Properties of the Primer-Binding Site and the Role of Magnesium Ion in Primer–Template Recognition by KB Cell DNA Polymerase α†

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ABSTRACT: The results of classical steady-state kinetic analyses and semiquantitative sedimentation binding assays have provided substantial definition of the important step of primer-terminus recognition by KB cell DNA polymerase α. The polymerase is capable of binding a base-paired 3′-primer stem following a prerequisite step of binding to single-stranded polydeoxynucleotide template [Fisher, P. A., & Korn, D. (1981) *Biochemistry*, preceding paper in this issue]. Efficient priming with a bimolecular primer–template requires an octadeoxynucleotide, and this length requirement appears to be independent of both base composition and temperature. Both ribo and deoxyribo termini bearing 3′-hydroxyl groups are recognized with equal facility and support similar rates of catalysis. 2′,3′-Dideoxy termini, while unable to support catalysis, are bound by the polymerase similarly to 3′-hydroxyl termini. By exploiting the phenomenon of dideoxy primer induced dNTP inhibition [Fisher, P. A., & Korn, D. (1981) *Biochemistry*, preceding paper in this issue], we demonstrate that a single terminally mismatched primer residue blocks detectable binding of the primer stem. Mg^{2+} plays an important role in the interaction of the polymerase with both template and primer. Increasing concentrations of Mg^{2+} lead to dramatic increases in the affinity of polymerase α for pyrimidine deoxynucleotide homopolymer and copolymer templates, as well as for natural single-stranded DNA, but over the same concentration range, Mg^{2+} has little or no effect on the binding of purine polydeoxynucleotide templates. From this result, it has been possible with the hook template–primer $(dA)_n$-$(dT)_m$ to demonstrate that free Mg^{2+} produces a highly parabolic pattern of competitive inhibition, and the measured Hill coefficient of 3.9 supports the interpretation that at least four Mg^{2+}-binding sites are involved. The effects of Mg^{2+} on the binding of template and primer have been shown by sedimentation binding analyses to be qualitatively independent of the presence or absence of dNTPs and thus to reflect a property of the polymerase–nucleic acid interaction that is independent of catalysis. The combined results are compatible with the proposal that DNA polymerase α binds a terminally base-paired primer stem essentially independently of the nature of the 3′-terminal sugar residue and that it does so via a Mg^{2+} chelate of the phosphodiester backbone that involves the participation of four primer-binding subsites.

The catalytic interaction of KB cell DNA polymerase α with its substrates obeys a rigidly ordered sequential terreactant mechanism that entails initial recognition of single-stranded polydeoxynucleotide template, followed by binding of a suitably base-paired 3′-primer moiety and, finally, binding of the correct (template-sequence-specified) dNTP (Fisher & Korn, 1979a,b, 1981; Fisher et al., 1981). We have described in some detail the initial step of template recognition by the polymerase and have shown it to be highly base composition (or sequence) dependent and to appear to involve at least two separate but positively interactive binding sites on the polymerase molecule (Fisher & Korn, 1979a; Fisher et al., 1981). Because our understanding of the step of primer binding by the enzyme was by comparison markedly incomplete, we undertook the experiments that are described in this manuscript. From the results of these studies, we are now able to present a detailed description of primer recognition by DNA polymerase α and, in conjunction, to provide substantial clarification of the role of Mg^{2+} in both the template-binding and the primer-binding steps of the polymerase–nucleic acid interaction.

Materials and Methods

The sources of most materials, and many of the methods, were as previously described (Fisher & Korn, 1977, 1979a,b, 1981; Fisher et al., 1979, 1981). [^{3}H]ATP was from New England Nuclear, ribonucleoside triphosphates were from Boehringer, and poly(dG), $d(pT)_4$, $d(pC)_4$, $d(pG)_4$, $d(pG)_6$, $d(pG)_8$, and $d(pG)_{10}$ were from Collaborative Research. The synthetic oligomers were used as supplied. Oligo(deoxythymidylate) polymers of defined length, and all other defined nucleic acid molecules, were prepared, purified, and characterized as described below. Terminal deoxynucleotidyl transferase (TdT), purified from calf thymus, was the generous gift of Dr. R. L. Ratliff (Los Alamos Scientific Laboratory).

