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Observations on template-specific conditions for DNA synthesis by avian myeloblastosis virus DNA polymerase

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ABSTRACT

The effects of Mg^{++}, Mn^{++}, and KCl addition, individually and in combination, on the rate of DNA- and RNA-primed DNA synthesis by avian myeloblastosis virus DNA polymerase (reverse transcriptase) using a variety of natural and synthetic template-primer combinations were examined. Optimal divalent cation concentrations were found to vary by as much as 10-fold depending upon the template-primer used to direct synthesis. Addition of KCl to reaction mixtures containing optimal divalent cation concentrations produced stimulation or inhibition of DNA synthesis which was also template-specific. DNA synthesis on the modified template poly (2'-O-methylcytidylate) was uniquely stimulated by combinations of divalent cations. With Mg^{++} as divalent cation, deviations from classical Michaelis-Menten kinetics of substrate saturation were observed with all template-primers tested.

INTRODUCTION

The ability of avian myeloblastosis virus (AMV) DNA polymerase (reverse transcriptase) to catalyse DNA synthesis on a wide variety of natural and synthetic template-primer combinations has been well documented[1-7]. Although the presence of divalent cations is obligatory for DNA synthesis, the exact nature of the role played by these ions is not clearly understood[8]. We have undertaken a systematic biochemical analysis of the ionic conditions required for DNA synthesis by the AMV enzyme using a number of synthetic and natural RNA and DNA templates. For the various template-primer combinations examined, the optimal concentration of divalent cation (as Mg^{++} or Mn^{++}) was first determined. The effects of combinations of divalent cations and the addition of monovalent cation on rates of DNA synthesis were then determined.

The synthetic homopolymeric templates $(rA)_{>300}$ and $(dA)_{>300}$ were primed with appropriate ribo- and deoxyribo-oligonucleotides, while $(rC)_{>300}$ and $(dC)_{>300}$ were primed with $(dG)_{12-18}$. A template prepared from a chemically modified precursor (2'-O-methylcytidylate)[9,10] was included in this study in order to determine whether such a structural modification would influence the response of the AMV DNA polymerase to changes in its ionic environment. Salmon

sperm DNA ("activated" by limited treatment with DNase I) and AMV genomic 70S RNA were the naturally-occurring heteropolymeric templates used in this study.

The biochemical analyses carried out as described above revealed template-specific ionic conditions required for optimal rates of DNA synthesis by the AMV DNA polymerase, and changes in ionic requirements as a result of template structural alterations.

MATERIALS AND METHODS

AMV reverse transcriptase. AMV was supplied as plasma from infected chickens by Life Sciences, Incorporated, St. Petersburg, Florida, prepared under contract NO1CP33291, Virus Cancer Program, National Cancer Institute. Reverse transcriptase was purified by affinity chromatography on polycytidylate-agarose as described by Marcus et al.[11] and also by classical methods of protein fractionation[12]. Enzyme prepared by both methods was over 90% pure as determined by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, free of contaminating exonuclease activity, and produced identical results in these studies. The αβ form [11] was used in all experiments.

Template-primers. The synthetic homopolymeric templates and oligodeoxynucleotide primers used in all assays were purchased from P-L Biochemical Laboratories. Polyadenylic acid $(rA)_{>300}$ or polydeoxyadenylic acid $(dA)_{>300}$ were annealed with oligodeoxythymidylic acid $(dT)_{10}$ before use at an equimolar nucleotide ratio by heating the solution to 60° for 5 minutes and then allowing it to cool slowly to room temperature. Oligouridylic acid $(U)_9$, was purchased from Boehringer Manneheim, Incorporated, and was annealed to $(rA)_{>300}$ and $(dA)_{>300}$ by the same procedure used for annealing of those templates with $(dT)_{10}$. Polycytidylic acid $(rC)_{>300}$, polydeoxycytidylate $(dC)_{>300}$, or poly (2'-O-methylcytidylate) $(rCm)_{>300}$, were annealed to oligodeoxyguanylate $(dG)_{12-18}$ at a 5:1 molar nucleotide ratio of polymer to oligomer. Annealing was carried out at 80° for 15 minutes, after which the solutions were allowed to cool slowly to room temperature. Poly (2'-O-methylcytidylate)[10] was a generous gift of Dr. F. Rottman. Activation of salmon sperm DNA by limited DNase treatment was carried out according to the procedure of Aposhian and Kornberg[13]. AMV 70S RNA was obtained by SDS-phenol extraction of purified virus and further purification of the RNA accomplished on glycerol gradients as previously described[14].

