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Metal Activation of Synthetic and Degradative Activities of ϕ29 DNA Polymerase, a Model Enzyme for Protein-Primed DNA Replication†

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ABSTRACT: Analysis of metal activation on the synthetic and degradative activities of ϕ29 DNA polymerase was carried out in comparison with T4 DNA polymerase and *Escherichia coli* DNA polymerase I (Klenow fragment). In the three DNA polymerases studied, both the polymerization and the 3′→5′ exonuclease activity had clear differences in their metal ion requirements. The results obtained support the existence of independent metal binding sites for the synthetic and degradative activities of ϕ29 DNA polymerase, according with the distant location of catalytic domains (N-terminal for the 3′→ 5′ exonuclease and C-terminal for DNA polymerization) proposed for both Klenow fragment and ϕ29 DNA polymerase. Furthermore, DNA competition experiments using ϕ29 DNA polymerase suggested that the main differences observed in the metal usage to activate polymerization may be the consequence of metal-induced changes in the enzyme–DNA interactions, whose strength distinguishes processive and nonprocessive DNA polymerases. Interestingly, the initiation of DNA polymerization using a protein as a primer, a special synthetic activity carried out by ϕ29 DNA polymerase, exhibited a strong preference for Mn^{2+} as metal activator. The molecular basis for this preference is mainly the result of a large increase in the affinity for dATP.

It has long been known that metal ions are required cofactors for the catalytic activities of DNA polymerases, i.e., DNA polymerization and exonuclease activity (Bessman et al., 1958; Lehman & Richardson, 1964). Mg^{2+}-activated catalysis has been the one most extensively studied, since, in general, it yields the highest activity. However, it has been demonstrated that other metal ions like Mn^{2+}, Co^{2+}, Ni^{2+}, or Zn^{2+} can also serve as activators for DNA polymerases in vitro (Sirover & Loeb, 1976; Burgers & Eckstein, 1979), being presently unknown which metal or metals are used in vivo.

Determination of metal ion requirements of DNA polymerases becomes complex by the fact that metals not only bind and activate DNA polymerases but also bind to DNA and dNTPs, leading to different template and substrate complexes depending on the metal ion and on its concentration (Murray & Flessel, 1976; Marzilli et al., 1980; Sigel, 1987).

The location of the polymerization active site of *Escherichia coli* DNA polymerase I Klenow fragment (pol I K) relies on biochemical and genetic studies (Joyce & Steitz, 1987; Polesky et al., 1990), as well as on recent X-ray crystallographic analysis showing dCTP bound to the enzyme; in this case, Gln-708 and Glu-710 are involved in metal ion binding (L. Beese, J. Friedman, and T. A. Steitz, personal communication). Furthermore, using a different approach, a single metal binding site has been detected in the presence of dGTP (Mullen et al., 1990). In the C-terminal domain of ϕ29 DNA polymerase and other α-like DNA polymerases from distantly related organisms, a highly conserved amino acid sequence motif (YGDTDS) has been proposed to be part of the metal binding site for polymerization (Argos, 1988; Bernad et al., 1990). However, the role for this metal in binding dNTPs and primer substrates and/or catalysis is not yet clear.

In the case of the 3′→ 5′ exonuclease activity, a detailed understanding of the structural basis of the metal ion requirements has been provided by crystallographic analysis of pol I K complexed with ssDNA (Freemont et al., 1988). The pol I K exonuclease active center contains two binding sites for metal ions: one metal ion (site A) is coordinated by the carboxylate groups of Asp-355, Glu-357, and Asp-501, the fourth ligand being the phosphate of the bond to be cleaved; a second metal ion (site B) is located between this phosphate and the carboxylate of Asp-424. Further support of the relevance of these residues involved in metal binding is the fact that they are conserved in many prokaryotic and eukaryotic DNA polymerases (Bernad et al., 1989). Thus, the putative residues involved in metal binding for both polymerase and exonuclease activities appear among the best conserved ones

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[1] Abbreviations: pol I K, *E. coli* DNA polymerase I Klenow fragment; ssDNA, single-stranded DNA; dsDNA, double-stranded DNA; p3–DNA, ϕ29 DNA–terminal protein complex; DTT, dithiothreitol; BSA, bovine serum albumin; SDS, sodium dodecyl sulfate.

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in DNA polymerases. By mutagenesis studies (Derbyshire et al., 1988), it has been established that metal A is mainly involved in substrate binding and metal B in catalysis of the 3′→ 5′ exonuclease reaction. The affinity of these two sites for Mn^{2+} has been studied quantitatively in solution (Mullen et al., 1990).

Here we report a study of the metal ion requirements of ϕ29 DNA polymerase, a model enzyme for protein-primed DNA replication (Salas, 1991), in comparison with T4 DNA polymerase and pol I K. These latter two DNA polymerases were selected taking into account their significant differences with ϕ29 DNA polymerase in terms of 3′→ 5′ exonuclease strength and processivity of DNA polymerization. ϕ29 and T4 DNA polymerases have a strong 3′→ 5′ exonuclease activity, whereas pol I K exhibits a weak one. Concerning the polymerization activity, ϕ29 DNA polymerase is a highly processive enzyme (Blanco et al., 1989), pol I K being a distributive one (Mizrahi et al., 1985). The processivity of T4 DNA polymerase is strongly dependent on the presence of accessory proteins (Jarvis et al., 1991). In addition, ϕ29 and T4 DNA polymerases belong to the same family (α-like DNA polymerases), whereas pol I K is less related to them, regarding the polymerization domain (Blanco et al., 1991).

In the three DNA polymerases, synthetic and degradative activities were proposed to reside in different domains of the enzyme (Freemont et al., 1988; Bernad et al., 1989; Derbyshire et al., 1988; Reha-Krantz, 1988), and, therefore, they could be expected to show different metal ion requirements. According to that, we have studied metal activation of both synthetic and degradative activities separately. We have also proposed that the special synthetic activity carried out by ϕ29 DNA polymerase, that is, the ability to use a protein as a primer (Salas, 1988), resides in the same domain as DNA polymerization (Bernad et al., 1990). Therefore, we have studied the metal ion requirements for this protein-primed initiation activity.

Materials and Methods

Nucleotides and Metal Salts. Unlabeled nucleotides were from Pharmacia P-L Biochemicals; [α-^{32}P]dATP (400 Ci/mmol) and [γ-^{32}P]ATP (3000 Ci/mmol) were obtained from Amersham International Plc. $MgCl_2$, $MnCl_2$, $ZnCl_2$, $FeCl_3$, and $CaCl_2$ were purchased from Merck, and $FeSO_4$, $CdCl_2$, $NiCl_2$, $BeSO_4$, $CuCl_2$, and $HgCl_2$ were from Aldrich.

Proteins. T4 polynucleotide kinase, T4 DNA polymerase, pol I K, and restriction enzyme *Eco*RI were purchased from New England Biolabs; fungal proteinase K was from Merck. ϕ29 DNA polymerase was purified as described (Blanco & Salas, 1984) from *E. coli* NF2690 cells harboring plasmid pLBw2 (Blanco et al., 1984). The exonuclease-deficient ϕ29 DNA polymerase (D12AD66A), obtained by site-directed mutagenesis (Bernad et al., 1989), was overproduced under the control of the T7 RNA polymerase ϕ10 promoter (Tabor & Richardson, 1985) in *E. coli* BL21 DE3 strain (Studier & Moffat, 1986) and purified essentially as described for the wild-type ϕ29 DNA polymerase. ϕ29 terminal protein (p3) was purified as described (Prieto et al., 1984) from *E. coli* N99λCI857 cells transformed with plasmid pKC30A1 (Garcia et al., 1983).

