+} or Mg^{2+} (*10*). Relatively little is known about the role of the p58 subunit during primer synthesis (*11*). It lacks detectable phosphodiester bond formation activity, although it greatly stabilizes the p49 subunit. The p58 subunit greatly enhances primer synthesis by p49 purified in the presence of a divalent cation and is essential for primer synthesis if no divalent cations were present during purification of the p49 (*10*). In the four subunit pol α-primase complex, an additional role of p58 is to tether p49 to the 180 kDa pol α subunit (*12*).

Functional aspects of primase in vitro have been well-studied and have helped define a minimal kinetic model (*12*−*14*). The catalytic cycle of primase consists of three segments: initiation (dinucleotide formation), elongation (converting the dinucleotide into a primer typically 7−10 nucleotides long), and termination (movement of the primer to the pol α active site and reactivation of primase for another round of primer synthesis). Under in vitro assay conditions, the initiation step typically limits the overall rate of primer synthesis. Presently, however, there exist dramatic and unresolved differences between the characteristics of primase activity in vitro and in whole cells. For example, primase is strongly inhibited by physiological levels of salts (*6*), and the rates of primer synthesis measured for purified primase would barely be sufficient to support the in vivo rates of replication fork movement (*14*).

Mn^{2+} is a physiologically relevant cation that alters the activity of a variety of DNA polymerases (e.g., DNA polymerase β and Klenow fragment (*15*, *16*)) and has been shown to be able to replace Mg^{2+} for calf thymus primase (*6*). Mn^{2+} has typically been examined in terms of its mutagenic properties, although it has also been shown to increase significantly the rate of dNTP polymerization for some polymerases. In light of these effects, we examined in detail the effects of Mn^{2+} on human DNA primase. Low concentrations of Mn^{2+} significantly increase the rate of both initiation and elongation in a template dependent manner and decrease the sensitivity of primase to inhibition by anions. In addition, Mn^{2+} alters the sequence specificity for primer synthesis such that the in vitro specificity now more closely matches that observed in vivo.

MATERIALS AND METHODS

Materials. Unless noted, all materials and methods were as described previously (*14*, *17*). Both primase and the p49 subunit were overexpressed in an *Escherichia coli* JM105-(DE3) strain deficient in exonuclease I and purified via Ni-NTA (Qiagen) chromatography (*12*). Human pol α-primase was immunopurified from baculovirus-infected insect cells as described previously (*18*, *19*). Synthetic oligonucleotides of defined sequence were from Integrated DNA Technologies, Inc. and Oligos Etc., Inc. (Table 1). Poly(dT) and poly-

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[1] Abbreviations used: dsDNA, double-stranded DNA; Pol α, DNA polymerase α; Pol β, DNA polymerase β; ssDNA, single-stranded DNA.

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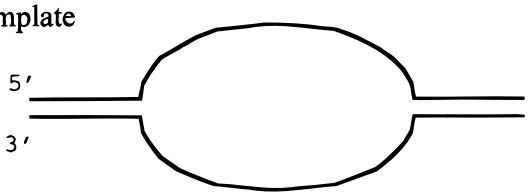
Table 1: DNA Templates Used

d(ACT)$_{20}$	(5′)ACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACTACT
d(ACC)$_{20}$	(5′)ACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACCACC
d(TC)$_{30}$	(5′)TCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTCTC
(dC)$_{40}$	(5′)CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC

"Bubble" Template



(dC) were from Sigma. The concentrations of ssDNAs were determined spectrally and are expressed in terms of total nucleotide. $MnCl_2$ and $MgCl_2$ concentrations were determined by ethylenediamine tetraacetic acid titrimetric analysis (*20*). All other reagents were of the highest purity available.

Methods.—Primase Activity Assays. Reactions (10 μL) typically contained 50 mM tris(hydroxylmethyl)aminomethane, pH 7.9 (HCl salt), 60 μM ssDNA template (total nucleotide), 100 nM primase, 0.05 mg mL^{-1} bovine serum albumin, 1 mM dithiothreitol, 100−200 μM [α-^{32}P]NTPs, and 0−2 mM $MnCl_2$ and/or 5 mM $MgCl_2$. Under these conditions, the NTP concentration is nonsaturating. Reactions were initiated by adding an enzyme and incubating at 37 °C for 1 h. After the assays were quenched by adding 2.5 volumes of gel-loading buffer (90% formamide), the products were separated by denaturing polyacrylamide gel electrophoresis (18% polyacrylamide, 8 M urea) and analyzed via phosphorimagery (Molecular Dynamics). Intensity of product bands were determined individually via area integration and converted to pmol of product (5′-ends). Unless noted otherwise, all metals used in assays were present as the Cl^- salts.

p49 Activity Assays. Reaction mixtures were similar to those in primase activity assays with the exception that the DNA substrate was 150 μM poly(dT)/(rA)$_{12}$ (20:1), and primase was replaced with 500 nM p49. Reactions were initiated by adding enzyme and incubated at 30 °C for 7.5 min. Reactions were quenched and the products were separated and quantified as above.

Misincorporation Assays. Assays to measure misincorporation during primer synthesis were performed as described previously on the template d(ACC)$_{20}$ (*17*).

Start-Site-Selection Assays. Start-site selection of human pol α-primase was determined using a "bubble" template 458 nucleotides long (Table 1) and analyzed as published previously (*21*, *22*).

RESULTS

We initially determined that Mn^{2+} could both support human primase activity in the absence of Mg^{2+} as well as increase its activity in assays already containing 5 mM Mg^{2+}. Consistent with previous studies showing that primase is a Mg^{2+}-dependent enzyme (*6*), we found that omitting Mg^{2+} from primase activities resulted in no detectable activity (Figure 1). Titration of Mn^{2+} into assays lacking Mg^{2+} gave high levels of activity at low concentrations, but then resulted in partial inhibition at higher concentrations (Figure 1). Interestingly, addition of low levels of Mn^{2+} to assays containing 5 mM Mg^{2+} also gave increased activity followed by inhibition. Stimulation was observed both in terms of the total moles of product synthesized (all length products) as well as the amount of product $\geq$7 nucleotides long (i.e., primers that pol α can elongate, see below). To control for the possibility that the changes in total metal concentration affected Mn^{2+} stimulation, the effects of Mn^{2+} were also measured under conditions where the sum of Mn^{2+} plus Mg^{2+} was kept constant at 5 mM. The results were virtually identical to those shown in Figure 1. Mn^{2+} stimulated both full length and total product formation at low concentrations, with maximal stimulation occurring at 100 and 400 μM Mn^{2+}, respectively, followed by inhibition at higher Mn^{2+} concentrations (data not shown). These effects likely reflect specific binding of Mn^{2+}, since two other divalent cations, Ca^{2+} and Zn^{2+}, were unable to either replace Mg^{2+} or stimulate activity in the presence of 5 mM Mg^{2+}.