DNA Polymerase Assays. Reaction mixtures (in a total volume of 0.1 ml) for synthetic homopolymeric template-primers consisted of 50 mM Tris-HCl, pH 7.8, 2 mM dithiothreitol, 25 μg bovine serum albumin, 80 μM (^{3}H)-dTTP or (^{3}H)-dGTP, and 0.5 μg of the desired template-primer combination. Purified AMV reverse transcriptase (0.02 μg) was present in all such assays with the exception of $(rCm)_{>300}$,

$(dG)_{12-18}$-directed reactions represented in Figures 2 and 3, which contained 0.1 µg of enzyme. Reaction mixtures containing activated DNA or AMV 70S RNA as template-primers were identical to those above except that unlabeled dATP, dGTP, and dCTP were added to a final concentration of 80 µM each, while (^{3}H)-dTTP was at 10 µM concentration. Activated DNA (2.5 µg per assay) or AMV 70S RNA (0.5 µg per assay) were used as template-primers together with 0.2 µg of purified reverse transcriptase. Specific activities of (^{3}H)-dTTP and (^{3}H)-dGTP in assays utilizing synthetic template-primers were adjusted to 60-120 cpm per picomole and 50-100 cpm per picomole of deoxynucleoside triphosphate, respectively, while that of (^{3}H)-dTTP in reaction mixtures containing activated DNA or 70S RNA was 1000 cpm per picomole. Concentrations of Mg^{++} (as $MgCl_2$), Mn^{++} (as $MnCl_2$), and KCl are specified in the legends to figures and tables. Unless otherwise noted, all reactions were incubated at 37° for 30 minutes, during which polymerization was linear with respect to time. Reactions were terminated by the addition of 5% (w/v) trichloroacetic acid solution containing 0.01 M sodium pyrophosphate. Acid-insoluble material was collected onto Whatman GF/B glass fiber filters by vacuum filtration. After drying the filters were placed in toluene-based scintillation fluid and the radioactivity measured using a liquid scintillation counter.

RESULTS

The experimental results are outlined below and grouped according to the type of homopolymeric or heteropolymeric ribo- or deoxyribonucleotide template examined.

$(rA)_{>300}$ and $(dA)_{>300}$ primed with $(dT)_{10}$ or $(U)_9$. Optimal divalent cation concentrations and resulting rates of DNA synthesis on these template-primer combinations are given in Table I. In the presence of Mg^{++}, $(rA)_{>300}.(dT)_{10}$-directed DNA synthesis catalysed by the AMV DNA polymerase proceeded at a rate 60-fold that observed with $(dA)_{>300}.(dT)_{10}$ as template-primer, in agreement with previous reports[7]. Mg^{++} was the preferred divalent cation for $(rA)_{>300}.(dT)_{10}$-directed synthesis, while Mn^{++} was preferred for DNA synthesis directed by $(dA)_{>300}.(dT)_{10}$ (Table I). Although Mn^{++} could partially substitute for Mg^{++} in $(rA)_{>300}.(dT)_{10}$-directed synthesis, the addition of as little as 0.05 mM Mn^{++} to reaction mixtures containing *optimal* concentrations of Mg^{++} produced significant *inhibition* of catalysis (Fig. 1A). On the other hand, addition of increasing quantities of Mg^{++} to Mn^{++}-containing reaction mixtures utilizing $(rA)_{>300}.(dT)_{10}$ stimulated rates of synthesis, although levels obtained with Mg^{++} alone were never reached. For $(dA)_{>300}.(dT)_{10}$-directed reactions, Mn^{++} was the preferred divalent cation, and Mg^{++} added to reaction mixtures containing Mn^{++}

TABLE I

Divalent Cation Requirements and Optimal Rates of DNA Synthesis by AMV Reverse Transcriptase on Various Template-Primers