DNA Templates and Substrates. p3–DNA was obtained as described (Peñalva & Salas, 1982). ϕ29 DNA was obtained by treating phage particles with SDS and proteinase K (Inciarte et al., 1976). After proteinase K digestion, the DNA was phenol-extracted, ethanol-precipitated, and digested with *Eco*RI to generate fragments with 3′-recessive ends suitable as templates for DNA polymerase activity. M13mp2 ssDNA was obtained as described (Kunkel, 1985), and, when indicated, it was used as challenger DNA in the polymerization assay on *Eco*RI-digested ϕ29 DNA. As substrate for 3′→ 5′ exonuclease activity on dsDNA, *Eco*RI-digested ϕ29 DNA was 3′-labeled with [α-^{32}P]dATP in a partial filling-in reaction carried out by the exonuclease-deficient ϕ29 DNA polymerase (Bernad et al., 1989). The incubation mixture contained, in 250 μL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/mL BSA, 10 mM $MgCl_2$, 0.5 μM [α-^{32}P]dATP (50 μCi), 4.4 μg of *Eco*RI-digested ϕ29 DNA, and 250 ng of exonuclease-deficient ϕ29 DNA polymerase. After incubation for 30 min at 30 °C, the reaction mixture was phenol-extracted and filtered twice through Sephadex G-50 spun columns; the specific activity obtained was 2.6×10^5 cpm/pmol of 3′ end. For 3′→ 5′ exonuclease assays on ssDNA, a 15-mer oligonucleotide was prepared with a DNA synthesizer from Applied Biosystems as described (Strauss et al., 1986). The oligonucleotide was purified by electrophoresis in 20% polyacrylamide gels containing 8 M urea, ethanol-precipitated, and 5′-labeled with [γ-^{32}P]ATP and T4 polynucleotide kinase. Further separation by polyacrylamide gel electrophoresis was necessary to purify the full-size 5′-labeled oligonucleotide; the specific activity obtained was 1.6×10^4 cpm/pmol. 17-mer M13 forward sequencing primer was purchased from Amersham, and it was used in the primed-M13 DNA replication assay.

Nonprocessive DNA Polymerase Assay (Filling-In Reaction). The incubation mixture contained, in 25 μL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/mL BSA, 0.1 μM [α-^{32}P]dATP (400 Ci/mmol), 0.2 mM dGTP and dTTP, 0.5 μg of *Eco*RI-digested ϕ29 DNA as template, either 5 ng of ϕ29 DNA polymerase, 0.1 ng of pol I K, or 0.05 ng of T4 DNA polymerase, and the indicated type and concentration of metal ion. The samples were incubated for 2 min at 30 °C (these conditions were shown to be linear with time and enzyme amount). Reactions were stopped by adding 10 mM EDTA and 0.1% SDS; the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the excluded volume was counted (Cerenkov radiation) and analyzed by agarose gel electrophoresis and autoradiography. In some cases, and depending on the metal activator used, nonincorporated [α-^{32}P]dATP was present in the excluded fractions. In these cases, the activity values were corrected by densitometry of autoradiographs (nonincorporated radioactivity migrates separately from labeled DNA fragments, allowing a direct comparison with other fractions in which nonincorporated radioactivity did not appear in the excluded volume).

To analyze the association–dissociation of ϕ29 DNA polymerase from the *Eco*RI-digested ϕ29 DNA, a challenger experiment was performed, in which a mixture containing all the components of the final reactions, with the exception of the metal activator, was preincubated for 15 min at 4 °C to allow the formation of the DNA polymerase–template/primer complex. The reaction was initiated by addition of the metal activator and the challenger DNA (4 μg of M13 ssDNA), and it was allowed to proceed as indicated above. As controls, parallel reactions were performed in which the challenger DNA was mixed with the *Eco*RI-digested ϕ29 DNA before addition of ϕ29 DNA polymerase.

Processive DNA Polymerase Assay (Replication of Primed-M13 DNA). The incubation mixture contained, in 25 μL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/mL BSA, 20 μM each of dCTP, dGTP, dTTP, and [α-^{32}P]dATP (1 μCi), 0.5 μg of primed-M13mp2 ssDNA,

Table I: Summary of Metal Ion Requirements for the Three Catalytic Activities of ϕ29 DNA Polymerase[a]

	Mg^{2+}	Mn^{2+}	Co^{2+}	Zn^{2+}	Fe^{2+}	Cd^{2+}	Ni^{2+}	Fe^{3+}	Ca^{2+}	Be^{2+}	Cu^{2+}	Hg^{2+}
polymerization[b] (nonprocessive)	100	91	21	3	11	2	1	6	nd[e]	nd	nd	nd
initiation[c]	2	100	8	10	18	1	nd	nd	nd	nd	nd	nd
exonuclease[d]	92	100	18	20	9	2	3	10	nd	nd	nd	4

[a] DNA polymerase assay (*Eco*RI-digested ϕ29 DNA filling-in), initiation reaction (p3–dAMP complex formation), and 3′→5′ exonuclease activity (on dsDNA), catalyzed by ϕ29 DNA polymerase, were as described under Materials and Methods. The maximal value obtained for each activity was considered as 100%. [b] As metal activators, 10 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $CoCl_2$, 2 mM $ZnCl_2$, 0.5 mM $FeSO_4$, 2 mM $CdCl_2$, 10 mM $NiCl_2$, 2 mM $FeCl_3$, 10 mM $CaCl_2$, 1 mM $BeSO_4$, 1 mM $CuCl_2$, or 1 mM $HgCl_2$, shown to be the optimal ones, were used. [c] As metal activators, 10 mM $MgCl_2$, 1 mM $MnCl_2$, 0.5 mM $CoCl_2$, 2 mM $ZnCl_2$, 1 mM $FeSO_4$, or 2 mM $CdCl_2$, shown to be the optimal ones, were used. [d] As metal activators, 10 mM $MgCl_2$, 20 mM $MnCl_2$, 1 mM $CoCl_2$, 2 mM $ZnCl_2$, 0.5 mM $FeSO_4$, 2 mM $CdCl_2$, 2 mM $NiCl_2$, 2 mM $FeCl_3$, 10 mM $CaCl_2$, 1 mM $BeSO_4$, 1 mM $CuCl_2$, or 1 mM $HgCl_2$, shown to be the optimal ones, were used. [e] nd, not detected.

either 25 ng of ϕ29 DNA polymerase, 15 ng of T4 DNA polymerase, or 2 ng of pol I K, and the indicated type and concentration of metal ion. After incubation for the indicated times at 30 °C, the reactions were stopped by adding 10 mM EDTA and 0.1% SDS; the samples were filtered through Sephadex G-50 spun columns in the presence of 0.1% SDS, and the excluded volume was analyzed by alkaline 0.7% agarose gel electrophoresis and autoradiography.

Assay for the Formation of the p3–dAMP Complex. The incubation mixture contained, in 25 μL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/mL BSA, 20 mM ammonium sulfate, 0.1 μM [α-^{32}P]dATP (400 Ci/mmol), 0.5 μg of p3–DNA as template, 20 and 25 ng of purified ϕ29 DNA polymerase and terminal protein, respectively, and the indicated type and concentration of metal ion. After incubation for 2 min at 30 °C, the reaction was stopped by adding 10 mM EDTA and 0.1% SDS, and the samples were filtered as above. The excluded fractions were subjected to SDS electrophoresis in 10% polyacrylamide gels, and the p3–dAMP complex was detected by autoradiography. Quantitation was done by excising from the gel the radioactive band corresponding to the p3–dAMP complex and counting the Cerenkov radiation, or by densitometry of the autoradiograph. For the steady-state analysis of dATP K_M and V_{max} values, a 10-fold molar excess of p3 was used, and the reaction was measured at different times to obtain an accurate estimation of the reaction velocity.

Replication Assay (Initiation plus Elongation) with p3–DNA as Template. The incubation mixture contained, in 25 μL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/mL BSA, 20 mM ammonium sulfate, 20 μM each of dCTP, dGTP, dTTP, and [α-^{32}P]dATP (1 μCi), 0.5 μg of p3–DNA, 20 and 25 ng of purified ϕ20 DNA polymerase and terminal protein, respectively, and the indicated type and concentration of metal ion. After incubation for the indicated times at 30 °C, the reaction was stopped by adding 10 mM EDTA and 0.1% SDS and filtered as above, and the Cerenkov radiation was counted. The samples were subjected to alkaline 0.7% agarose gel electrophoresis and autoradiography.