Similar assays were performed with the four subunit pol α-primase complex to ensure that the above results do not involve the His-tag on the p49/p58 primase complex and that they also occur with the physiologically relevant pol α-primase complex. Similar to the effects on the p49/p58 complex, Mn^{2+} significantly stimulated primase activity of the pol α-primase complex, with maximum stimulation of full length product (4.4-fold) and of total product (3.1-fold) occurring at 200 μM Mn^{2+} in the presence of 5 mM Mg^{2+}. Thus, the effects of Mn^{2+} on the p49/p58 complex are not due to the His-tag and are relevant to the pol α-primase complex.

To demonstrate that stimulation by Mn^{2+} is a general effect and not limited to poly(dT), we examined stimulation of primase activity on several additional templates. Table 2 shows that on each template examined, adding Mn^{2+} to assays containing 5 mM Mg^{2+} significantly stimulated activity. Interestingly, the maximum extent by which Mn^{2+}

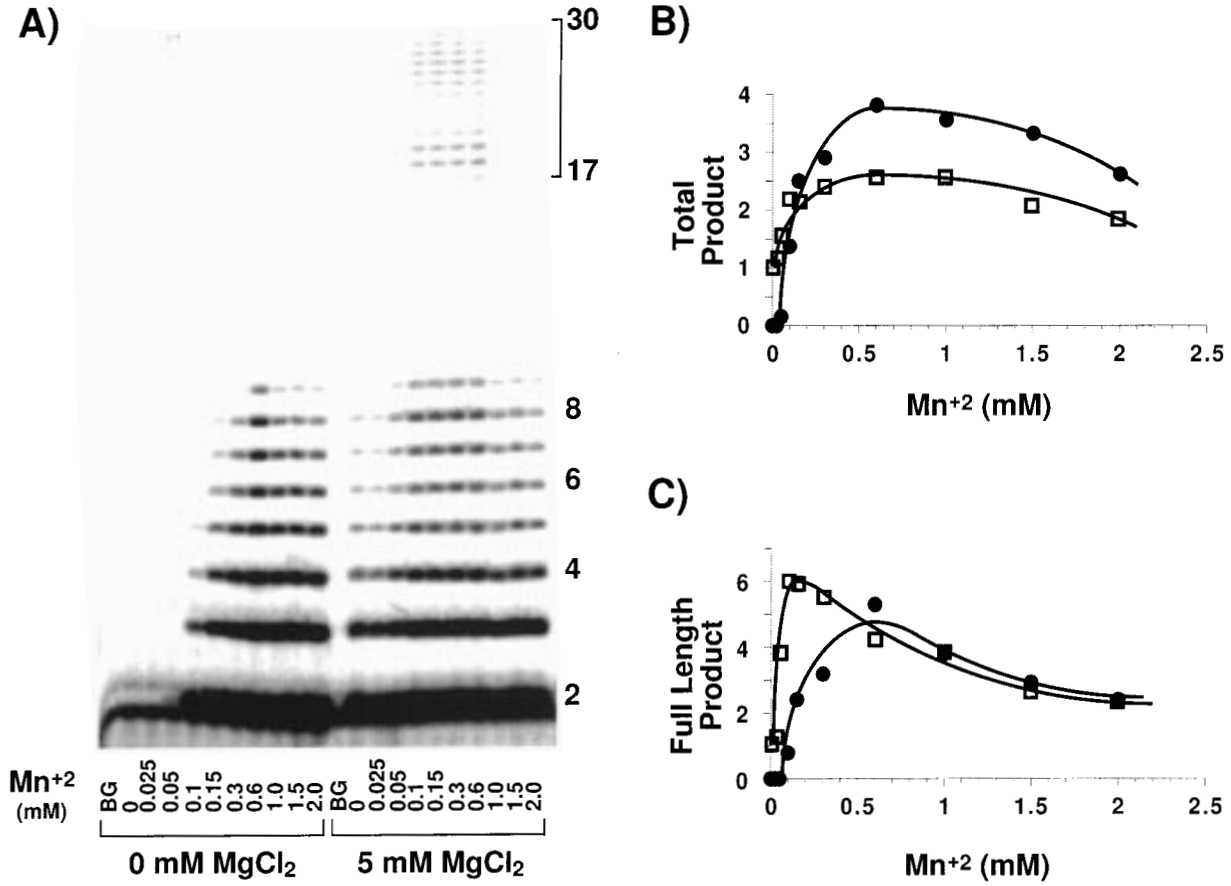


FIGURE 1: Mn^{2+} stimulates primase activity on poly(dT). (A) Phosphorimage of the products from primase assays containing the indicated concentrations of Mn^{2+} and Mg^{2+}. Assays were performed as described under Materials and Methods. The numbers to the right of the panel refer to the length of each product. BG = background. (B) The total moles of primers synthesized in the absence (●) or presence (□) of 5 mM $MgCl_2$ are shown. (C) The total moles of full length primers (i.e., ≥7 nucleotides long) in the absence (●) or presence (□) of 5 mM $MgCl_2$ are shown. In both B and C, the amount of primers was normalized to the amount produced in the presence of 5 mM Mg^{2+} (0 Mn^{2+}). This corresponds to 22.6 and 0.18 pmol in B and C, respectively.