Template-primer	Mg^{++}[a] (mM)	Optimal rate of DNA synthesis[b]	Mn^{++}[a] (mM)	Optimal rate of DNA synthesis[b]	$\left(\frac{\text{Opt. rate } Mg^{++}}{\text{Opt. rate } Mn^{++}}\right)$[c]
$(rA)_{>300}\cdot(dT)_{10}$	5-30	300	0.1-1	100	3.0
$(dA)_{>300}\cdot(dT)_{10}$	5-10	5	2.0	400	0.0125
Activated DNA	30-70	42	0.05	12	3.5
$(rC)_{>300}\cdot(dG)_{12-18}$	30-50	800	0.05-0.5	40	20
$(dC)_{>300}\cdot(dG)_{12-18}$	5-50	500	0.05-2	130	3.85
$(rCm)_{>300}\cdot(dG)_{12-18}$	20-40	40	0.5-2	28	1.42
$(rA)_{>300}\cdot(U)_9$	30-50	5.5	2.0	4.5	1.22
$(dA)_{>300}\cdot(U)_9$	5-20	4.0	0.5-1.0	40	0.10
AMV 70S RNA	10-30	4.0	0.5-1.0	2.7	1.48

Table I. Conditions of assay are those described in Materials and Methods.

[a] Divalent cation concentrations are those which allow 90-100% of maximal rate of DNA synthesis.

[b] Rates of DNA synthesis are expressed as picomoles of precursor nucleotide incorporated during incubation periods of 30 minutes at 37°. Rates of synthesis on activated DNA and 70S RNA are expressed as picomoles of a single nucleotide precursor incorporated.

[c] No significant change in optimal divalent concentrations was noted when substrate concentrations were varied from 10 to 400 μM.

produced slight stimulation of synthesis (Fig. 1C). As expected, addition of Mn^{++} to reaction mixtures containing Mg^{++} greatly stimulated DNA synthesis for this template-primer (Fig. 1A).

When $(U)_9$, an oligomeric RNA, was used as a primer molecule in place of $(dT)_{10}$, the efficiency of DNA synthesis on both $(rA)_{>300}$ and $(dA)_{>300}$ as template by the AMV DNA polymerase was sharply decreased (Table I). Interestingly, divalent cation preferences for DNA synthesis on these templates appeared to be relatively unaffected by the use of either a DNA or RNA primer molecule

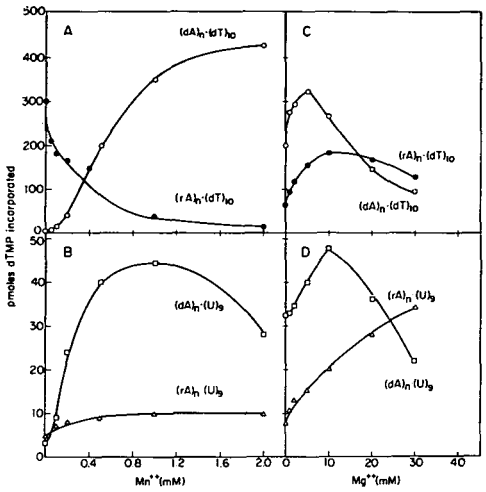

Fig. 1. Effect of divalent cation combinations on RNA- and DNA-primed DNA synthesis directed by $(rA)_n$ and $(dA)_n$. (A) and (B): increasing Mn^{++} concentrations added to optimal Mg^{++} concentrations; (C) and (D): increasing Mg^{++} concentrations added to optimal Mn^{++} concentrations. Fixed, optimal concentrations of divalent cations: $(rA)_n.(dT)_{10}$ 5 mM Mg^{++}, 0.2 mM Mn^{++}; $(dA)_n.(dT)_{10}$ 5 mM Mg^{++}, 2 mM Mn^{++}; $(rA)_n.(U)_9$ 40 mM Mg^{++}, 2 mM Mn^{++}; $(dA)_n.(U)_9$ 5 mM Mg^{++}, 1 mM Mn^{++}. In this and subsequent figures and figure legends, the subscript n denotes chain length of greater than 300 nucleotides.