3′→ 5′ Exonuclease Assay on dsDNA. The incubation mixture contained, in 25 μL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/mL BSA, either 20 ng of ϕ29 DNA polymerase, 0.5 ng of T4 DNA polymerase, or 10 ng of pol I K, and the indicated type and concentration of metal ion. 3′-labeled *Eco*RI-digested ϕ29 DNA [5.3×10^3 cpm (20.5 fmol of 3′ end)$^{-1}$ (32 ng)$^{-1}$], obtained as indicated above (see DNA Templates and Substrates), was used as substrate. After incubation for 5 min at 25 °C (20 min when pol I K was used), the reaction was stopped by adding 10 mM EDTA. Released dAMP was determined by poly(ethylenimine)–cellulose thin-layer chromatography and autoradiography; the chromatogram was developed with 0.15 M lithium formate, pH 3, conditions in which dAMP migrates whereas DNA remains at the origin. After autoradiography, quantitation was done by densitometry of the [^{32}P]dAMP spots relative to the total amount of ^{32}P-labeled substrate. These relative values were used to calculate the amount of released [^{32}P]dAMP.

3′→ 5′ Exonuclease Assay on ssDNA. The incubation mixture contained, in 12.5 μL, 50 mM Tris-HCl, pH 7.5, 1 mM DTT, 4% glycerol, 0.1 mg/mL BSA, 1.25 ng of ϕ29 DNA polymerase, and the indicated type and concentration of metal ion. As substrate, a 5′-labeled 15-mer oligonucleotide (2400 cpm/0.15 pmol/0.72 ng) was used. After incubation for 1.5 min at 25 °C, the reactions were directly quenched with 3 μL of gel loading buffer and analyzed by electrophoresis in a 20% polyacrylamide gel in the presence of 8 M urea, and autoradiography.

Results

Synthetic Activities

It has been reported that several metal ions can be used as activators of the polymerization activity of several DNA polymerases (Sirover & Loeb, 1976; Burgers & Eckstein, 1979); however, the optimal ion concentration as well as the efficiency of the metal-activated reaction varies substantially among different systems. Clearly, different reaction conditions can account, at least partially, for the heterogeneity of these results, and presumably, the nature of the DNA substrate is one of the most relevant factors. This is especially true when several DNA polymerases with different replication properties are compared. In this case, the metal ion requirements may reflect the efficiency of the polymerization reaction, as well as the efficiency of template usage. To achieve a comprehensive understanding of the polymerization reaction in the presence of different metals, we have studied the synthetic activity of ϕ29 DNA polymerase in comparison with T4 DNA polymerase and pol I K (clearly different in processiveness and primer usage) in two kinds of assays: filling-in reaction on *Eco*RI-digested ϕ29 DNA (a nonprocessive assay) and replication of primed-M13 DNA (an assay in which a processive replication is allowed to occur).

Nonprocessive DNA Polymerization. The filling-in assay (see Materials and Methods) is especially useful to get some insight into the efficiency of the insertion reaction, due to the short elongation reaction it can support, allowing it to overcome the effects of metal ions on DNA polymerase processiveness (Hohn & Grosse, 1987). The ability of several metal ions to activate polymerization by ϕ29 DNA polymerase was tested. As shown in Table I, Mg^{2+} and Mn^{2+} yielded the highest activities. Other metal ions, like Co^{2+} and Fe^{2+}, were also able to activate ϕ29 DNA polymerase, the activity obtained at their optimal concentration being 21% and 11%, respectively, of the Mg^{2+}-activated reaction.

The fact that Mn^{2+} can substitute for Mg^{2+} in DNA replication assays has been widely described; however, the ion concentration and the efficiency of the Mn^{2+}-activated reaction varied substantially when different systems were used (Hohn

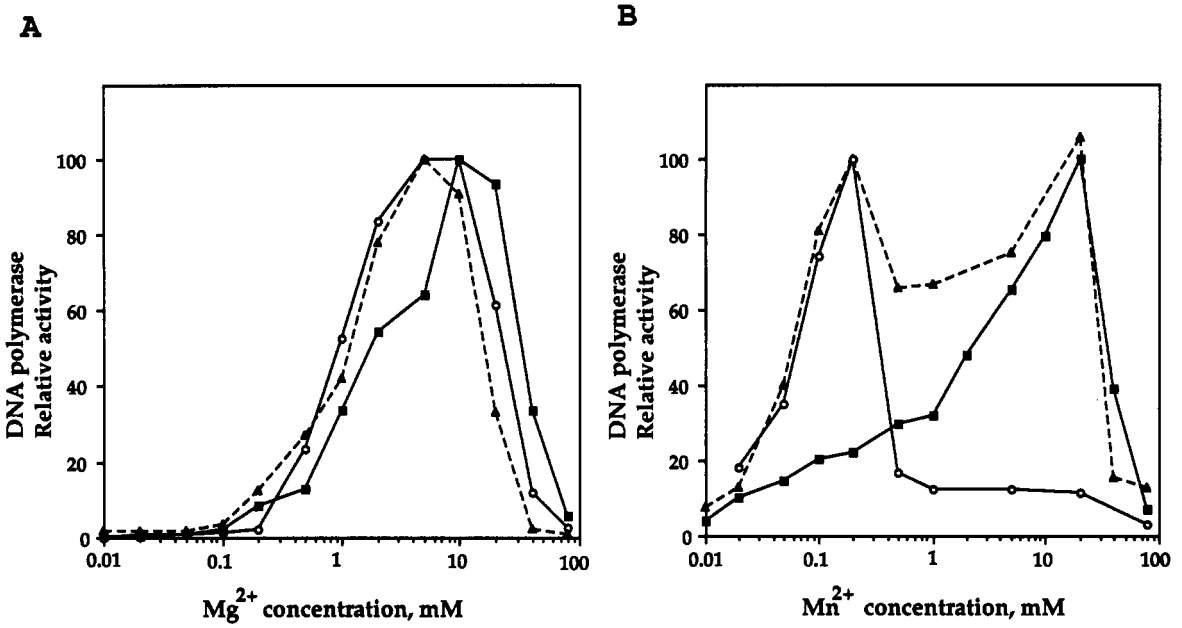

FIGURE 1: Effect of Mg^{2+} and Mn^{2+} concentration on ϕ29 DNA polymerase, T4 DNA polymerase, and pol I K in nonprocessive DNA polymerization. DNA polymerase assay was as described under Materials and Methods, using ϕ29 DNA polymerase (■), pol I K (○), or T4 DNA pol (▲). As metal activators, $MgCl_2$ (A) or $MnCl_2$ (B) at the indicated concentrations were used. The maximal values obtained with each DNA polymerase, considered as 100%, were as follows: ϕ29 DNA polymerase, 37.5 fmol of incorporated [α-^{32}P]dAMP at 10 mM $MgCl_2$ and 35.6 fmol at 20 mM $MnCl_2$; T4 DNA polymerase, 50 fmol at 5 mM $MgCl_2$ and 6.5 fmol at 20 mM $MnCl_2$; pol I K, 25 fmol at 10 mM $MgCl_2$ and 7.8 fmol at 0.2 mM $MnCl_2$.

