

# *In vitro* replication of bacteriophage PRD1 DNA. Metal activation of protein-primed initiation and DNA elongation

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## ABSTRACT

**Bacteriophage PRD1 replicates its DNA by means of a protein-primed replication mechanism. Compared to Mg<sup>2+</sup>, the use of Mn<sup>2+</sup> as the metal activator of the phage DNA polymerase results in a great stimulation of the initiation reaction. The molecular basis of the observed stimulatory effect is an increase in the velocity of the reaction. The phage DNA polymerase is also able to catalyze the formation of the initiation complex in the absence of DNA template. Although the presence of Mn<sup>2+</sup> does not affect either the polymerization activity or the processivity of the DNA polymerase, this metal is unable to activate the overall replication of the phage genome. This can be explained by a deleterious effect of Mn<sup>2+</sup> on the 3'-5'-exonucleolytic and/or the strand-displacement activity, the latter being an intrinsic function of the viral DNA polymerase required for protein-primed DNA replication.**

## INTRODUCTION

Bacteriophage PRD1 belongs to a group of closely related viruses infecting a wide variety of Gram-negative bacteria harboring P, N or W incompatibility group plasmids (1–5). Among the hosts are *Salmonella typhimurium* and *Escherichia coli*. The phage particle consists of an external protein shell surrounding a lipid membrane inside which resides the phage genome. For a review on the PRD1 system see (6).

The PRD1 genome is a linear, double-stranded DNA molecule of 14,925 kb (7,8) which has 110 bp long inverted terminal repeats (9), and a specific viral terminal protein covalently linked at each 5'-end (10,11). The replication of the genome uses a protein-primed mechanism (for a review, see 12) in which the terminal protein (protein P8) functions as a primer in the initiation of DNA replication whereby the phage-encoded DNA polymerase (protein P1) catalyzes the formation of a phosphodiester bond between Tyr<sup>190</sup> residue of P8 and dGMP (13), the 5'-terminal nucleotide at both DNA ends. Subsequent to initiation, elongation of the initiation complex takes place catalyzed by the viral DNA polymerase itself. The fact that both activities, protein-primed initiation and DNA polymerization, are carried out by the same DNA polymerase molecule guarantees an efficient coupling

between the initiation and the elongation steps. DNA with 5'-covalently attached proteins has been discovered in several groups of viruses and linear plasmids of both prokaryotic and eukaryotic origin (reviewed in 12).

*Bacillus subtilis* phage  $\phi$ 29 is, together with adenovirus, the best understood protein-primed replication system (12,14,15). The availability of a highly purified *in vitro* replication system has allowed the detailed characterization of both  $\phi$ 29 terminal protein and DNA polymerase, as well as the basis for the  $\phi$ 29 DNA replication mechanism (12). Recent studies indicate that, in addition to Mg<sup>2+</sup>-activated catalysis, other metal ions, such as Zn<sup>2+</sup>, Co<sup>2+</sup>, and especially Mn<sup>2+</sup>, are able to serve as activators of protein-primed initiation of replication (16). The  $\phi$ 29 DNA polymerase, in the presence of Mn<sup>2+</sup> and without any DNA template, is able to covalently link any of the four deoxynucleoside monophosphates to the terminal protein (17).

Previous work on the replication of bacteriophage PRD1 DNA resulted in the purification of proteins P1 and P8 (18,19), as well as in the purification of the phage genome (20), and in the development of an *in vitro* replication system that used purified components (19). Preliminary studies carried out using this *in vitro* system showed that P1 possessed DNA polymerase and protein-priming activities, as well as 3'-5'-exonuclease activity, and the replication of the phage DNA yielded full-length products (19). In the present study, we have continued the characterization of PRD1 DNA replication *in vitro*. We show that Mn<sup>2+</sup> greatly stimulates the formation of the P8-dGMP replication initiation complex, and that this complex can be formed in the absence of the DNA template.