(Table I), e.g. $(dA)_{>300}$-directed synthesis proceeded most efficiently in the presence of Mn^{++}, while $(rA)_{>300}$-directed synthesis proceeded at maximal rates in the presence of Mg^{++}. For $(dA)_{>300}.(U)_9$-directed synthesis, addition of increasing concentrations of one divalent cation to assay mixtures containing an optimal concentration of the other divalent cation did not increase rates of synthesis above those observed with Mn^{++} alone. These results are similar to those obtained with $(dA)_{>300}$ primed with $(dT)_{10}$. Increasing concentrations of Mn^{++} added to reaction mixtures containing $(rA)_{>300}.(U)_9$ and optimal levels of Mg^{++} stimulated synthesis nearly 2-fold (Fig. 1B) in marked contrast to the inhibition observed under similar conditions with $(rA)_{>300}.(dT)_{10}$. Addition of increasing Mg^{++} to $(rA)_{>300}.(U)_9$-directed reactions in the presence of optimal levels of Mn^{++} stimulated synthesis 4-fold (Fig. 1D). Thus, for this synthetic RNA-primed RNA template, Mg^{++} and Mn^{++} appear to act in a synergistic manner to increase the net synthesis.

The addition of monovalent cation to reaction mixtures has been reported to stimulate the reverse transcription of oncornaviral RNA[7,15]. At individually optimal concentrations of divalent cations, increasing concentrations of KCl up to 0.1M did not significantly affect rates of DNA synthesis (less than 10% stimulation or inhibition) by AMV DNA polymerase on $(rA)_{>300}$ or $(dA)_{>300}$ primed by RNA or DNA oligomers (data not shown).

$(dC)_{>300}.(dG)_{12-18}$, $(rC)_{>300}.(dG)_{12-18}$, and $(rCm)_{>300}.(dG)_{12-18}$. Mg^{++} was found to be the preferred divalent cation for DNA synthesis on all three templates (Table I). Mn^{++} could partially substitute for Mg^{++}, although the degree of substitution varied with the template. The AMV DNA polymerase could utilize $(rC)_{>300}$ and $(dC)_{>300}$ as templates with less than a 2-fold preference for the ribohomopolymeric template. The methylated polycytidylate template $(rCm)_{>300}$, has been reported to be a highly selective template for the detection of RNA-directed DNA polymerase when primed with $(dG)_{12-18}$[10]: High levels of Mg^{++} (20-30 mM) are required for optimal rates of DNA synthesis on both $(rC)_{>300}$ and $(rCm)_{>300}$, although $(rCm)_{>300}$ was found to be only 5% as effective a template as $(rC)_{>300}$. Poly dG synthesis directed by $(rCm)_{>300}$ also exhibits the least preference for Mg^{++} over Mn^{++} of all three templates (Table I). Further studies indicated that $(rCm)_{>300}$ may interact in a unique fashion with divalent cations. We observed that addition of Mn^{++} to Mg^{++}-containing reaction mixtures inhibited DNA synthesis directed by $(rC)_{>300}$ and $(dC)_{>300}$ (Fig. 2A). In contrast to this finding, rates of $(rCm)_{>300}$-directed synthesis were increased over 3-fold by the addition of increasing concentrations of Mn^{++} to reaction mixtures containing optimal levels of Mg^{++} (Fig. 2A). This stimulation appeared synergistic, since rates of synthesis obtained by combinations of divalent cations were twice those which would have resulted from simple additive effects. Addition of increasing quantities of Mg^{++} to Mn^{++}-containing reaction mixtures (Fig. 2B), did not restore rates of $(rC)_{>300}$- or $(dC)_{>300}$-directed synthesis to those levels observed with Mg^{++} alone.

Addition of increasing concentrations of KCl to $(rC)_{>300}$ and $(rCm)_{>300}$-directed reactions at optimal Mg^{++} concentrations inhibited DNA synthesis (Fig. 3A). These two templates are similar in that relatively high concentrations of Mg^{++} (20-30 mM) are required for efficient synthesis (Table I). At sub-optimal Mg^{++} concentrations (5 mM), however, 50 mM KCl stimulated rates of DNA synthesis on these template-primers almost 2-fold (data not shown). At optimal Mg^{++} levels (10 mM), $(dC)_{>300}$-directed DNA synthesis was stimulated almost 2-fold by 100 mM KCl (Fig. 3A). In the presence of optimal Mn^{++} concentrations (Fig. 3B), DNA synthesis on each of three template-primers was stimulated at