Table 2: The Effects of Mn^{2+} on Initiation and Elongation in the Presence of 5 mM Mg^{2+}

	maximum stimulation by Mn^{2+}		
template	total products[a]	full length products[a]	full length products[b]/total products (0.1 mM Mn^{2+})
poly(dT)[c]	3.0 (0.5 mM)	5.0 (0.1 mM)	2.5
$d(ACT)_{20}$	2.8 (0.5 mM)	1.4 (0.1 mM)	0.8
$d(TC)_{30}$	2.8 (0.5 mM)	1.4 (0.1 mM)	0.9
$d(ACC)_{20}$	6.8 (0.5 mM)	3.7 (0.1 mM)	1.0
poly(dC)[c]	6.0 (1.0 mM)	6.3 (1.0 mM)	1.0

[a] The maximum-fold stimulation by Mn^{2+} and the concentration of Mn^{2+} at which this occurs are given. [b] This ratio is calculated from the effects of 0.1 mM Mn^{2+} on total products and full length products for each of the templates. [c] Poly(dT) and poly(dC) are both >1000 nucleotides long.

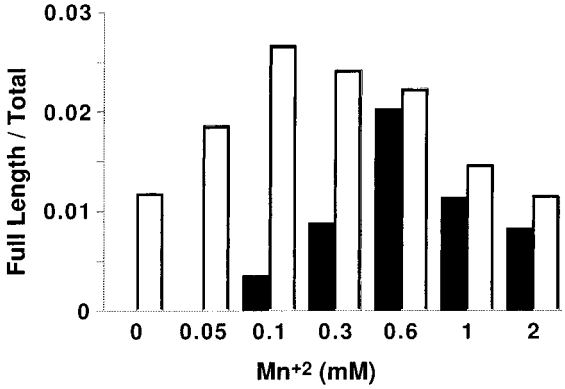


FIGURE 2: Effect of Mn^{2+} on primer elongation on poly(dT). The effect of Mn^{2+} on the fraction of primers that become competent for elongation by pol α (i.e., full length primers) in the absence (black bars) and presence (open bars) of 5 mM $MgCl_2$ is shown.

stimulated initiation varied significantly among the different templates.

Mn^{2+} Alters the Processivity of Primase. The effects of Mn^{2+} on processivity were determined by comparing the amount by which Mn^{2+} stimulates the synthesis of primers ≥7 nucleotides long (i.e., those that pol α can elongate) versus stimulation of initiation (i.e., total primers). This ratio will be >1 if Mn^{2+} stimulates elongation since the frequency with which a newly initiated primer will reach a length ≥7 nucleotides long will now be increased. Alternatively, inhibition of full length primer production would give a ratio <1. When poly(dT) was used as the template, Mn^{2+} stimulated elongation since a ratio of 2.5 was obtained (Table 2).

The ability of Mn^{2+} to affect processivity was significantly altered by the presence of 5 mM Mg^{2+}. The maximum processivity of primase was slightly higher when assays contained both Mn^{2+} and Mg^{2+}, and this maximum occurred with a 6-fold lower concentration of Mn^{2+} as compared to assays that lacked Mg^{2+} (Figure 2). At concentrations of Mn^{2+} higher than the optimal value, the processivity of

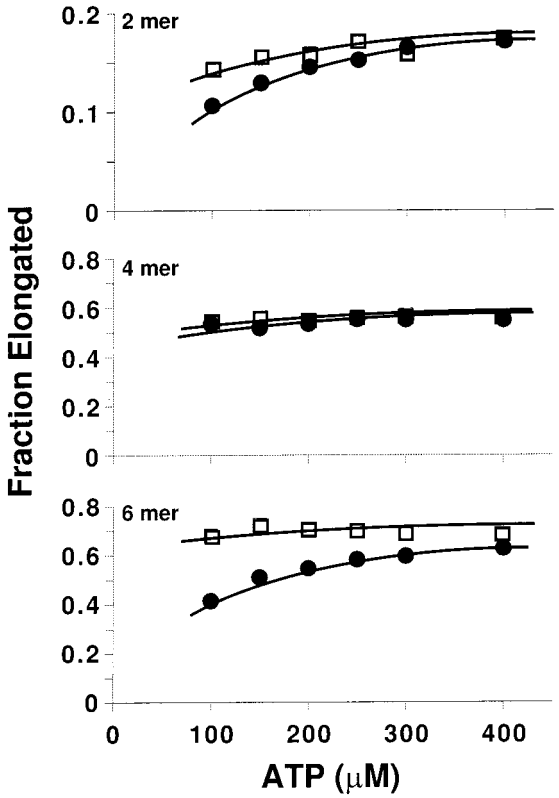


FIGURE 3: Mn^{2+} stimulates primase processivity on poly(dT). Assays contained 0.1 mM Mn^{2+}, while the concentration of ATP was varied as shown in the absence (●) or presence (□) of 5 mM $MgCl_2$. The fraction of the two-mer, four-mer, and six-mer elongated was quantified as described under Experimental Procedures.

primase decreased substantially in both the presence and absence of Mg^{2+}.

We further examined the mechanism by which Mn^{2+} increased the processivity of primase when the template was poly(dT) by measuring the fraction of primers of various lengths that were elongated as a function of ATP concentration. As shown in Figure 3 and Table 2, adding 100 μM Mn^{2+} to assays containing 5 mM Mg^{2+} and 200 μM ATP increased the processivity of primase, although the effects varied with different length primers.[2] However, raising the concentration of ATP to 400 μM greatly decreased the ability of Mn^{2+} to enhance the processivity of primase, indicating that the effects of Mn^{2+} on processivity only occur at low-NTP concentrations.

In contrast to the results with poly(dT), Mn^{2+} had either little effect ($\pm$10% on $d(ACC)_{20}$ and poly(dC)) or slightly inhibited ($d(ACT)_{20}$ and $d(TC)_{30}$) the production of full length primers relative to total products when we examined a series of templates containing increased amounts of deoxycytidylate. This decrease in processivity on $d(ACT)_{20}$ and $d(TC)_{30}$ was much greater at higher Mn^{2+} concentrations: with 600 μM Mn^{2+} and 5 mM Mg^{2+}, the ratio of full length products to total products is 0.2 for $d(ACT)_{20}$ and 0.1 for $d(TC)_{30}$, whereas raising the Mn^{2+} concentration had no effect on processivity with the other templates. The absence of processivity effects on $d(ACC)_{20}$ and poly(dC) may reflect the inherently higher processivity of primase on templates rich in deoxycytidylate (*14*). Since high processivity indicates that the rate of NTP polymerization is much faster than the rate of primer dissociation, further increasing the NTP polymerization rate would not be expected to further increase processivity, the observed result.