& Grosse, 1987; Sirover et al., 1979; Kunkel & Loeb, 1979; El-Deiry et al., 1984, 1988; Tabor & Richardson, 1989). In an attempt to rationalize these differences, we have comparatively assayed the polymerization activity of ϕ29 DNA polymerase, T4 DNA polymerase, and pol I K over a wide range of concentrations of each metal ion. Whereas the three DNA polymerases shared roughly the same Mg^{2+} concentration requirements, the optimal concentration being around 10 mM (Figure 1A; see also Table II), the Mn^{2+} requirements were very different: the Mn^{2+} concentration needed to give an optimal reaction with ϕ29 DNA polymerase differed by 2 orders of magnitude from the optimal concentration with pol I K (Figure 1B; see also Table II); T4 DNA polymerase showed bimaximal activity, sharing the two maxima obtained with ϕ29 DNA polymerase and pol I K. The data obtained showed another relevant difference between ϕ29 DNA polymerase and the other two polymerases: the amount of reaction obtained with ϕ29 DNA polymerase at the optimal Mn^{2+} concentration (20 mM) was similar to the one obtained at the optimal Mg^{2+} concentration (10 mM) ($[Mn]_{opt}$ reaction/$[Mg]_{opt}$ reaction $\approx$ 1) (Figure 1, legend; see also Table II), whereas with T4 DNA polymerase and pol I K, the reaction obtained at the optimal Mn^{2+} concentration was low, relative to the Mg^{2+}-driven reaction ($[Mn]_{opt}$ reaction/$[Mg]_{opt}$ reaction $\approx$ 0.1 for the T4 DNA polymerase and 0.3 for pol I K) (Figure 1, legend; see also Table II).

Therefore, the polymerization activity of ϕ29 DNA polymerase is characterized by a high optimal Mn^{2+} concentration and by an efficient Mn^{2+}-activated reaction. As discussed below, the efficiency of template binding and dissociation (as a way to utilize new template/primer molecules) are important factors to give a maximal reaction on this kind of nonprocessive assay, due to the few catalytic reactions supported by each template molecule. Thus, we could not rule out the possibility that the high metal concentration required for this nonprocessive assay by ϕ29 DNA polymerase [a highly processive enzyme (Blanco et al., 1989)] might be related to template binding and/or dissociation. In fact, ϕ29 DNA polymerase does not dissociate easily from DNA even at the DNA terminus (unpublished results).

Table II: Summary of Metal Ion Requirements for ϕ29 DNA Polymerase, T4 DNA Polymerase, and Pol I K Catalytic Activities[a]

		ϕ29 DNA pol	T4 DNA pol	pol I K
$[Mg^{2+}]_{opt}$ (mM)	initiation	5–10		
	nonproc polym	10	5	5–10
	proc polym	10	20	5
	exonuclease	20	5–10	10–20
$[Mn^{2+}]_{opt}$ (mM)	initiation	0.5–1		
	nonproc polym	20	(0.2, 20)	0.2
	proc polym	0.3	0.1	0.1
	exonuclease	10–20	10–20	1–2
$[Mn^{2+}]_{opt}$/ $[Mg^{2+}]_{opt}$	initiation	100		
	nonproc polym	1	0.1	0.3
	proc polym	0.5	7.8	0.2
	exonuclease	1.1	1	1.6

[a] The initiation reaction (p3–dAMP complex formation), catalyzed by ϕ29 DNA polymerase, was assayed as described under Materials and Methods; the data correspond to the experiments shown in Figure 4. Nonprocessive polymerization (nonproc polym) (*Eco*RI-digested ϕ29 DNA filling-in), processive polymerization (proc polym) (oligonucleotide-primed M13 DNA replication), and 3′-5′ exonuclease (on dsDNA), catalyzed by ϕ29 DNA polymerase, T4 DNA polymerase, and pol I K, were assayed as described under Materials and Methods. The indicated data correspond to the experiments shown in Figures 1, 3, and 6.

To evaluate how many polymerization events are mediated by dissociation of ϕ29 DNA polymerase from a filled-in primer/template molecule and reassociation to another nonfilled one, and how this process is affected by metal concentration, we performed the reaction with an 8-fold molar excess of primer/template over ϕ29 DNA polymerase at the optimal concentration and at one suboptimal metal concentration in the presence of a challenger DNA (see Materials and Methods). M13 ssDNA was chosen as challenger DNA due to its strong efficiency to bind ϕ29 DNA polymerase (unpublished results). Thus, when the challenger DNA and template/primer were simultaneously added, no labeling of template/

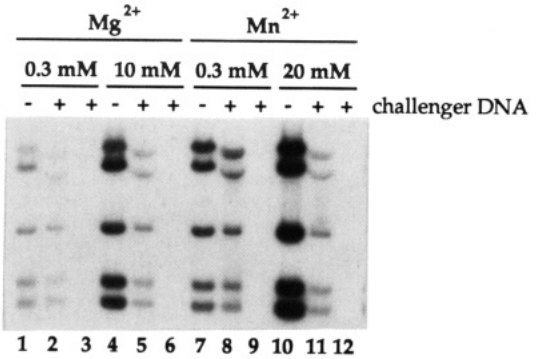

FIGURE 2: Challenger DNA assay showing ϕ29 DNA polymerase/template dissociation and reassociation. The filling-in reaction was performed as described under Materials and Methods. The metal activators and their concentrations are indicated. Lanes 1, 4, 7, and 10, reactions without challenger DNA. Lanes 2, 5, 8, and 11, the challenger DNA was added just when the reaction started by addition of the metal ion. Lanes 3, 6, 9, and 12, the challenger DNA was present before the reaction started.

primer molecules occurred (Figure 2, lanes 3, 6, 9, and 12). When ϕ29 DNA polymerase was allowed to interact with the template/primer substrate, and the reaction was started by addition of the metal activator and the challenger DNA, a clear reaction was observed (Figure 2, lanes 2, 5, 8, and 11). With this experimental design, if dissociation of the ϕ29 DNA polymerase–template complex occurs, all the enzyme molecules will become bound to the challenger DNA and not to another template/primer molecule, limiting the reaction to a single enzyme–DNA binding event. As controls, the reaction yield obtained in the absence of challenger DNA is shown in lanes 1, 4, 7, and 10. The presence of the challenger DNA did not strongly affect the reaction yield obtained at the low metal concentration (compare lanes 1 and 2, 35% inhibition; lanes 7 and 8, 30% inhibition); however, at the optimal (high) metal concentration, the reaction was severely decreased by the presence of the challenger DNA (compare lanes 4 and 5, 75% inhibition; lanes 10 and 11, 85% inhibition). Therefore, it can be concluded that the dissociation process is favored at high metal concentration, the optimal reaction obtained at the high metal concentration being mainly the result of multiple enzyme–DNA association events. Consequently, the low reaction obtained at the suboptimal metal concentration is mainly due to the fact that the reaction is limited to a single enzyme–DNA association event.

Processive DNA Polymerization. ϕ29 DNA polymerase has been described as a highly processive enzyme (Blanco et al., 1989), in contrast with T4 DNA polymerase (Jarvis et al., 1991) and pol I K (Mizrahi et al., 1985), which present moderate processivity. Thus, these polymerases were assayed for primed-M13 DNA replication over a wide range of Mg^{2+} or Mn^{2+} concentrations, in order to investigate the effect of these metal ions on their intrinsic processiveness. Considering the previous results, metal-induced dissociation of DNA polymerase–template complexes (occurring not only at the DNA terminus) is expected to strongly affect processive DNA polymerization. As shown in Table II, the optimal Mg^{2+} concentration in this assay was similar in all cases to the one observed in the nonprocessive assay, but significant differences were obtained for ϕ29 DNA polymerase in the presence of Mn^{2+}; in this case, a strong inhibition was observed at Mn^{2+} concentrations higher than 0.3 mM (results not shown). Therefore, low Mn^{2+} concentrations seem to be needed for a processive DNA polymerization.