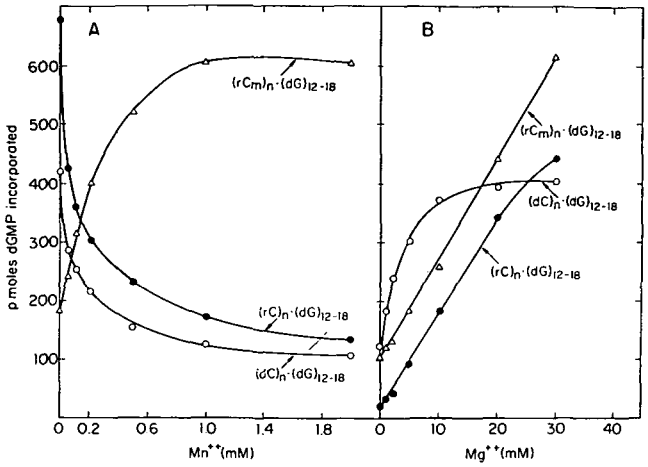

Fig. 2. Effect of divalent cation combinations on $(rC)_n.(dC)_n$, and $(rCm)_n$-directed DNA synthesis. (A): increasing Mn^{++} concentrations added to optimal concentrations of Mg^{++}. (B): increasing Mg^{++} concentrations added to optimal Mn^{++} concentrations. Fixed, optimal concentrations of divalent cations: $(rC)_n.(dG)_{12-18}$ 30 mM Mg^{++}, 0.2 mM Mn^{++}; $(dC)_n.(dG)_{12-18}$ 10 mM Mg^{++} 0.05 mM Mn^{++}; $(rCm)_n.(dG)_{12-18}$ 30 mM Mg^{++}, 1 mM Mn^{++}. Assays performed using $(rCm)_n.(dG)_{12-18}$ each contained 0.1 µg of AMV DNA polymerase; all other assays contained 0.02 µg enzyme.

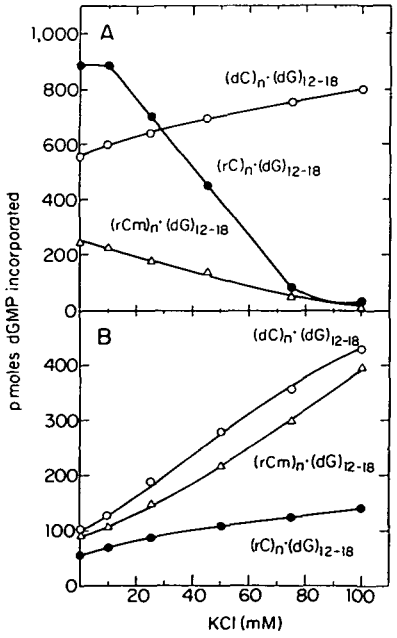

Fig. 3.

Effect of KCl on rates of DNA synthesis directed by $(rC)_n.(dG)_{12-18}$, $(dC)_n.(dG)_{12-18}$, and $(rCm)_n.(dG)_{12-18}$ in the presence of optimal Mg^{++} concentrations (A) and optimal Mn^{++} concentrations (B). Divalent cation concentrations: $(rC)_n.(dG)_{12-18}$ 30 mM Mg^{++}, 0.2 mM Mn^{++}; $(dC)_n.(dG)_{12-18}$ 10 mM Mg^{++}, 0.5 mM Mn^{++}, $(rCm)_n.(dG)_{12-18}$ 30 mM Mg^{++}, 1 mM Mn^{++}. Assays performed using $(rCm)_n.(dG)_{12-18}$ each contained 0.1 µg of AMV DNA polymerase; all other assays contained 0.02 µg enzyme.

least 3-fold by the addition of KCl.

Activated DNA and AMV 70S RNA. DNA synthesis on these heteropolymeric templates required the presence of all four deoxynucleoside triphosphates. DNA synthesis directed by AMV 70S RNA in the presence of Mn^{++} was 70-80% the rate observed with Mg^{++}, while synthesis on activated DNA templates exhibited a greater preference for Mg^{++} (Table I). A very sharp Mn^{++} optimum of 0.05 mM for activated DNA-directed DNA synthesis was observed. Concentrations of Mn^{++} greater than 0.05 mM proved inhibitory to DNA-directed synthesis. High concentrations of Mg^{++} ($\gtrsim$40 mM) were required for the expression of optimal rates of DNA synthesis directed by activated DNA, although Mn^{++} concentrations which were optimal for the majority of template-primers tested inhibited the DNA-directed reaction. The effects of various mono- and divalent cation combinations on DNA synthesis using these natural templates are summarized in Table II. No significant effect on the rate of RNA or DNA-directed DNA synthesis was produced by divalent cation combinations or by the addition of KCl to individually optimal divalent cation concentrations. However, a combination of Mg^{++}, Mn^{++} and KCl produced significant stimulation of RNA-directed synthesis.