## MATERIALS AND METHODS

### Nucleotides, proteins and templates

Unlabeled nucleotides, poly (dC)<sub>250</sub>, and oligo (dG)<sub>10</sub> were purchased from Pharmacia PL-Biochemicals. ( $\alpha$ -<sup>32</sup>P)dNTPs (400 Ci/mmol) were obtained from Amersham. The 15-mer oligonucleotide SP1 (3'-CATGAGTGACACTAG-5'), labeled at the 5'-end (2400 cpm/0.15 pmol), was synthesized and purified as previously reported (21). *Eco*RI-treated phage  $\phi$ 29 DNA was prepared as described (22). Bacteriophage PRD1 terminal protein and DNA polymerase were purified as described (18,19). The

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DNA polymerase preparation (17 ng/ $\mu$ l) contained 0.05% (v/v) Triton X-100. PRD1 P8-DNA was isolated after SDS treatment and phenol extraction (20).

#### Assay for the formation of the P8-dGMP complex

The standard incubation mixture contained, in 25  $\mu$ l, 50 mM Tris-HCl, pH 7.5, 1 mM dithiothreitol (DTT), 5% glycerol, 0.1 mg/ml bovine serum albumin (BSA), 20 mM ammonium sulphate, 0.25  $\mu$ M ( $\alpha$ - $^{32}$ P)dGTP (2.5  $\mu$ Ci), 0.5  $\mu$ g of P8-DNA as template, 34 ng and 100 ng of purified DNA polymerase P1 and terminal protein P8, respectively, and the metal ion, either  $Mg^{2+}$  or  $Mn^{2+}$ , at the indicated concentration. When indicated, particular components were omitted. After incubation for the indicated time at 30°C the reaction was stopped by adding 10 mM EDTA and 0.1% SDS. The samples were then filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the excluded volume was analyzed by SDS-10% polyacrylamide gel electrophoresis and subsequent autoradiography. Quantitation was done by excising the radioactive band corresponding to the P8-dGMP complex from the gel and measuring the Cerenkov radiation, or by densitometry of the autoradiographs.

#### Replication assay (initiation plus elongation)

The incubation mixture contained, in 25  $\mu$ l, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5% glycerol, 0.1 mg/ml BSA, 20 mM ammonium sulphate, 50  $\mu$ M each dCTP, dATP, dTTP, and ( $\alpha$ - $^{32}$ P)dGTP (2.5  $\mu$ Ci), 0.5  $\mu$ g P8-DNA, 34 ng of DNA polymerase P1, 100 ng of terminal protein P8, and the indicated type and concentration of metal ion. After incubation at 37°C for the indicated time, the reaction was stopped and filtered as above, and the Cerenkov radiation measured. The samples were subjected to alkaline 0.7% agarose gel electrophoresis (23) and autoradiography.

#### Filling-in assay

The reaction mixture contained, in a volume of 25  $\mu$ l, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5% glycerol, 0.1 mg/ml BSA, 100  $\mu$ M each dGTP and dTTP, 0.25  $\mu$ M ( $\alpha$ - $^{32}$ P)dATP, 0.5  $\mu$ g of *Eco*RI-digested phage  $\phi$ 29 DNA as template, 3 ng of DNA polymerase P1, and the metal ion at the indicated concentration. After incubation for 5 min at 30°C, samples were treated and filtered as above. The radioactivity of the excluded volume was measured (Cerenkov radiation), and the samples analyzed by native 0.7% agarose gel electrophoresis and autoradiography.

#### Assay for the elongation of poly (dC)<sub>250</sub>-oligo (dG)<sub>10</sub>

The incubation mixture contained, in 25  $\mu$ l, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5% glycerol, 0.1 mg/ml BSA, 20  $\mu$ M ( $\alpha$ - $^{32}$ P)dGTP (2.5  $\mu$ Ci), 1  $\mu$ g poly (dC)<sub>250</sub>, 0.5  $\mu$ g oligo (dG)<sub>10</sub>, 10 ng of DNA polymerase P1, and the metal ion at the indicated concentration. Samples were incubated for 15 min at 30°C, and treated as above. The radioactivity of the excluded volume was determined (Cerenkov radiation), and the samples analyzed by alkaline 0.7% agarose gel electrophoresis and autoradiography.