A direct comparison between ϕ29 DNA polymerase, T4 DNA polymerase, and pol I K at their optimal Mg^{2+} and Mn^{2+}

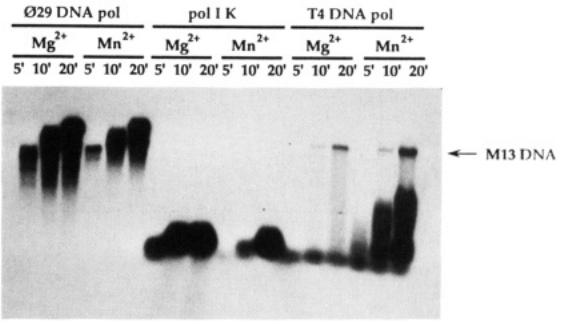

FIGURE 3: Effect of Mg^{2+} and Mn^{2+} on primed-M13 DNA replication catalyzed by ϕ29 DNA polymerase, T4 DNA polymerase, and pol I K. DNA polymerase assay was as described under Materials and Methods. Mg^{2+} and Mn^{2+} were used as metal activators at the optimal concentration for each DNA polymerase: 10 mM Mg^{2+} and 0.3 mM Mn^{2+} for ϕ29 DNA polymerase, 5 mM Mg^{2+} and 0.1 mM Mn^{2+} for pol I K, and 20 mM Mg^{2+} and 0.1 mM Mn^{2+} for T4 DNA polymerase. The incubation was performed at 30 °C for the indicated times. The migration position of unit-length M13 DNA is indicated by arrows. (The unit-length band observed in the lanes corresponding to T4 DNA polymerase is the result of a terminal labeling of linearized molecules.)

concentrations for this processive assay is shown in Figure 3. It is interesting to note that Mn^{2+} is a better activator for T4 DNA polymerase than Mg^{2+}, just the inverse behavior to that obtained in the nonprocessive assay, whereas for pol I K and ϕ29 DNA polymerase, the ratios between the Mn^{2+}-activated reaction and the Mg^{2+}-activated one are quite similar in the nonprocessive and processive assays (see Figure 1 legend and Table II).

Metal Activation of the Initiation Activity of ϕ29 DNA Polymerase. A specific reaction carried out by ϕ29 DNA polymerase is the formation of a covalent linkage between the viral terminal protein and 5′dAMP [reviewed in Salas (1991)]. This activity, also observed in other replication systems like adenovirus (Stillman, 1983), phage Cp-1 (Garcia et al., 1986), and phage PRD1 (Bamford & Mindich, 1984), involves the formation of a phosphoester bond between the α-phosphorus group of an incoming nucleotide and the OH group of a specific amino acid residue in the terminal protein. Thus, this protein-primed initiation reaction provides the first 5′-nucleotide at both ends as donor of the 3′-OH group needed to start elongation until complete replication of both strands is achieved. 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 elongation steps.

The protein-primed initiation activity of this class of DNA polymerases has been studied in detail with ϕ29 and adenovirus-purified proteins, and in both cases, a strict requirement for metal ions was reported. In vitro initiation of DNA replication has been described to require Mg^{2+} as metal activator (Lichy et al., 1981; Blanco & Salas, 1985a), although Mn^{2+} was shown to significantly stimulate the initiation reaction using adenovirus subviral particles as template and partially purified proteins (Leith et al., 1989). Site-directed mutagenesis studies on the putative polymerization active site of ϕ29 DNA polymerase (Bernad et al., 1990; Salas et al., 1988; Blanco et al., 1991) indicated a similar location of both activities, protein-primed initiation and DNA polymerization, in the primary structure of the enzyme. Therefore, it was of interest to examine the metal ion requirements of ϕ29 DNA polymerase for this particular synthetic activity, in comparison with its requirements for DNA polymerization.

Surprisingly, Mg^{2+} was one of the poorest activators of the initiation reaction, Mn^{2+} being about 50-fold more efficient

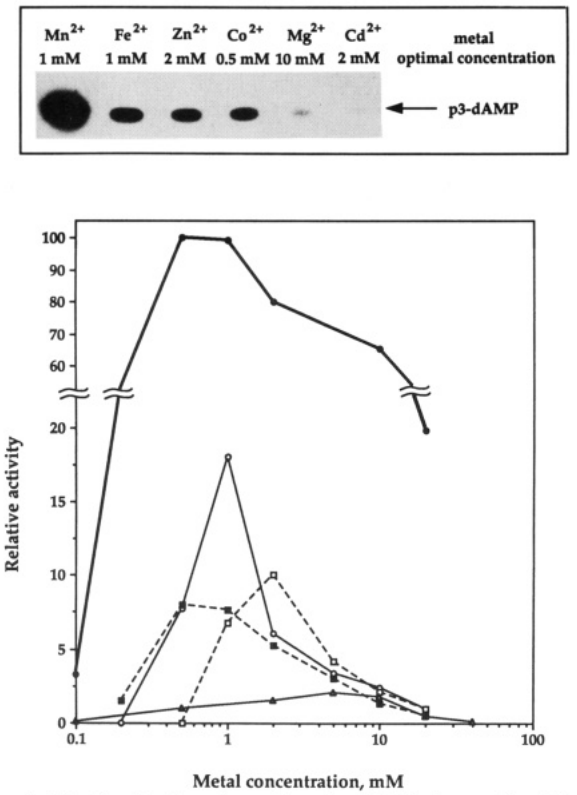

FIGURE 4: Metal activation of protein-primed initiation catalyzed by ϕ29 DNA polymerase. The metal requirement for the formation of the p3–dAMP complex (initiation reaction) was assayed as described under Materials and Methods. (A) SDS–polyacrylamide gel electrophoresis analysis of p3–[^{32}P]dAMP formed in the presence of $MnCl_2$, $FeSO_4$, $ZnCl_2$, $CoCl_2$, $MgCl_2$, or $CdCl_2$, at the indicated concentrations. The p3–dAMP band was excised from the gel and quantitated as described under Materials and Methods. The absolute values were 14.7 fmol (Mn^{2+}), 3.1 fmol (Fe^{2+}), 2.1 fmol (Zn^{2+}), 1.7 (Co^{2+}), 0.3 fmol (Mg^{2+}), and 0.2 fmol (Cd^{2+}). (B) Effect of metal ion concentration on p3–dAMP formation. The metal activators used were Mn^{2+} (●), Fe^{2+} (○), Zn^{2+} (□), Co^{2+} (■), and Mg^{2+} (△). The maximal value obtained with Mn^{2+} was considered 100% [see (A) for absolute values].

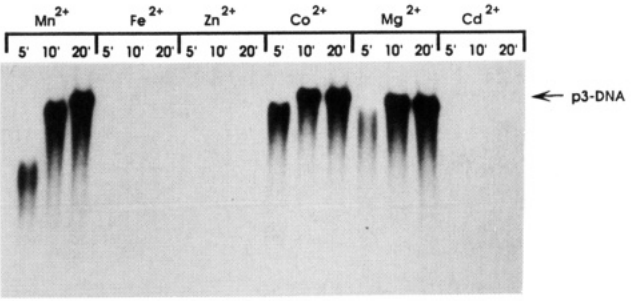

FIGURE 5: Analysis of metal activators in the replication of p3–DNA by ϕ29 DNA polymerase. Analysis by alkaline agarose gel electrophoresis of DNA synthesized by ϕ29 DNA polymerase using ϕ29 terminal protein as primer and p3–DNA as template, as indicated under Materials and Methods, in the presence of 1 mM $MnCl_2$, 1 mM $FeSO_4$, 2 mM $ZnCl_2$, 0.5 mM $CoCl_2$, 10 mM $MgCl_2$, or 2 mM $CdCl_2$ as metal activators. The incubation times are indicated. The migration position of unit-length p3–DNA is indicated by an arrow.

than Mg^{2+} (Figure 4A; see also Table I). Other activators of this reaction were Fe^{2+} (9-fold more efficient than Mg^{2+}), Zn^{2+} (5-fold), Co^{2+} (4-fold), and Cd^{2+} (0.5-fold). These values correspond to the optimal concentration for each metal ion (Figure 4B). The relative efficiency of these metals in the initiation reaction (see Table I) is notably different from that in the polymerization reaction (especially the large activity supported by Mn^{2+}). On the other hand, it is important to note the broad range of Mn^{2+} concentration (0.5–10 mM) able to activate the initiation reaction without a significant decrease in activity (Figure 4B), supporting the idea of a good tolerance of ϕ29 DNA polymerase for this metal in assays which do not require processivity.