The decreased processivity on $d(ACT)_{20}$ and $d(TC)_{30}$ may be due to the assays containing multiple NTPs. As will be described below in greater detail, Mn^{2+} can increase the binding of noncognate NTPs during primer synthesis and, thereby, inhibit polymerization of the next correct NTP. For polymerization of each NTP on the templates $d(ACT)_{20}$ and $d(TC)_{30}$, either one or two noncognate NTPs are present (e.g., during polymerization of an ATP on $d(ACT)_{20}$, the noncognate GTP, and UTP are also present). The decreased rate of cognate NTP polymerization due to binding of these transiently noncognate NTPs would account for the decreased processivity on $d(ACT)_{20}$ and $d(TC)_{30}$.

Mn^{2+} Primarily Enhances NTP Utilization. The effects of Mn^{2+} on K_M and V_{max} for primer synthesis on $(dC)_{40}$ were measured (Table 3). As compared to assays containing just 5 mM Mg^{2+}, including 500 μM Mn^{2+} had little effect on V_{max} but significantly decreased the K_M for GTP. Since dinucleotide formation is rate limiting during primer synthesis (*14*), the lack of a V_{max} effect indicates that Mn^{2+} does not increase the rate of this process. The minimal effects of Mn^{2+} on the rate of dinucleotide synthesis at high-NTP concentrations are analogous to the reduced effects of Mn^{2+} on processivity at higher NTP concentrations, suggesting that the primary mechanism by which Mn^{2+} stimulates primase activity is by enhancing NTP binding/utilization during both initiation and elongation.

Mn^{2+} Interacts with the α-Phosphate of the Elongating NTP. In models for the active site of polymerases, a metal ion interacts with the α-phosphate of the incoming (d)NTP during phosphodiester bond formation (*23*, *24*). To provide evidence that this can also occur with primase, we performed thio-rescue experiments (*25*). This approach compares the ability of Mn^{2+} and Mg^{2+} to overcome the inhibition of (d)-NTP polymerization activity often observed when one of the nonbridging oxygens of the α-phosphate is replaced with sulfur. If the metal interacts with this phosphate, then Mn^{2+} will restore activity more efficiently than Mg^{2+} due to the significantly greater thiophylicity of Mn^{2+} than Mg^{2+} (*25*). Primase activity was measured in assays containing $d(ACT)_{20}$, 200 μM [α-^{32}P]ATP, and either 200 μM GTP or 200 μM α-thioGTP. Since assays lack UTP, primase can only synthesize the pppApG dinucleotide. In assays containing 1 mM Mg^{2+}, replacing GTP with α-thioGTP decreased the rate of pppApG synthesis by 82% (Table 4). Importantly, the effects of the α-thio substitution could not be overcome by increasing the Mg^{2+} concentration since including 10 mM Mg^{2+} resulted in only a slight recovery of activity. In contrast, increasing the Mn^{2+} concentration restored most of the activity lost due to substitution of GTP with α-thioGTP; with 0.2 mM Mn^{2+} in the assay, activity was inhibited by 80%, whereas with 1 mM Mn^{2+}, inhibition was reduced to 36% (Table 4). Together, these results indicate

[2] The different effects of Mn^{2+} on elongation of different length primers may indicate that the length of the primer alters the interactions of primase with the next NTP to be polymerized. For example, Mn^{2+} did not significantly alter the fraction of 4-mer further elongated. This lack of an effect indicates that Mn^{2+} does not alter the net rate constant for polymerization over the range of ATP concentrations examined, perhaps because of more efficient binding of ATP to the E·4-mer·DNA complex.

Table 3: The Effects of Mn^{2+} on K_M and V_{max} for NTPs[a]

varied substrate (GTP)	K_M (μM)	V_{max} ((pmol primers)/(h·pmol primase))	V_{max}/K_M ((pmol primers)/(h·pmol primase·μM))
5 mM Mg^{2+}	300 ± 6	120 ± 10	0.40 ± 0.02
5 mM Mg^{2+} + 0.5 mM Mn^{2+}	76 ± 9	180 ± 5	2.4 ± 0.4

[a] Assays were performed using $(dC)_{40}$ as template.

Table 4: Rescue of Primase Activity in the Presence of α-Thio(S_P) GTP

	activity with α-Thio(S_P) GTP/activity with GTP[a]				
[M^{2+}]	0.2 mM	0.5 mM	1 mM	5 mM	10 mM
Mg^{2+}			0.18	0.20	0.27
Mn^{2+}	0.20	0.43	0.64		

[a] Assays contained $d(ACT)_{20}$, [α-^{32}P]ATP, either GTP or α-Thio(S_P) GTP, and the indicated concentration of divalent metal. Activity was determined from the amount of pppApG dinucleotide formed and is given as the ratio of activity when the assay contained α-thioGTP versus when it contained GTP.

that Mn^{2+} interacts with the α-phosphate of the incoming NTP when it is the only metal present.

Mn^{2+} Stimulates Activity of the p49 Subunit. The observation that Mn^{2+} interacts with the α-phosphate of the incoming NTP predicts that the catalytic p49 subunit must contain at least one Mn^{2+} binding site. Consistent with this hypothesis, Mn^{2+} dramatically stimulates p49 activity (Figure 4). This stimulation is apparent at even low concentrations of Mn^{2+}: adding 50 μM Mn^{2+} to assays containing 5 mM Mg^{2+} increased the rate of NTP polymerization by 6-fold.