#### 3'-5' exonuclease assay on ssDNA

The incubation mixture contained, in a volume of 12.5  $\mu$ l, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 5% glycerol, 0.1 mg/ml BSA, 0.075 ng of 5'-labeled SP1 oligonucleotide as substrate, 3 ng of DNA polymerase P1, and the metal ion at the indicated concentration. The samples were incubated at 25°C for 3 min,

and the reaction stopped by adding 3  $\mu$ l of sequencing gel loading buffer. The analysis was done by electrophoresis in 20% polyacrylamide gels in the presence of 8 M urea, followed by autoradiography.

## RESULTS

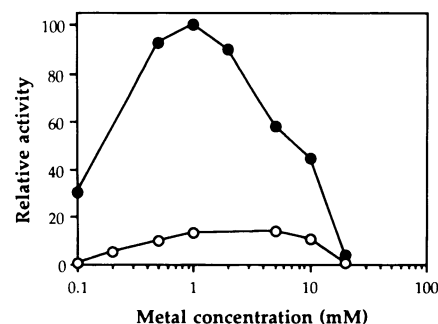
### Metal activation of the initiation reaction

The activity of the PRD1 DNA polymerase was earlier shown to be dependent on the presence of  $Mg^{2+}$  (18,19). When the effects of  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Fe^{3+}$ ,  $Zn^{2+}$ ,  $Co^{2+}$ ,  $Ni^{2+}$ ,  $Hg^{2+}$ ,  $Ca^{2+}$ ,  $Cu^{2+}$ , and  $Be^{2+}$  on the initiation step of replication were investigated, we found that only  $Mn^{2+}$  and  $Co^{2+}$  were able to support the reaction. The activation due to  $Co^{2+}$  was practically identical to that observed with  $Mg^{2+}$  and was not further investigated. However, a 6 to 10-fold stimulation relative to  $Mg^{2+}$  was observed with  $Mn^{2+}$  as the metal ion (Fig. 1). As can be seen,  $Mg^{2+}$  can support the initiation reaction over a rather large concentration range (from 0.5 mM to 10 mM), the optimal concentration being around 5 mM. With  $Mn^{2+}$ , a more defined peak of activity was detected, at concentrations between 0.5 mM and 2 mM, with an optimum at about 1 mM. With both metal ions at their optimal concentration and at 30°C, the initiation reaction proceeded linearly for up to 20 min (not shown).

A further investigation of the molecular basis underlying the activation of the initiation reaction obtained with  $Mn^{2+}$  was undertaken. To investigate the possibility that  $Mn^{2+}$  affected the  $K_M$ , the  $V_{max}$ , or both, the replication initiation assay was carried out at different dGTP concentrations. As can be seen in Fig. 2, with either  $Mg^{2+}$  or  $Mn^{2+}$  and P8-DNA as substrate, the initiation reaction reached a value close to its maximum at a dGTP concentration of 1  $\mu$ M, suggesting that  $Mn^{2+}$  does not exert its activating effect through lowering the  $K_M$  but acting on the  $V_{max}$  of the reaction. A double-reciprocal plot of the same data (not shown) indicated that the reactions with both metals have an identical apparent  $K_M$  of about 0.5  $\mu$ M. Indeed,  $Mn^{2+}$  produced a relatively moderate increase of the  $V_{max}$  (13.3 fmol/min) compared to  $Mg^{2+}$  (1.8 fmol/min).

### DNA-independent initiation reaction

Recent studies have shown that, in the absence of DNA template, the phage  $\phi$ 29 DNA polymerase is able to catalyze the

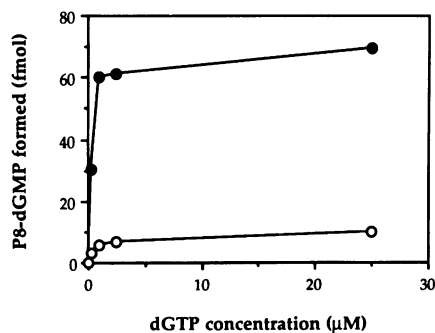


**Figure 1.** Effect of metal ion concentration on the formation of the initiation complex. The metal activators used were  $Mn^{2+}$  (●) and  $Mg^{2+}$  (○). After 10 min at 30°C, the formation of the P8-dGMP initiation complex was analyzed as described in Materials and Methods. The maximal value obtained with  $Mn^{2+}$  was considered 100%, and corresponded to 25.1 fmoles of P8-dGMP complex.