Template-Independent Reaction Properties:

Alteration of template:primer ratio. The effects of altering the molar nucleotide ratio of template:primer on the kinetics of substrate incorporation in the presence of optimal and sub-optimal concentrations of a preferred divalent cation are illustrated in Figure 4. Incorporation of dGMP into tricholoroacetic

TABLE II

The Effects of Cation Combinations on DNA Synthesis by AMV Reverse Transcriptase

Template-primer	pmoles dTMP incorporated					
	Mg^{++} alone	Mn^{++} alone	Mg^{++} + KCl	Mn^{++} + KCl	Mg^{++} + Mn^{++}	Mg^{++} + Mn^{++} + KCl
AMV 70S RNA[a]	3.5	1.9	3.8	2.8	3.8	5.1
Activated DNA[b]	62.2	18.3	68.4	34.6	57.5	58.2

[a] Mg^{++} = 10mM; Mn^{++} = 0.5mM; KCl = 50mM
[b] Mg^{++} = 40mM; Mn^{++} = 0.05mM; KCl = 50mM
Assays were carried out as described in Materials and Methods.

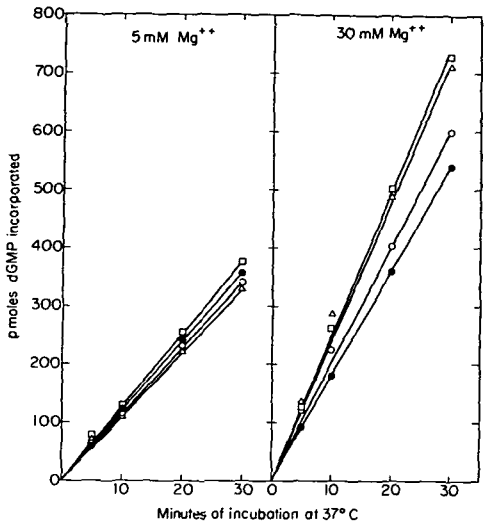

Fig. 4. Effect of sub-optimal (5 mM) and optimal (30 mM) Mg^{++} levels and varying template-to-primer ratios on kinetics of (^{3}H)-dGMP incorporation. Annealing of template-primer combinations was carried out as described in Methods. Molar nucleotide ratios of $(rC)_n$ to $(dG)_{12-18}$: (●) = 1:1; (o) = 2:1; (Δ) = 7.5:1; (□) = 15:1.

acid-insoluble material catalysed by AMV reverse transcriptase and directed by $(rC)_{>300}.(dG)_{12-18}$ was linear with time over a period of 30 minutes. Varying the template to primer ratio over a 15-fold range did not alter the rates of synthesis over the time interval used by more than 50%. The differential effect of 5 mM Mg^{++} (sub-optimal) and 30 mM Mg^{++} (optimal) on the rate of DNA synthesis is clearly shown and is not appreciably altered by changes in the template:primer ratio. Similar results were also obtained for $(rA)_{>300}.(dT)_{10}$ and the other synthetic template-primer combinations used for this study. A very high template:primer ratio is apparently not required for efficient DNA synthesis by the AMV enzyme. Thus, the values expressed in Table I truly reflect differential rates of DNA synthesis.

Divalent Cations Affect Apparent K_m Values for Substrates. To determine whether the use of different divalent cations for DNA synthesis might have an effect on the concentration of deoxynucleoside triphosphates required for maximal rates of DNA synthesis, substrate saturation experiments were carried out. The re-

sults of such an experiment in the form of a double reciprocal plot are shown in Fig. 5. With $(rA)_{>300}.(dT)_{10}$ as template-primer, and Mn^{++} as the divalent cation (the least preferred by the enzyme for this template), classical Michaelis-Menten kinetics are observed, with an apparent K_m value for dTTP of 20 μM. However, when this determination is carried out in the presence of Mg^{++} as the divalent cation, a biphasic plot is obtained, with apparent K_m values for dTTP of 20 μM and 250 μM, respectively, by extrapolation of the linear portions of the plot. That portion of the plot containing those values which extrapolate to the high apparent K_m and V_{max} values is shown in more detail in Fig. 5 (insert) with values from a similar plot obtained using $(rC)_{>300}$ as template with Mg^{++} as divalent cation. Similar plots were obtained which were of a biphasic nature for $(rC)_{>300}$, $(dC)_{>300}$, activated DNA and AMV 70S RNA only when Mg^{++} was the divalent cation present. This finding may be of functional significance in the re-