Mn^{2+} Slightly Decreases the Fidelity of Primase. Previous studies have demonstrated that Mn^{2+} significantly reduces the fidelity of DNA polymerases (*15*, *16*, *26*). We extended these studies to primase, perhaps the least accurate of all known template-directed nucleotide polymerizing enzymes (*17*). In assays containing $d(ACC)_{20}$, 5 mM Mg^{2+}, 10 μM UTP and GTP, primase synthesizes a series of products that consist of both UTP and GTP (Figure 5 and (*17*)). Increasing the concentration of GTP results in the appearance of products with altered mobility that largely comigrate with primase products that only contain guanylate (Figure 5 and (*17*)), indicating that primase polymerized GTP in place of UTP. When the same titration was performed in assays containing 5 mM Mg^{2+} and 500 μM Mn^{2+}, the extent of misincorporation with only 300 μM GTP was similar to that obtained with 1 mM GTP in the assays lacking Mn^{2+}. Under both conditions, the relative amounts of products that comigrate with the normal products (i.e., no misincorporation) and those that consist of only guanylate were comparable. Thus, Mn^{2+} increases misincorporation by approximately 3-fold on $d(ACC)_{20}$, and similar small effects on fidelity were observed with other DNA templates (Data not shown).[3] The extent of misincorporation with only Mn^{2+} present was similar to the extent of misincorporation when both Mn^{2+} and Mg^{2+} were present (Figure 5), indicating that a 10-fold molar excess of Mg^{2+} did not protect primase from the mutagenic effect of Mn^{2+}. These modest effects of Mn^{2+} on fidelity suggest that it may be difficult to further decrease the accuracy of an enzyme that normally is only marginally accurate.

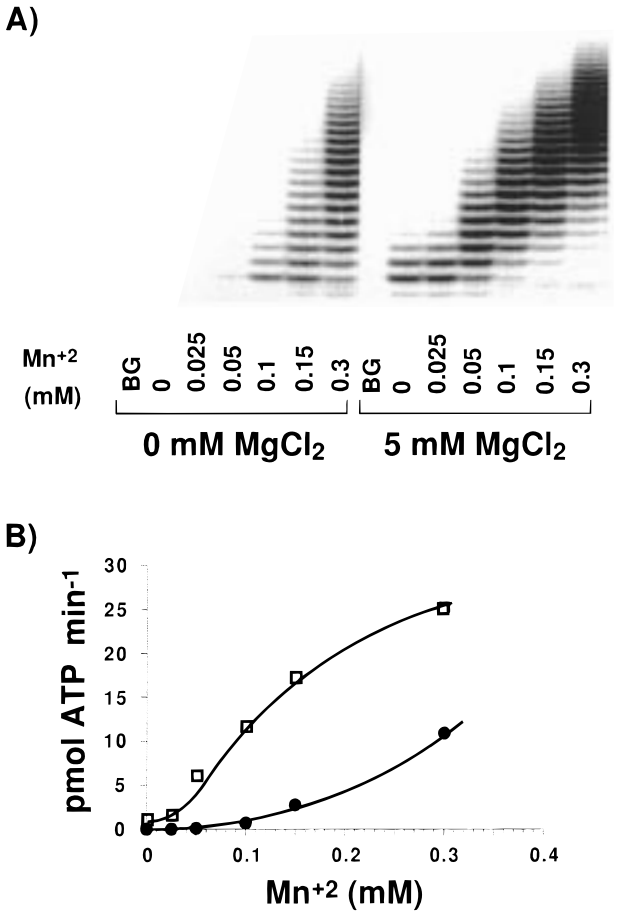


FIGURE 4: Mn^{2+} stimulates p49 activity. p49 activity assays were performed as described under Materials and Methods. (A) Phosphorimage of the products synthesized on poly(dT):$(rA)_{12}$ at the noted Mn^{2+} and Mg^{2+} concentrations. (B) The amount of ATP polymerized in the absence (●) and presence (□) of 5 mM $MgCl_2$ was quantified.

In addition to slightly increasing the rate of NTP misincorporation, Mn^{2+} can enhance the binding of noncognate NTPs to the primase−DNA complex. We measured the IC_{50} of CTP, GTP, and UTP in assays containing poly(dT) as a template and 100 μM [α-^{32}P]ATP. The data in Table 5 show that the addition of 100 μM Mn^{2+} to assays containing 5 mM Mg^{2+} decreases the IC_{50} by approximately 2-fold for each of the noncognate NTPs (under these conditions, the ATP concentration is much less than the K_M (5−10 mM, data not shown)). Separation of the products by electrophoresis followed by phosphorimager analysis revealed that no products of altered mobility were generated in the presence of a noncognate NTP, indicating that the effects of noncognate NTPs on the rate were not due to incorporation

[3] This value is an estimate in the change in mis-incorporation since we cannot measure the number of guanylates that were polymerized in place of uridylate. For a primer nine nucleotides long, up to three noncognate guanylates can be incorporated.

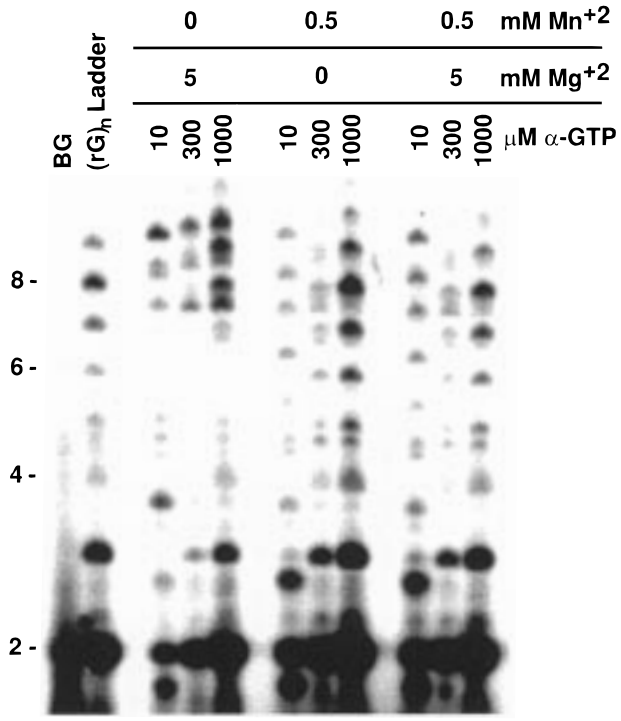


FIGURE 5: Mn^{2+} slightly decreases primase fidelity on $d(ACC)_{20}$. Assays contained 60 μM $d(ACC)_{20}$, 10 μM UTP, and the indicated concentration of GTP, Mn^{2+}, and Mg^{2+}. The oligo(G) ladder was synthesized in assays containing $d(ACC)_{20}$ and only [α-^{32}P]GTP (17), and the lengths of each species are noted. BG = background.