The strong activation of the initiation reaction obtained in the presence of Mn^{2+} led us to further investigate the molecular basis of this effect. To check the possibility that Mn^{2+} was affecting the K_M or V_{max} values of some of the ligands or substrates involved, protein-primed initiation was analyzed in steady-state conditions. The Mn^{2+}-driven reaction/Mg^{2+}-driven reaction was kept roughly constant from limiting to saturating levels of ϕ29 DNA polymerase, terminal protein, and p3–DNA (results not shown). On the contrary, Mn^{2+} produced a 25-fold increase in the affinity for dATP with respect to Mg^{2+} (the K_M value was 0.2 μM with Mn^{2+} versus 5.3 μM with Mg^{2+}). Mn^{2+} also produced a moderate increase of V_{max} with respect to Mg^{2+} (6.8 fmol/min with Mn^{2+} versus 1.8 fmol/min with Mg^{2+}). The efficiency of nucleotide insertion can be estimated as V_{max}/K_M, and, therefore, the Mn^{2+} versus Mg^{2+} activation ratio can be expressed as $f(Mn/Mg) = [(V_{max}/K_M)_{Mn}]/[(V_{max}/K_M)_{Mg}]$. Using this parameter, it can be concluded that Mn^{2+} is about a 100-fold better activator of the initiation reaction than Mg^{2+}.

Metal Activation of p3–DNA Replication. Replication of p3–DNA involves protein-primed initiation at both DNA ends and subsequent elongation of the initiation complex to produce full-length ϕ29 DNA (Blanco & Salas, 1985a). ϕ29 DNA polymerase and free terminal protein (acting as a primer) are the only proteins needed to carry out this process due to the fact that ϕ29 DNA polymerase is a highly processive enzyme and able to produce strand displacement (Blanco et al., 1989). The metal ion requirements for p3–DNA replication were studied using only those metals that were active in the initiation reaction. As shown in Figure 5, the elongation rate, determined as an increase in the length of labeled DNA, was very similar in the presence of Co^{2+} and Mg^{2+}, being a little slower with Mn^{2+}; Fe^{2+}, Zn^{2+}, and Cd^{2+} were completely inactive, despite the fact that Fe^{2+} and Zn^{2+} were good activators of the initiation reaction. However, we cannot rule out the possibility that the optimal concentration of these metals for the initiation reaction may be toxic for a processive DNA replication. On the other hand, it is important to note that the high efficiency of the Mn^{2+}-activated initiation is not reflected in this replication assay. This is likely due to the high nucleotide concentration at which the p3– DNA replication assay is carried out (20 μM). This concentration is high enough to achieve a maximal velocity of the initiation reaction.

Degradative Activities

Metal Activation of the 3′→ 5′ Exonuclease Activity. Like the majority of DNA-dependent DNA polymerases, ϕ29 DNA polymerase has a 3′→ 5′ exonuclease activity (Watabe et al., 1984; Blanco & Salas, 1985b). In the case of pol I K, it has been shown that this activity resides in a different domain from the polymerase activity (Freemont et al., 1988; Derbyshire et al., 1988). On the basis of conservation of the critical amino acid residues forming the 3′→ 5′ exonuclease active site, and site-directed mutagenesis studies, the same structural arrangement has been proposed for ϕ29 DNA polymerase (Bernad et al., 1989; Blanco et al., 1991) and for T4 DNA polymerase (Reha-Krantz, 1989; Blanco et al., 1991). The physical separation between the two domains and the specificity of DNA binding in each domain (dsDNA for polym-

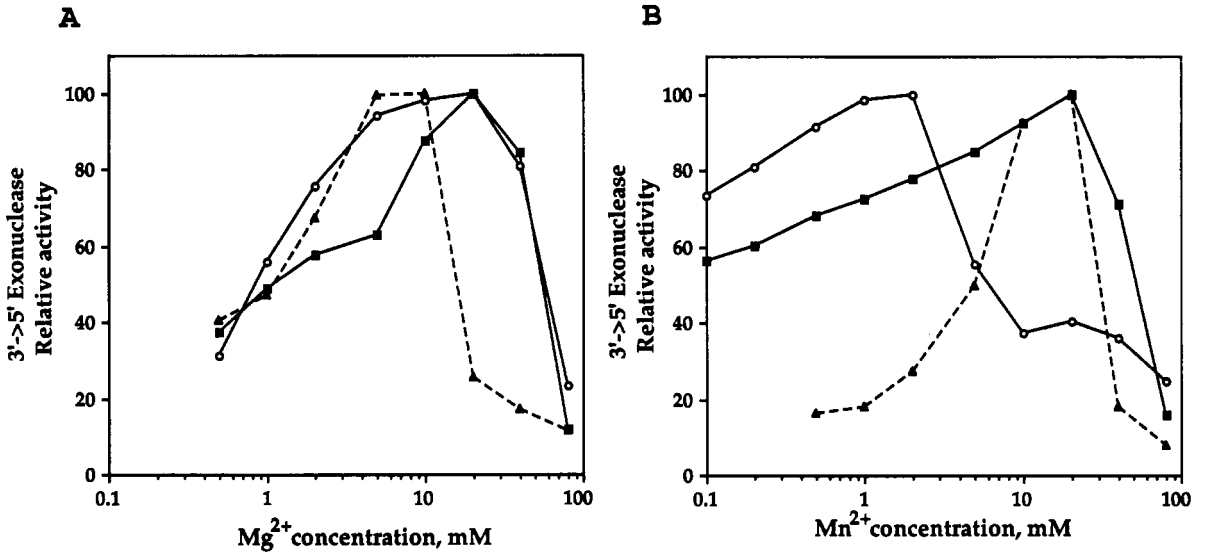

FIGURE 6: Effect of Mg^{2+} and Mn^{2+} concentration on the 3′→ 5′ exonuclease activity on dsDNA of ϕ29 DNA polymerase, T4 DNA polymerase, and pol I K. 3′→ 5′ exonuclease activity on dsDNA was assayed and quantitated as described under Materials and Methods, using ϕ29 DNA polymerase (■), pol I K (○), or T4 DNA polymerase (▲). As metal activators, $MgCl_2$ (A) or $MnCl_2$ (B) were used at the indicated concentrations. The maximal values obtained with each DNA polymerase, considered as 100%, were the following: ϕ29 DNA polymerase, 9.2 fmol of [^{32}P]dAMP released at 20 mM $MgCl_2$ and 9.7 fmol at 20 mM $MnCl_2$; T4 DNA polymerase, 6.1 fmol at 10 mM $MgCl_2$ and 5.8 fmol at 20 mM $MnCl_2$; pol I K, 8.2 fmol at 20 mM $MgCl_2$ and 12.7 fmol at 2 mM $MnCl_2$.

erization and ssDNA for exonuclease) led to the proposition of the molecular basis of proofreading as a mechanism involving melting of the DNA to allow the 3′ terminus to bind to the exonuclease active site (Freemont et al., 1988; Cowart et al., 1989), located about 25 Å apart according to the three-dimensional structure of pol I K. In agreement with this model, a template/primer molecule can be used as substrate for 3′→ 5′ exonucleolysis. Therefore, the same *Eco*RI-digested DNA used in the polymerase assay, labeled at its 3′ end (see Materials and Methods), was used as substrate to analyze the metal activation of the 3′→ 5′ exonuclease of ϕ29 DNA polymerase, in comparison with T4 DNA polymerase and pol I K.