deoxynucleotidylation of the  $\phi 29$  terminal protein (17). We investigated the possibility that such DNA-independent deoxyguanylation of the PRD1 terminal protein (protein P8) by PRD1 DNA polymerase (protein P1) occurred. As shown in Fig. 3A, a  $^{32}\text{P}$ -labeled band running in the position of P8 was found when P1 and P8 were incubated with ( $\alpha\text{-}^{32}\text{P}$ )dGTP, in the presence of 1 mM  $\text{Mn}^{2+}$  (lane e). No labeled band was detected in the absence of either P1 (lane a), P8 (lane b), or  $\text{Mn}^{2+}$  (lane c). As with the initiation reaction done with P8-DNA as template (18), the DNA-independent reaction was stimulated by the presence of ammonium ions (optimal concentration about 20 mM; compare lanes d and e). This DNA-independent reaction was only detected with  $\text{Mn}^{2+}$  and proceeded linearly for more than 6 hours. However, it was only between 0.5% and 1% of the initiation reaction obtained in the presence of the P8-DNA template at the same metal ion concentration. As shown in Fig. 3B, the reaction increased linearly with increasing dGTP concentration, up to 100  $\mu\text{M}$ , indicating a very high  $K_M$  value for the DNA-independent deoxyguanylation of the terminal protein P8.

### Nucleotide specificity of the initiation reaction

As mentioned earlier, the protein-primed initiation of PRD1 DNA replication results in the formation of a P8-dGMP covalent complex. To test whether the use of a different metal ion could alter the specificity of this reaction, the effect of each one of the four different ( $\alpha\text{-}^{32}\text{P}$ )-dNTPs was tested. As shown in Fig. 4A, the initiation reaction, in the presence of 5 mM  $\text{Mg}^{2+}$  and P8-DNA template, is by far more efficient with dGTP than with any of the other three dNTPs. By densitometry of overexposed autoradiographs this relative efficiency was calculated to be over 200-fold. Carrying out the reaction in the presence of 1 mM  $\text{Mn}^{2+}$  resulted in an increase of the P8-dAMP, P8-dCMP, and P8-dTMP formed (Fig. 4B), the amounts being about 4%, 6%, and 10%, respectively, of that obtained as P8-dGMP, as determined by densitometry of autoradiographs obtained at different exposure times. In the absence of P8-DNA, and with  $\text{Mn}^{2+}$  as the activator, the initiation reaction had a similar efficiency with each of the four P8-dNTP complexes, the formation of P8-dAMP and P8-dTMP being somehow more favored (1.5 and 1.3-fold relative to P8-dGMP; Fig 4C).