Table 5: Mn^{2+} Enhances Binding of Noncognate NTPs

	IC_{50} of NTP[a] (mM)	
NTP	5 mM Mg^{2+}	5 mM Mg^{2+} + 0.1 mM Mn^{2+}
CTP	130	60
GTP	100	55
UTP	115	60

[a] Assays contained 100 μM [α-^{32}P]ATP and were performed on poly(dT).

into products.[4] Thus, the presence of low levels of Mn^{2+} enhances the ability of noncognate NTPs to inhibit the primase−DNA complex. This inhibition will likely affect primer synthesis on templates that require polymerization of different NTPs because, as noted earlier, enhanced binding of noncognate NTPs can result in decreased processivity on these templates.

Mn^{2+} Decreases the Ability of Anions to Inhibit Primase. Previous studies have shown that elevated salt concentrations can inhibit primase (*6*, *27*), but little is known about the inhibitory process. Primase activity was measured on poly(dT) in assays containing increasing concentrations of the Na^+, K^+, and NH_4^+ forms of Cl^-, OAc^-, and HPO_4^{2-}. Table 6 shows that for any given anion, the IC_{50} remained constant regardless of the cation. Furthermore, the IC_{50}s varied from 1 mM for HPO_4^{2-} to 50 mM for OAc^-, indicating that inhibition is not due to ionic strength effects but is rather due to the anion binding to primase.[5]

[4] We have previously found that incorporation of a nucleotide other than adenylate during primer synthesis results in a small but detectable shift in the band (*1*).

[5] Ionic strengths were determined assuming ideal conditions with the activity coefficient = 1.

Table 6: Salt Inhibition of Primase and the p49 Subunit

		IC_{50} of salt (mM)[a]		
	ion	Cl^-	OAc^-	HPO_4^{2-}
	Primase			
5 mM Mg^{2+}	Na^+	35	55	1.1
	K^+	25	50	0.9
	NH_4^+	30	45	1.1
5 mM Mg^{2+} + 0.1 mM Mn^{2+}	Na^+	70	105	4.5
	K^+	70	105	4.3
	NH_4^+	65	90	4.5
	p49			
5 mM Mg^{2+}	Na^+	18	15	3.0
5 mM Mg^{2+} + 0.1 mM Mn^{2+}	Na^+	20	nd[b]	3.2

[a] Assays contained 200 μM [α-^{32}P]ATP and the indicated divalent metal(s) and were performed on either poly(dT) (primase) or poly(dT)-oligo(rA) (p49). [b] nd = not done.

Just as salts inhibit primer synthesis by the p49/p58 primase complex, salts also inhibit elongation of poly(dT)/oligo(rA) by the isolated p49 subunit (Table 6). Interestingly, NaOAc and NaCl inhibited the p49 subunit somewhat more potently than they inhibited the p49/p58 complex, whereas Na_2HPO_4 inhibited complexation slightly. These differences in the potency of inhibition may be due to the more complex reaction catalyzed by the p49/p58 complex (initiation and elongation) as compared to p49 alone (just elongation), or they could indicate that binding of p58 to the p49 subunit results in a perturbed and/or novel anion binding site.

The ability of Mn^{2+} to influence inhibition by anions was measured by including 100 μM Mn^{2+} in the assays (5 mM Mg^{2+} was still present). Table 6 shows that including Mn^{2+} in the assays reduced the salt sensitivity of the p49/p58 complex. In the cases of Cl^- and OAc^-, the IC_{50}s increased by a factor of 2, while in the case of HPO_4^{2-} the IC_{50} increased by a factor of 4. In contrast to the large effect on salt inhibition of the p49/p58 complex. Mn^{2+} had virtually no effect on inhibition of p49 by either NaCl or Na_2HPO_4 (Table 6).

The effects of HPO^{2-} on the pol α-primase complex were also measured to ensure that the results described above were relevant to the physiologically more important form of primase. In the presence of only 5 mM Mg^{2+}, the IC_{50} of HPO_4^{2-} was 2 mM, but upon the addition of 200 μM Mn^{2+}, the IC_{50} increased to 5 mM. Thus, phosphate inhibits both the p49/p58 primase and the four subunit pol α-primase complexes, and Mn^{2+} reduces the potency of inhibition in ease case.

In vitro Start-Sites Appear more in vivo like in the Presence of Mn^{2+}. The observations that Mn^{2+} stimulates primase activity and that the magnitude of stimulation is template dependent predicts that Mn^{2+} will alter where primase initiates synthesis on a more natural template. Therefore, we examined how low concentrations of Mn^{2+} affect where primase initiates primer synthesis on a 458 nucleotide long "bubble" template derived from the plasmid FP5 (Table 1, (*22*)). In these assays, primase synthesizes an RNA primer that pol α then elongates via dNTP polymerization to the end of the template. Hence, product length defines where on the template primase synthesized a primer. Figure 6 demonstrates a typical in vitro primase start-site pattern in assays containing 1 mM Mg^{2+}, the optimal concentration on this template. Initiation primarily occurs