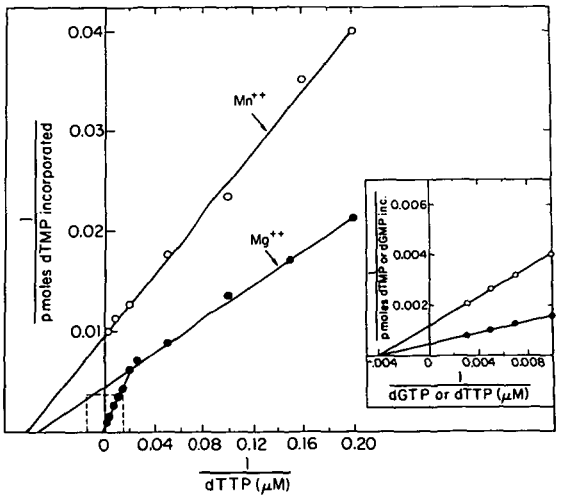

Fig. 5. Double reciprocal plots of 1/velocity vs. 1/deoxyribonucleoside triphosphate concentrations. Units of velocity are expressed as picomoles incorporated per 30-minute interval under standard assay conditions (see Methods). Effect of divalent cations on $(rA)_n.(dT)_{10}$-directed (^3H)-dTMP incorporation, in the presence of 5 mM Mg^{++} or 0.2 mM Mn^{++}. Identical effects were observed with $(rC)_n.(dG)_{12-18}$-directed synthesis in the presence of 30 mM Mg^{++} or 0.2 mM Mn^{++} (data not shown in Figure). The area enclosed by dotted lines has been enlarged in the Insert to include data from $(rA)_n$-directed (^3H)-dTMP incorporation in the presence of 5 mM Mg^{++} (o) and $(rC)_n$-directed (^3H)-dGMP incorporation in the presence of 30 mM Mg^{++} (•).

verse transcription of oncornaviral genomic RNA, since the lower value for the K_m of all four deoxynucleoside triphosphates for DNA synthesis on AMV 70S RNA in the presence of Mg^{++} was 15 μM, while the higher value was 740 μM (data not shown).

DISCUSSION

While the importance of the ionic environment in determining optimal rates of DNA synthesis by reverse transcriptase has been recognized[1-7], reaction conditions with respect to metal ion type and concentration have often been generalized in the literature[1-3,8,16]. We have attempted to carry out a critical analysis of the contributions of divalent and monovalent cation, singly and in combination, to the process of DNA synthesis catalysed by AMV DNA polymerase on a variety of natural and synthetic template-primers.

We have found that optimal divalent cation concentrations may vary as much as 10-fold, depending upon the template-primer used to direct DNA synthesis (Table I). The nature of the template appears to be of critical importance in determining divalent cation preference since synthesis directed by $(rA)_{>300}$ and primed with either $(dT)_{10}$ or $(U)_9$ proceeds at optimal rates in the presence of Mg^{++}, while $(dA)_{>300}$-directed synthesis primed with either molecule was best carried out in the presence of Mn^{++}. Although $(dA)_{>300}.(dT)_{10}$ has been described as an inefficient template for AMV DNA polymerase in the presence of Mg^{++} due to the formation of a triplex structure at equimolar template and primer nucleotide ratios[16], we have found that this template-primer is efficiently utilized in the presence of Mn^{++}. We do not know whether the Mn^{++}-specific nature of this response is due to a change in template-primer structure or is mediated through a metal-specific enzyme-Mn^{++} interaction.