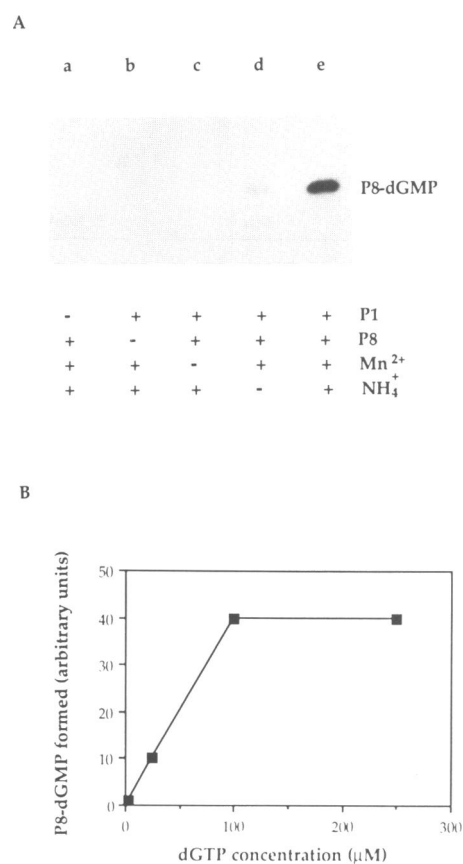


**Figure 2.** Effect of dGTP concentration on the initiation reaction. The formation of the P8-dGMP complex was assayed under standard conditions with an incubation time of 5 min, P8-DNA as template, and either  $\text{Mg}^{2+}$  (○) or  $\text{Mn}^{2+}$  (●) as metal activators. The concentrations of dGTP employed were: 0  $\mu\text{M}$ , 0.25  $\mu\text{M}$ , 1  $\mu\text{M}$ , 2.5  $\mu\text{M}$ , and 25 mM. Quantitation was done by measuring the Cerenkov radiation corresponding to the  $^{32}\text{P}$ -labeled P8-dGMP band, and by densitometry of the autoradiographs.

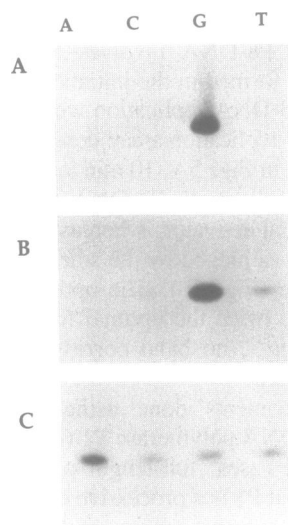
### Metal activation of P8-DNA replication

The replication of P8-DNA involves the elongation of the P8-dGMP complex formed in the initiation step. The metal ion requirements for P8-DNA replication were studied with  $\text{Mg}^{2+}$  and  $\text{Mn}^{2+}$  using the replication assay described in Materials and Methods. As shown in Fig. 5A, 10 min incubation at 37°C were sufficient to reach full-length P8-DNA synthesis when  $\text{Mg}^{2+}$  was used as the metal activator, whereas  $\text{Mn}^{2+}$  appeared to be much less efficient, as judged by the smear seen after a 25-fold longer gel exposure (Fig. 5B). The optimal concentration for  $\text{Mg}^{2+}$  was 10 mM, twice the optimal for initiation. With 0.2 mM to 20 mM  $\text{Mn}^{2+}$ , no band corresponding to full-length P8-DNA was detected.

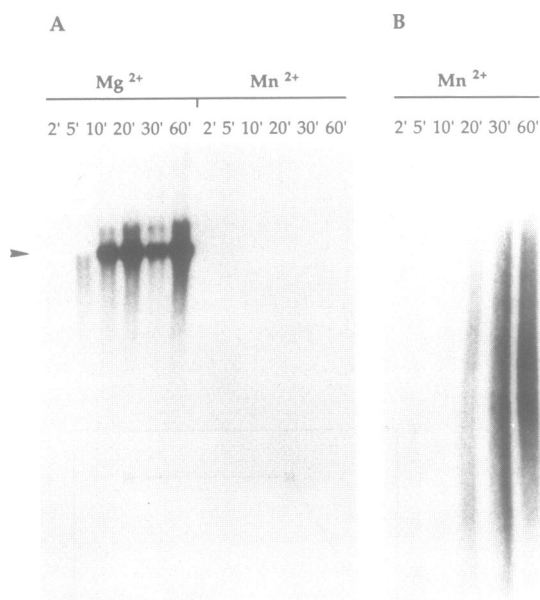
Replication experiments done using  $\text{Mg}^{2+}$  and variable amounts of PRD1 DNA polymerase P1 (from 34 ng to 0.34 ng) showed that, in all cases, full-length products were formed, clearly indicating that P1 is a processive enzyme. There was the possibility that the negative effects of  $\text{Mn}^{2+}$  were due to an alteration of the insertion reaction itself, the processivity of the polymerase, or both. To address these questions, filling-in assays