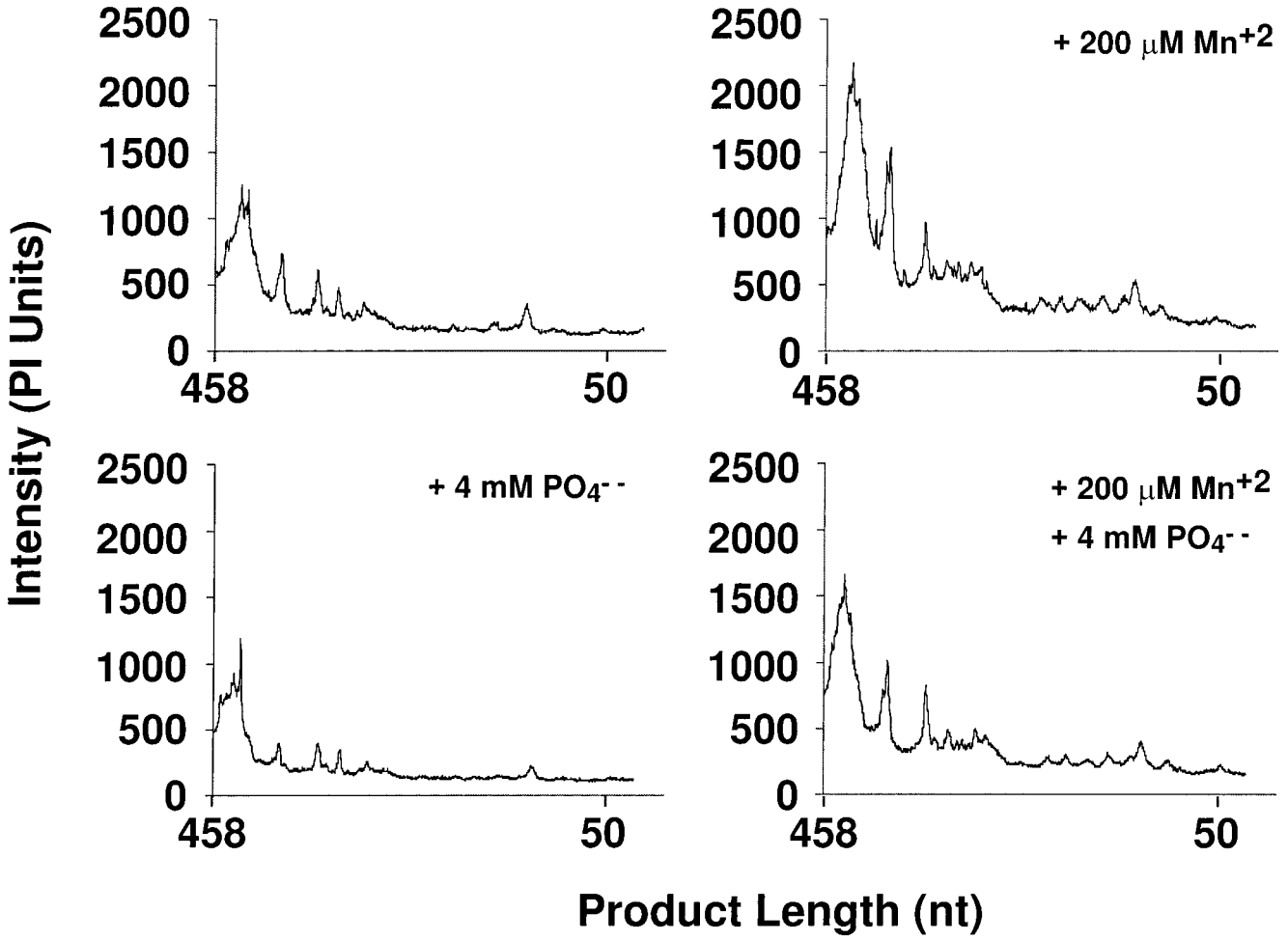


FIGURE 6: Mn^{2+} alters primase start-site selection in the presence and absence of physiological concentrations of phosphate. Primase-coupled pol α assays were performed as described under Materials and Methods. All assays contained 1 mM $MgCl_2$ and the indicated addition of $MnCl_2$ and Na_2HPO_4. Products were purified on an 8% denaturing polyacrylamide gel and quantified using phosphorimager analysis.

in template regions that are pyrimidine rich and is consistent with previous studies showing that primase greatly prefers pyrimidine rich templates (*28*, *29*). Addition of 200 μM Mn^{2+} to the assay effects two prominent changes (Figure 6). First, the amount of product increases 2-fold, consistent with the ability of Mn^{2+} to stimulate primase activity. Second, the enzyme generates products due to initiation in all regions of the template, not just the pyrimidine-rich regions. Importantly, utilization of the entire template is the result observed during DNA replication in whole cells (*30*, *31*).

Mn^{2+} also overcomes the inhibitory effects of phosphate on this more natural template. In assays containing only Mg^{2+}, 4 mM phosphate inhibits product formation by 30% (Figure 6), similar to the effects when poly(dT) was the template but does not change the sites used for initiation. Upon addition of Mn^{2+}, the amount of product increases 2-fold and initiation occurs in all regions of the template, similar to the results when phosphate was not present in the assays.

DISCUSSION

We have found that low concentrations of Mn^{2+} have multiple effects on primase activity, including more efficient utilization of NTPs, resistance to inhibition by anions, and altered start-site selectivity with respect to template sequence. These effects of Mn^{2+} are manifested in the presence of a large excess of Mg^{2+}, indicating that the sites to which Mn^{2+} binds are quite specific for Mn^{2+}. While the precise location of the Mn^{2+} binding sites have not been determined, the p49 subunit must contain at least one Mn^{2+} binding site as evidenced by the ability of Mn^{2+} to affect activity of both the p49/p58 primase complex as well as isolated p49.

Mn^{2+} appears to primarily stimulate primase activity by enhancing the ability of primase to utilize NTPs. When measuring the rate of primer synthesis, this results in a decreased K_M for NTPs. Since the rate limiting step during overall primer synthesis is initiation (i.e., dinucleotide formation), this decreased K_M indicates that Mn^{2+} enhances utilization of the initiating NTPs. The relatively small change in V_{max} indicates that Mn^{2+} does not increase the rate limiting step during initiation, which is likely either a conformational change or phosphodiester bond formation (*14*).

Mn^{2+} also increases the processivity of primase at low-NTP concentrations, indicating that Mn^{2+} enhances the utilization of NTPs during the elongation steps but probably has little effect on the rates of either primer−template dissociation or phosphodiester bond formation during elongation. Processivity is defined by a competition between polymerization of the next NTP versus dissociation of the E−RNA−DNA complex and can be defined kinetically as

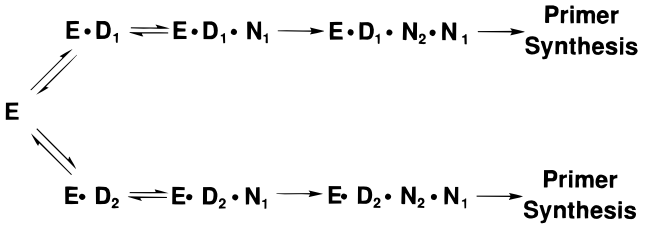
$k_{pol}/(k_{pol} + k_{diss})$ where k_{pol} and k_{diss} are the net rate constants for polymerization and dissociation, respectively. Thus, enhanced processivity could result from either a decrease in the net rate constant for complex dissociation or an increase in the net rate constant for polymerization of the next NTP. The observation that the effects of Mn^{2+} are minimal at 400 μM ATP indicates that enhanced processivity is primarily due to an increased net rate constant for polymerization. Since only k_{pol} should contain terms for ATP concentration, only k_{pol} will be affected by changing the ATP concentration. If Mn^{2+} had altered the frequency with which a given length primer is elongated by changing k_{diss}, then varying the ATP concentration should not have eliminated the effects of Mn^{2+}. Furthermore, this lack of an effect at high-NTP concentrations also suggests that Mn^{2+} does not affect the rate of phosphodiester bond formation. Since net rate constants contain terms for all steps up to and including the first irreversible step (*32*), one would expect k_{pol} to contain terms for the phosphodiester bond-forming step. If Mn^{2+} had increased this rate, then processivity should have increased even at high-NTP concentrations.