The 3′→ 5′ exonuclease activity of ϕ29 DNA polymerase was assayed using different metal ions as activators. As shown in Table I, Mg^{2+} and Mn^{2+} were the best activators, whereas other metal ions (Co^{2+}, Zn^{2+}, Fe^{2+}, Fe^{3+}) yielded between 10% and 20% of the Mg^{2+}- and Mn^{2+}-activated reactions. Figure 6A shows that, as in the case of DNA polymerization, the 3′→ 5′ exonuclease activity of ϕ29 DNA polymerase, T4 DNA polymerase, and pol I K shared roughly similar Mg^{2+} requirements, the optimal concentration being around 10–20 mM. However, they showed different requirements of Mn^{2+} concentration (Figure 6B): the Mn^{2+} concentration needed to give an optimal reaction with ϕ29 DNA polymerase and T4 DNA polymerase (20 mM) differed by 1 order of magnitude from the optimal concentration with pol I K (1–2 mM). On the other hand, the amount of reaction obtained at the optimal Mn^{2+} concentration was similar to the one obtained at the optimal Mg^{2+} concentration in all three DNA polymerases ($[Mn]_{opt}$ reaction/$[Mg]_{opt}$ reaction = 1.1 for ϕ29 DNA polymerase, 0.9 for T4 DNA polymerase, and 1.5 for pol I K) (Figure 6 legend). Having used the same substrate in the polymerase and in the exonuclease assays, it can be concluded that these two activities have different metal ion requirements for their catalytic functions (see also Table II).

The exonuclease activity of ϕ29 DNA polymerase is characterized by its high processivity (Garmendia et al., 1992), in contrast with the exonuclease activites of T4 DNA polymerase (Garmendia et al., 1992) and pol I K (Joyce, 1989). Therefore, in the case of ϕ29 DNA polymerase, the effects of metal ion in substrate binding and in catalytic efficiency can be precisely discriminated. Thus, we assayed the exonuclease activity of ϕ29 DNA polymerase, in the presence of Mg^{2+} and Mn^{2+}, using a 5′ ^{32}P-labeled oligonucleotide as substrate, to examine the efficiency of substrate usage as well as the intermediates of the exonucleolytic degradation. As shown in Figure 7, at a Mg^{2+} concentration lower than 1 mM, only a low percent of the substrate was degraded, and the extent of degradation was also very low (measured as the length reduction of the oligonucleotide). At a Mg^{2+} concentration higher than 1 mM, nearly all the substrate was used, and the extent of degradation was almost complete. A different behavior was obtained with Mn^{2+}: although high concentrations of Mn^{2+} were also needed to use the substrate efficiently, quite low concentrations were enough to achieve a complete degradation of the molecules used.

Discussion

In this paper, we have analyzed the ability of different metal ions to activate the enzymatic activities of ϕ29 DNA polymerase.

Nonprocessive versus Processive DNA Polymerization. Distinct metal ion requirements were observed when nonprocessive polymerization and processive polymerization were compared (Table II). Nonprocessive DNA polymerization catalyzed by ϕ29 DNA polymerase (filling-in reaction) was well accomplished using Mg^{2+} or Mn^{2+} as metal activators (Table I), their optimal concentrations being 10 and 20 mM, respectively. This study was also carried out with pol I K and T4 DNA polymerase for comparative purposes. It is noteworthy the high optimal Mn^{2+} concentration and the high efficiency of the Mn^{2+}-activated reaction of ϕ29 DNA polymerase (Figure 1 and Table II). Using a challenged filling-in reaction, we showed that the high optimal metal concentration for ϕ29 DNA polymerase was reflecting the optimization of template usage by increasing dissociation of the enzyme from the template, enabling reassociation to occur (Figure 2). Furthermore, when the reactions obtained in the presence of the challenger DNA are compared, the catalytic efficiency at different metal concentrations can be inferred. It can be observed that the catalytic efficiency increases at high Mg^{2+}

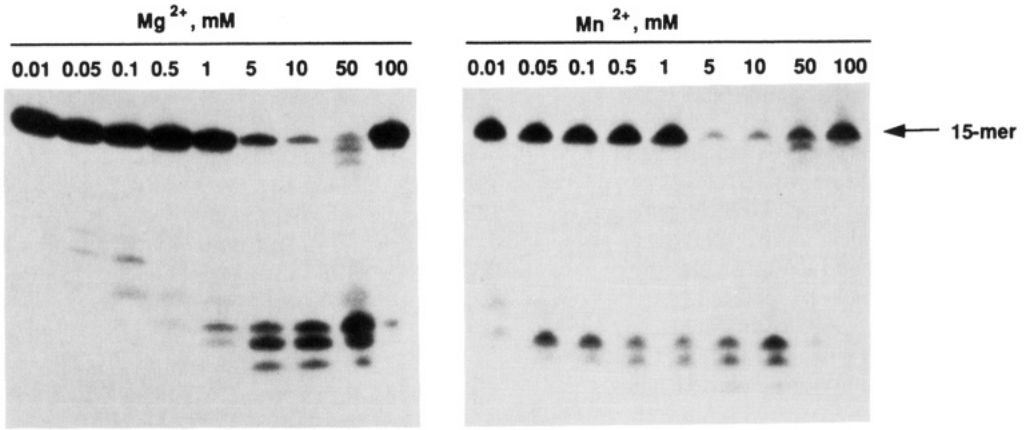

FIGURE 7: Effect of Mg^{2+} and Mn^{2+} concentration on the exonucleolytic degradation of ssDNA, carried out by ϕ29 DNA polymerase. The 3′→ 5′ exonuclease activity on ssDNA was assayed as described under Materials and Methods, using the indicated concentrations of $MgCl_2$ or $MnCl_2$ as metal activators. The extent of substrate usage and degradation, measured by a decrease of the amount and size, respectively, of the 5′-labeled oligonucleotide (15-mer), was analyzed by electrophoresis in 8 M urea–20% polyacrylamide gels and autoradiography.

concentration (Figure 2, compare lanes 2 and 5) whereas it slightly diminishes at high Mn^{2+} concentration (Figure 2, compare lanes 8 and 11).

T4 DNA polymerase and pol I K are intrinsically distributive enzymes (Jarvis et al., 1991; Mizrahi et al., 1985); therefore, their low optimal Mn^{2+} concentration and their poor Mn^{2+}-activated reaction (Figure 1 and Table II) could reflect the metal ion requirement for catalysis. The bimaximal activity observed with T4 DNA polymerase (Figure 1) may be the consequence of a moderate influence of metal-induced dissociation of the enzyme from the template. Thus, the behavior with respect to Mn^{2+} activation showed by the three DNA polymerases may be the result of two opposite effects: whereas a high Mn^{2+} concentration could be needed to give an optimal template usage, this concentration may be toxic for the catalytic functions of the enzyme. Pol I K would represent a very sensitive enzyme to high Mn^{2+} concentration and ϕ29 DNA polymerase a tolerant one.

In agreement with this idea, when primed-M13 DNA replication was analyzed, the optimal concentration for Mn^{2+} dropped to submilimolar levels (0.3 mM for ϕ29 DNA polymerase and 0.1 mM for pol I K and T4 DNA polymerase; see Table II). In this assay, ϕ29 DNA polymerase was very sensitive to Mn^{2+} concentrations higher than 0.3 mM. This fact could be explained as a result of ϕ29 DNA polymerase dissociation from the template, a clearly harmful event for this processive assay. Accordingly, pol I K did not present a different optimal Mn^{2+} concentration for the processive and nonprocessive assays, likely due to its intrinsic low processivity.

The capability of metal ions to alter the processiveness of DNA polymerases can be due to metal–DNA interactions. It is well-known that metal ions interact with DNA and that this interaction differs substantially among different metals (Marzilli et al., 1980; Murray & Flessel, 1976). DNA polymerases are, likely, sensitive to metal-induced alterations on DNA conformation, which may, in turn, affect translocation efficiency as well as DNA polymerization fidelity (Goodman et al., 1983; Beckman et al., 1985). Metal–DNA polymerase interactions may also alter the intrinsic processiveness of the enzyme, being in part responsible for the distinct efficiencies of different metals in activating processive or nonprocessive DNA polymerization.