**Figure 3.** DNA-independent initiation reaction. (A): The assay was carried out in the absence of P8-DNA under the standard conditions described in Materials and Methods, except that 1  $\mu\text{M}$  ( $\alpha\text{-}^{32}\text{P}$ )dGTP (10  $\mu\text{Ci}$ ) and an incubation time of 3 h at 30°C was used. Particular components were omitted as indicated in the lower part of the figure. (B): The DNA-independent formation of the P8-dGMP complex was assayed under standard conditions with an incubation time of 3 h, using  $\text{Mn}^{2+}$  as the metal activator. The concentrations of dGTP employed were: 2.5  $\mu\text{M}$ , 25  $\mu\text{M}$ , 100  $\mu\text{M}$  and 250  $\mu\text{M}$ . Quantitation was done by measuring the Cerenkov radiation corresponding to the  $^{32}\text{P}$ -labeled P8-dGMP band, and by densitometry of the autoradiographs.

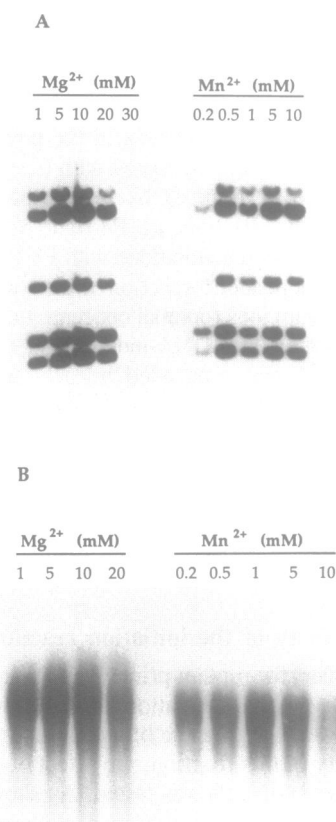


**Figure 4.** Nucleotide specificity of the initiation reaction. The formation of the P8-dNMP complex was assayed under standard conditions, with an incubation time of 10 min. Assays were carried out using P8-DNA as template, either with  $Mg^{2+}$  (A) or  $Mn^{2+}$  (B), or in the absence of P8-DNA with  $Mn^{2+}$  (C). Lanes A, C, G and T indicate assays performed in the presence of the corresponding dNTP. The exposure times of panels (A) and (C) are 8-fold and 36-fold of that of panel (B), respectively.



**Figure 5.** Effects of  $Mg^{2+}$  and  $Mn^{2+}$  on the kinetics of P8-DNA replication. (A): The assays (initiation plus elongation) were done as described in Materials and Methods. The concentration of the metal activator was 10 mM ( $Mg^{2+}$ ) and 0.5 mM ( $Mn^{2+}$ ). The incubation times (min) at 37°C are indicated at the top. The position of the band corresponding to unit-length P8-DNA is marked with an arrowhead. (B) 25-fold exposure of the lanes corresponding to the  $Mn^{2+}$ -containing reactions shown in (A).

and elongation of poly (dC)<sub>250</sub>-oligo (dG)<sub>10</sub> were carried out. The filling-in assay makes use of *EcoRI*-treated  $\phi$ 29 DNA, providing information on the efficiency of the insertion reaction and overcoming possible effects on the processiveness of the



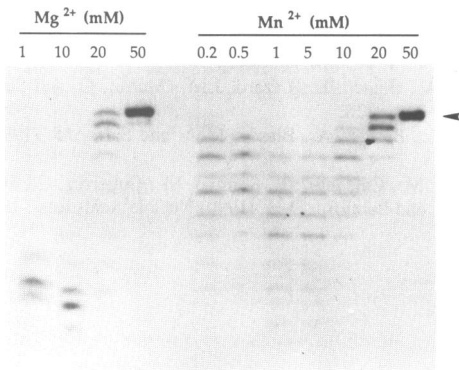
**Figure 6.** Effect of  $Mg^{2+}$  and  $Mn^{2+}$  on the insertion reaction and processivity of PRD1 DNA polymerase. (A): Filling-in reaction carried out with either  $Mg^{2+}$  or  $Mn^{2+}$ , and using *EcoRI*-treated  $\phi$ 29 DNA as template. The concentration of the metal is indicated in each case. (B): Elongation of poly (dC)<sub>250</sub>-oligo (dG)<sub>10</sub> with either  $Mg^{2+}$  or  $Mn^{2+}$ , at the concentrations indicated. The conditions for both assays are given in Materials and Methods.