Primase is inhibited by physiological concentrations of salt and the inhibitory species is the anion. Similar specific anion inhibition has been observed with other DNA polymerases (*6*, *27*), and another member of the class-X nucleotidyl transferase superfamily, pol β, contains an anion binding site in its active site (*15*). The very low concentration of HPO_4^{2-} required for inhibition is of particular interest, since intracellular HPO_4^{2-} concentrations are estimated at 1.4 mM (liver, (*33*)), levels that should give substantial inhibition in vivo. The ability of Mn^{2+} to mitigate this inhibition may well be important for primase activity under physiological conditions.

The unique ability of Mn^{2+} to reduce anion inhibition of the p49/p58 complex but not isolated p49 could be explained by three different mechanisms: (1) there is a Mn^{2+} binding site on the p58 subunit that when occupied, decreases the ability of anions to inhibit the p49/p58 primase complex, (2) binding of p58 to p49 alters the conformation of p49 such that now Mn^{2+} can bind and, consequently, reduces the binding of anions, and (3) inhibition of primer synthesis by anions occurs by very different mechanisms for the p49/p58 complex versus the isolated p49 subunit. Regardless of which mechanism is correct, the effects of Mn^{2+} at this site must be rather specific to Mn^{2+} since reduction of inhibition occurred in the presence of a 50-fold higher concentration of Mg^{2+}.

Three lines of evidence indicate that primase contains at least two metal binding sites. First, adding Mn^{2+} to assays results in stimulation of activity followed by inhibition, both in the presence and absence of Mg^{2+} (Figure 1). Stimulation of activity at low Mn^{2+} concentrations indicates the presence of at least one Mn^{2+} binding site. If Mn^{2+} only bound to this site, then increasing the occupancy of this site by increasing the Mn^{2+} concentration should not have resulted in decreased activity. Second, the concentration of Mn^{2+} that gave maximal stimulation of full length products was significantly lower than the concentration of Mn^{2+} that gave maximal stimulation of initiation (Table 2). If Mn^{2+} were binding to a single site and affecting both processes, one would have expected that both processes should have been maximal at the same Mn^{2+} concentration. Third, maximal processivity occurs at a lower Mn^{2+} concentration when Mg^{2+} is also present as compared to when only Mn^{2+} is present (Figure 2). If the effects of Mn^{2+} were simply due to Mn^{2+} binding to and displacing Mg^{2+} from a single site, then including Mg^{2+} in the assays should have increased, not decreased, the Mn^{2+} concentration at which the maximal processivity occurred due to the competition between Mn^{2+} and Mg^{2+} for binding to this site. Together, these data indicate that the primase reaction complex contains at least two metal binding sites.

Scheme 1



The presence of Mn^{2+} significantly altered the sequence specificity of primase during initiation of new primers. When only Mg^{2+} was present in assays, primase primarily initiated synthesis at pyrmidine-rich regions. Importantly, adding low levels of Mn^{2+} to these assays resulted in primase utilizing sequences throughout the template, qualitatively similar to what is observed during DNA replication in whole cells (*30*, *31*). The more effective utilization of NTPs in the presence of Mn^{2+} may well account for the altered start-sites. As described previously, a mechanism by which more efficient NTP utilization will dramatically affect where primase initiates primer synthesis is provided by Scheme 1 (*21*), a simplified situation where primase can synthesize a primer at either of two sites (D_1 or D_2). At low-NTP concentrations (i.e., V_{max}/K_M conditions), primer synthesis will occur at the more preferred site since primase will have the opportunity to bind to and dissociate from both potential primer synthesis sites prior to primer synthesis. Adding Mn^{2+} decreases the K_M for NTPs, an effect that is functionally equivalent to increasing the NTP concentrations. Now, two NTPs can bind to and "trap" primase at the first potential initiation site to which it initially binds by immediately converting the E−DNA binary complex into an E−DNA−NTP−NTP quaternary complex that is poised to initiate primer synthesis. This predicts that primase will initiate primer synthesis at sequences throughout the template, the observed result.

Is Binding of Mn^{2+} to Primase Physiologically Relevant? We have demonstrated that Mn^{2+} binds to and stimulates primase, decreases its' sensitivity to in vivo levels of anions, and results in in vitro start-sites appearing more whole cell-like. However, it remains unclear if this binding of Mn^{2+} is physiologically relevant since levels of Mn^{2+} in cells are typically 2−30 μM (*34*), and we do not see large effects on our in vitro assays until we reach levels of 50−100 μM. Interestingly, for a number of other enzymes that appear to be Mn^{2+}-dependent in vivo, optimal in vitro levels of Mn^{2+} range from 1−10 mM (e.g., galactosyltransferases, O-linked glycosyltransferases, N-acetylgalactosylaminyltransferase (*35*−*37*)), concentrations 10−100 fold higher than needed to affect primase activity. The effects of Mn^{2+} on primase activity occur in the presence of a 50 to 100-fold molar excess of Mg^{2+}, similar to the large excess of Mg^{2+} present in whole

cells, indicating that the Mn^{2+} binding site(s) on primase are quite specific. Thus, the levels of Mn^{2+} needed to alter in vitro primase activity are not inconsistent with Mn^{2+} influencing activity in vivo. Finally, unlike the situation with DNA polymerases where the mutagenic effects of Mn^{2+} would clearly be deleterious to their normal role, the effects of Mn^{2+} on primase fidelity are physiologically irrelevant since the RNA primer will not be present in the product DNA. In addition, the presence of mismatches in the primer should not affect their utilization by pol α since pol α readily elongates primase-synthesized primers containing multiple mismatches (*17*).

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