The effect of adding salt to reaction mixtures containing optimal concentrations of divalent cations was also examined. While synthetic template-primer directed DNA synthesis requiring concentrations of Mg^{++} >20 mM for optimal activity was inhibited by the addition of KCl to reaction mixtures, stimulation of DNA synthesis by KCl addition was observed at suboptimal Mg^{++} concentrations. This indicates that, for such template-primers, the AMV DNA polymerase requirement for high Mg^{++} levels is partly due to the necessity for a high-ionic-strength environment. For the reverse transcription of oncornaviral 70S RNA, we have found that KCl addition provides significant stimulation only in the presence of both Mg^{++} and Mn^{++} (Table II). Such results should be interpreted with caution, however, since a recent report[17] has shown that KCl stimulation only serves to increase the yield of partial cDNA transcripts of ovalbumin mRNA; omission of KCl was required in order to obtain full-length transcripts, while

lowering the total level of DNA synthesis.

Studies using the methylated template $(rCm)_{>300}$ revealed the divalent cation requirement and response to monovalent cation by AMV DNA polymerase using this template to be qualitatively similar to those of $(rC)_{>300}$-directed synthesis. However, Mg^{++} and Mn^{++}, in combination, were found to inhibit both $(rC)_{>300}$ and $(dC)_{>300}$-directed synthesis, while such combinations produced an apparent synergistic stimulation of $(rCm)_{>300}$-directed synthesis. This observation (Fig. 2) suggests that methylation of polycytidylate may affect the manner in which the template-primer combination interacts with divalent cations. Since the divalent-cation-specific alterations of nucleic acid conformation have been shown to exist[18], a similar effect may be responsible for the apparently unique response we have observed with $(rCm)_{>300}$. We have observed a similar alteration in divalent cation preference for $(rCm)_{>300}$ as compared with $(dC)_{>300}$ or $(rC)_{>300}$-directed DNA synthesis by the DNA polymerase purified from Rauscher leukemia virus (unpublished observations) as well as murine mammary tumor virus[19]. This finding supports the interpretation that, in this case, alterations in reaction requirements are not enzyme-specific but are due to structural alteration of the template. The use of this modified template for the detection and identification of reverse transcriptase in oncornavirus-infected cells[10] and in human tumor cells[20] emphasizes the importance of understanding the apparently unique manner in which this template interacts with the viral DNA polymerases.

A divalent cation-specific effect rather than a template-specific effect was observed in the biphasic double-reciprocal plots of velocity versus deoxynucleoside triphosphate concentration in the presence of Mg^{++} (Fig. 5). Classical kinetics were observed when Mn^{++} was used as the divalent cation. These results were obtained for natural heteropolymeric template-primers as well as synthetic homopolymeric template-primers, and thus may reflect a direct effect of the divalent cation on the enzyme molecule, enzyme-substrate complex, or enzyme-substrate-template-primer complex. Results obtained in a recent study on infidelity of homopolymer-directed DNA synthesis by AMV DNA polymerase indicate that this enzyme may contain multiple nucleotide binding sites[21]. The biphasic double-reciprocal plots we have observed with respect to substrate concentration are probably not due to multiple-site interactions, as such sites appear to be base specific[21]. It is unclear as to how Mg^{++} elicits this response from the AMV DNA polymerase, although this effect may result from the interactions of the α and β subunits in the presence of Mg^{++}. We intend to carry out experiments on the α form of the enzyme in order to determine whether the presence of the β subunit is essential for expression of this kinetic effect. In any event, these

results suggest that high DNA precursor concentrations may be essential for efficient reverse transcription of natural RNA molecules. Indeed, recent reports have shown that high substrate concentrations are needed to obtain a high percentage of full-length globin mRNA transcripts[22] as well as to produce full-length transcripts of avian oncornaviral genomic RNA[23].

The template-specific cation effects reported in this paper should not be generalized to other DNA polymerases. We have shown that E. coli DNA polymerase I can efficiently utilize $(rA)_{>300}.(dT)_{10}$ and $(dA)_{>300}.(dT)_{10}$ under identical conditions[24,25]. Eucaryotic, mammalian nuclear 3.4S DNA polymerase can copy $(rA)_{>300}.(dT)_{10}$ effectively only in the presence of Mn^{++} [25]. However, the large variety of templates which the AMV polymerase can copy makes it a useful tool for the study of enzyme-metal-template-primer interactions. It is hoped that future studies measuring divalent cation effects on template structure will eventually bring to light the exact nature of the manner in which templates participate in their own replication.

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