reaction, since it can only support a very short elongation. The results of these experiments showed that polymerization did take place in the presence of either metal ion,  $Mg^{2+}$  being about 20 to 30% more efficient than  $Mn^{2+}$  (Fig. 6A). The optimal concentration of  $Mg^{2+}$  was 10 mM, identical to that found for the replication of P8-DNA. Interestingly, two concentrations of  $Mn^{2+}$ , 0.5 mM and 5 mM, were found to be the best in supporting this polymerization reaction.

The elongation of a longer substrate, such as poly (dC)<sub>250</sub>-oligo (dG)<sub>10</sub>, should provide some information on the processivity of P1 with the two metal activators. As seen in Fig. 6B, both metal ions could support the elongation of the oligo (dG)<sub>10</sub> primer, with products ranging from 0.6 to 1.5 kb in length. This indicates that  $Mn^{2+}$  does not drastically affect the processivity of protein P1. The optimal concentration for this assay was 10 mM and 5 mM for  $Mg^{2+}$  and  $Mn^{2+}$ , respectively.

#### Effect of the metal ion on the 3'-5' exonuclease activity of the PRD1 DNA polymerase

Protein P1, as many other DNA polymerases, has a 3'-5'-exonuclease activity on ssDNA that is most probably used in the correction of misincorporated nucleotides (19). This



**Figure 7.** Effect of  $Mg^{2+}$  and  $Mn^{2+}$  on the 3'-5' exonucleolytic activity of PRD1 DNA polymerase. The assay was carried out as described in Materials and Methods, using the 15-mer single-stranded oligonucleotide SP1 as substrate. The concentrations of either  $Mg^{2+}$  or  $Mn^{2+}$  are indicated at the top, and the position of the SP1 band marked with an arrowhead.

exonuclease activity was assayed, in the presence of either  $Mg^{2+}$  or  $Mn^{2+}$ , using the 5'- $^{32}P$ -labeled SP1 oligonucleotide. As seen in Fig. 7, already at 1 mM nearly all the substrate was used and the extent of degradation, measured as the length reduction of the original oligonucleotide, was also very large. In the case of  $Mn^{2+}$ , however, it was evident that degradation was less efficient at the different concentrations tested, with a decrease in the processiveness of the reaction with respect to that using  $Mg^{2+}$  (Fig. 7).

## DISCUSSION

Several studies have shown that various metals can act as activators of DNA polymerases, although the extent and efficiency of this activation varies from system to system (24–27). This observation has also been made in the case of protein-primed replication. With adenovirus, it had been shown that the initiation of DNA replication required  $Mg^{2+}$ , and significant stimulation was observed with  $Mn^{2+}$  in a system using adenovirus subviral particles as template and partially purified proteins (28,29). In the case of phage  $\phi 29$ , it has been recently shown that, in addition to  $Mg^{2+}$ , also  $Mn^{2+}$ ,  $Fe^{2+}$ ,  $Co^{2+}$ , and, to a lower extent,  $Cd^{2+}$ , are able to activate the  $\phi 29$  DNA polymerase (16).  $Mn^{2+}$  was by far the best activator of the initiation reaction, with a 50 to 100-fold stimulation compared to  $Mg^{2+}$  (16). In the case of bacteriophage PRD1, we found that, in addition to  $Mg^{2+}$ , also  $Mn^{2+}$  and  $Co^{2+}$  were able to support the formation of the P8-dGMP initiation complex. The activation obtained with  $Co^{2+}$  was practically identical to that of  $Mg^{2+}$ , whereas  $Mn^{2+}$  had a 6 to 10-fold stimulatory effect relative to  $Mg^{2+}$  on the formation of the initiation complex.