Protein-Primed Initiation versus DNA Polymerization. Distinct metal ion requirements were also observed when the two synthetic reactions catalyzed by ϕ29 DNA polymerase were compared: protein-primed initiation and DNA polymerization (Tables I and II). These differences were particularly evident in the case of Mn^{2+}. This cation was the best activator for protein-primed initiation (Figure 4), mainly due to a significant increase of the affinity for dATP. This result contrasts with the null (or even slightly deleterious) effect of Mn^{2+} observed on DNA polymerization. Moreover, other good activators of the initiation reaction (Fe^{2+}, Zn^{2+}; see Figure 4) were completely unable to activate the replication of p3–DNA (Figure 5). Whereas Zn^{2+} was also a poor activator of nonprocessive DNA polymerization, Fe^{2+} had a moderate efficiency on the filling-in reaction (Table I). Therefore, bearing in mind that an efficient p3–DNA replication is strongly dependent on a processive DNA polymerization, it is likely that Fe^{2+} is actually decreasing the processivity of ϕ29 DNA polymerase (as Mn^{2+} at high concentration did). The inability of Zn^{2+} to activate any DNA polymerization (processive or nonprocessive), being a good activator of the initiation reaction, may reflect intrinsic differences between protein-primed initiation and DNA-primed polymerization. This is probably also the case of Mn^{2+}: whereas it decreases the K_M value for dATP in the initiation reaction, it has no effect on DNA polymerization of correct dNTPs (unpublished results).

Although it is likely that initiation and polymerization activities share the same catalytic site (Salas et al., 1988; Bernad et al., 1990; Blasco et al., 1990; Blanco et al., 1991), some specific differences in catalysis between these reactions could be expected. It is possible that the active site conformed by ϕ29 DNA polymerase, the terminal protein and p3–DNA as template, would be in some extent different from the active site conformed in the presence of a DNA molecule as template/primer. These variations in the insertion active site may be sensitive to variations on metal ion size, shape, and electronic properties, known to be important in the conformation of the metal–dNTP complex (Sigel, 1987; Sloan et al., 1975).

Metal Ion Requirements for Exonucleolysis. Slight differences in metal ion requirements for the exonuclease versus polymerase activities were observed for ϕ29 DNA polymerase (Tables I and II), these differences being more evident for T4 DNA polymerase and pol I K (Table II). This fact can be related to the existence of two independent domains for these activities, as it has been demonstrated for pol I K (Freemont et al., 1988; Derbyshire et al., 1988) and ϕ29 DNA polymerase (Bernad et al., 1989, 1990; Blanco et al., 1991), and extrapolated to other DNA polymerases based on sequence homology studies (Bernad et al., 1989; Blanco et al., 1991).

Taking advantage of the high processivity of the exonuclease activity of ϕ29 DNA polymerase, the metal ion requirements for substrate binding and catalysis were studied separately. Exonucleolytic catalysis was well accomplished at low Mn^{2+} concentration, whereas ssDNA binding required higher Mn^{2+} concentrations (Figure 7). These two different optima in Mn^{2+} concentration strongly suggest two separate roles of metal ions in exonucleolytic degradation by ϕ29 DNA polymerase: binding of DNA substrate and degradative catalysis of bound DNA. The presence of two metal ion molecules in the exonuclease domain of pol I K has been reported from crystallographic data (Freemont et al., 1988; Derbyshire et al., 1988). One of the metals (metal B) is involved in the exonuclease catalysis and the other one (metal A) in substrate binding. The results presented in this paper (Figure 7) seem to be a biochemical support for the existence of these two metal binding sites in ϕ29 DNA polymerase, probably having different affinities for Mn^{2+} ions. At low Mn^{2+} concentrations, only a few enzyme molecules would have the metal ion placed in site A, leading to poor substrate usage, but the high residence time of Mn^{2+} in site B (achieved by its high affinity) would lead to a complete degradation of the bound substrate.

These basic studies on metal ion activation of the different DNA polymerase catalytic functions may contribute to a better understanding of the complex DNA replication process, in which both synthetic and degradative activities must cooperate to guarantee the fidelity of DNA replication.

Registry No. Mn^{2+}, 7439-96-5; Mg^{2+}, 7439-95-4; Co^{2+}, 7440-48-4; Zn^{2+}, 7440-66-6; Cd^{2+}, 7440-43-9; Ni^{2+}, 7440-02-0; Fe^{3+}, 7439-89-6; Hg^{2+}, 7439-97-6; dATP, 1927-31-7.

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Growth State and Cell Cycle Dependent Phosphorylation of DNA Topoisomerase II in Swiss 3T3 Cells†

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ABSTRACT: We have investigated the amount of DNA topoisomerase II and phosphorylation of the enzyme in Swiss 3T3 cells during the transition from cell quiescence to proliferation. A relatively high level of phosphorylation was observed with proliferating cells while no or a very low level of phosphorylation was observed with quiescent cells. Phosphoamino acid analysis of the phosphorylated topoisomerase II revealed that the phosphorylated aminoacyl residue was serine. When quiescent cells were stimulated to grow by the addition of serum, DNA synthesis began to increase at 9 h after serum addition, reaching a maximum at 15 h and then declining. The amount of topoisomerase II began to increase at 6 h and reached a maximum at 22–27 h, corresponding to the G2 phase. The phosphorylation of topoisomerase II measured by pulse-labeling gradually increased from 6 to 18 h and reached a maximum at 22 h when the amount of the enzyme was maximum. The level of phosphorylation measured by continuous-labeling increased gradually up to 12 h and markedly up to 28 h, and then declined. The increase in the rate of phosphorylation in the G2 phase was affected by inhibiting DNA synthesis, but the increase in the amount of the enzyme was not. Thus, it was suggested that the regulation of phosphorylation of topoisomerase II differs from that of the amount of the enzyme.

DNA topoisomerase II is an enzyme that catalyzes the decatenation and the unknotting of topologically linked DNA circles and the relaxation of supercoiled DNA (Gellert, 1981; Wang, 1985; Vosberg, 1985). The enzyme is essential for the viability of eukaryotic cells because it is involved in chromosome segregation (Yanagida & Wang, 1987). It has been demonstrated that topoisomerase II is a major component of nuclear matrix (Berrios et al., 1985) and mitotic chromosome scaffold (Earnshaw et al., 1985) and that the enzyme is localized at the bases of the radial loop domains of mitotic chromosomes (Earnshaw & Heck, 1985; Gasser et al., 1986). Thus, the enzyme appears to play important roles in the construction of chromosome structure and in the organization of the nuclear matrix.

The activity and levels of topoisomerase II in proliferating cells are higher than those in quiescent cells (Miskimins et al., 1983; Duguet et al., 1983; Taudou et al., 1984; Sullivan et al., 1986; Heck & Earnshaw, 1986; Zwelling et al., 1987; Markovits et al., 1987; Nelson et al., 1987; Chow & Ross, 1987; Hsiang et al., 1988). During the development of *Drosophila*, the expression of topoisomerase II is high at the stages with increased mitotic activity (Fairman & Brutlag, 1988). Furthermore, it has been observed that both the amount and the stability of topoisomerase II are altered during the cell cycle (Heck et al., 1988).

Besides the fluctuation of enzyme levels, little is known about the mechanism to modulate the intracellular activity of topoisomerase II. In our previous study, we purified an unidentified protein kinase from mouse FM3A cells that phosphorylated purified topoisomerase II and observed that the phosphorylation of topoisomerase II by the protein kinase greatly stimulated the enzyme activity (Saijo et al., 1990). In addition, it has been reported that topoisomerase II is phosphorylated by casein kinase II or protein kinase C and that the modification by either kinase stimulates enzyme activity (Ackerman et al., 1985; Sahyoun et al., 1986). Thus, phosphorylation should be one of possible mechanisms that regulate intracellular topoisomerase II activity. Indeed, the enzyme appears to exist in eukaryotic cells as a phosphoprotein (Rottmann et al., 1987; Ackerman et al., 1988; Heck et al., 1989; Saijo et al., 1990).

In order to confirm the physiological meaning of phosphorylation of topoisomerase II, the changes in the level of phosphorylation of topoisomerase II as well as those in its amount during the transition of growth state were studied in the present work.

Materials and Methods

Materials. [^{32}P]Orthophosphate was purchased from NEN; [^{3}H]thymidine from ICN; protein A–Sepharose CL-4B from Pharmacia; anti-rabbit IgG from Medical & Biological Labs;

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