To our knowledge, no data are available on the molecular basis of the stimulation by  $Mn^{2+}$  in the case of adenovirus. In the case of phage  $\phi 29$  it has been shown that this metal acts mainly by increasing the affinity of the DNA polymerase for dATP, with a 20-fold decrease in the  $K_M$  of the reaction relative to that catalyzed by  $Mg^{2+}$ . In addition, a moderate increase of the  $V_{max}$  of the reaction was also observed (16). The experiments reported in this study indicate that, in the case of phage PRD1,  $Mn^{2+}$  does not affect the affinity of the DNA polymerase for dGTP, but increases the  $V_{max}$  of the reaction.

Earlier observations suggested that several DNA polymerases can add one nucleotide to the 3'-ends of dsDNA in the absence of any template information (30,31). Very recently, it has been shown that the  $\phi 29$  DNA polymerase is able to catalyze the deoxynucleotidylation of the terminal protein in the absence of DNA (17). As reported here, in the presence of  $Mn^{2+}$ , PRD1 DNA polymerase can also catalyze the deoxynucleotidylation of the PRD1 terminal protein in the absence of P8-DNA. This DNA-independent reaction is 100 to 200-fold lower than that obtained in the presence of the template, and could not be detected with  $Mg^{2+}$  as the activating ion. This reaction, with a high  $K_M$  for dGTP, is stimulated by ammonium ions in a similar manner than with the  $\phi 29$  system (17). As in the latter case, ammonium ions are most probably necessary for the stable formation of an equimolar terminal protein-DNA polymerase complex (32). The fact that each of the four dNTPs can be incorporated is in agreement with the general view on DNA polymerization suggesting that the presence of the DNA template is necessary to stabilize the incorporation of the complementary nucleotide, probably increasing the residence time at its binding site (33).

Despite the stimulatory effect of  $Mn^{2+}$  on the initiation reaction, this metal ion failed to support P8-DNA replication. Differences between initiation and elongation with regard to metal requirements have been previously observed. In the case of adenovirus, no DNA replication was observed in the presence of  $Mn^{2+}$  (28). With  $\phi 29$ , the excellent initiation activator  $Mn^{2+}$  had a null, and even slightly deleterious effect on DNA replication (16). Interestingly, the detailed study on the  $\phi 29$  system provided clear evidence that the metal activator employed could not only affect the processivity of the DNA polymerase but also the insertion reaction itself (16). The fact that  $Mn^{2+}$  can act as an activator in both the filling-in assay and the elongation of poly(dC)<sub>250</sub>-oligo(dG)<sub>10</sub> indicates that the failure of this metal ion in activating P8-DNA replication does not reside in either rendering the polymerase unable to carry out the polymerization reaction, or substantially modifying the high processivity of protein P1, essential to P8-DNA replication. It should be noted that the initiation reaction carried out with  $Mn^{2+}$  results in an increase of the misincorporation of A, C, and T compared to the reaction done with  $Mg^{2+}$ . The impairment by  $Mn^{2+}$  of the 3'-5'-exonucleolytic activity of P1, described in this study, could account, at least in part, for the failure of P1 to replicate the genome in the presence of this metal ion. It should be also borne in mind that, as reported with  $\phi 29$ , most likely PRD1 DNA replication starts at both ends of the genome, and proceeds by a strand-displacement mechanism (34). Therefore, the inactivity in P8-DNA replication could be also explained by a deleterious effect of  $Mn^{2+}$  on the efficiency of the strand-displacement coupled to polymerization carried out by PRD1 DNA polymerase.

In this investigation we have assessed the feasibility of an *in vitro* system to study the replication of bacteriophage PRD1 DNA. Making use of this system, we have further characterized the phage DNA polymerase and been able to compare its properties to those of the corresponding and well characterized phage  $\phi 29$  enzyme. This information is essential to carry out the construction of PRD1/ $\phi 29$  chimaeric polymerases in order to define the functions of the proposed conserved regions observed in  $\alpha$ -type DNA polymerases (35,36). Moreover, the development and characterization of the *in vitro* replication system described in this work should allow the study of the roles played by other viral early proteins (6,37) in the overall process of DNA

replication as well as the study of the involvement of host factors in this process. The fact that bacteriophage PRD1 is able to replicate in *E. coli* makes this system very suitable for such type of studies.

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