Preparation of $(dA)_{4000}$ and $(dT)_{3000}$.[1] Homopolymers $(dA)_{4000}$ and $(dT)_{3000}$ were prepared essentially as described for $(dA)_{100}$ and $(dT)_{100}$, respectively (Fisher et al., 1981), except that dNTP concentrations were increased appropriately.

Preparation of Diribonucleotide-Terminated Polydeoxynucleotides. Diribonucleotide-terminated polydeoxynucleotides were prepared with TdT as described (Roychoudhury & Kossel, 1971; Roychoudhury, 1972). The hook polymer $(dT)_{100}$-$(dA)_{25}$-$(A)_2$ was synthesized in a reaction (200 μL) that contained 500 μM (nucleotide) $(dT)_{100}$-$(dA)_{25}$, 40 mM sodium cacodylate, pH 7.2, 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, 1 mM ATP, and 3000 units of TdT. The progress of the terminal addition reaction was monitored in a sample that contained [^{3}H]ATP. The hook polymer $(dA)_{100}$-$(dT)_{25}$-$(U)_2$ was prepared in a reaction (400 μL) that contained 1 mM (nucleotide) $(dA)_{100}$-$(dT)_{25}$, 40 mM sodium cacodylate, pH 7.2, 2 mM $CoCl_2$, 1 mM 2-mercaptoethanol, 1 mM UTP, and 6000 units of TdT. The heteropolymer (dA, dG, dC, dT)$_{57}$-(A, G, C, U)$_2$ was synthesized in an incubation (500 μL) containing 1 mM (nucleotide) (dA, dG, dC, dT)$_{57}$ (Fisher et al., 1981), 40 mM sodium cacodylate, pH 7.2, 8 mM $MgCl_2$, 250 μM each of NTP, and 8000 units of TdT.

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[1] The lengths of synthetic deoxynucleotide polymers are designated by numerical subscripts that represent Poisson mean values.

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Progress of the latter two syntheses was monitored in sample aliquots as described above.

Preparation of Oligo(deoxythymidylate) Molecules of Defined Length. The oligomers $(dT)_5$ to $(dT)_{10}$ were prepared with TdT in a 1-mL incubation containing 2.85 mM (nucleotide) $d(pT)_4$, 200 mM sodium cacodylate, pH 7.2, 3 mM $CoCl_2$, 2.2 mM [^{3}H]dTTP at 20 μCi/mL, 1 mM 2-mercaptoethanol, and 6000 units of TdT. The reaction was allowed to proceed at 37 °C until 80% of the [^{3}H]dTTP had been incorporated into product [assessed by adsorption to DE-81 paper as previously described (Wang et al., 1977)]. Oligo(deoxythymidylate) products were separated according to size as described (Chang & Bollum, 1971).

Preparation of Dideoxynucleotide-Terminated Hook Polymers Bearing a Mismatched Terminal Base. Hook polymers $(dA)_{100}$-$(dT)_{25}$-(ddA, ddC, ddG)$_1$ and $(dC)_{100}$-$(dG)_{25}$-(ddA, ddC, ddT)$_1$ were prepared as described in the accompanying manuscript for the dideoxy-terminated hook polymers, except that the correct (base-pairable) ddNTP was replaced in the incubation by the three incorrect ddNTPs.

Sedimentation Binding Assays. Assays of binding of polymerase α to various DNA molecules were performed by sedimentation in linear glycerol gradients, essentially as described in the accompanying manuscript; specific parameters are indicated in the individual figures. In all cases, analyses were facilitated by the sedimentation of polymerase α alone under otherwise identical centrifugation conditions. This was done in order to define rigorously the area of the gradient subsumed by the peak of free polymerase, such that any nucleic acid induced shifts from this position could be quantitated accurately. The broken lines that are shown dividing the sedimentation profiles in Figures 4, 5, and 9 thus represent the lower boundary of sedimentation of free polymerase α in the absence of added DNA.

Assay of DNA Polymerase α. DNA polymerase assays and the methods of data analysis were as described in the accompanying manuscript, with specific variations as indicated in the individual figure legends.

Results

Effect of 3′-Terminal Sugar Structure on Primer Recognition and Utilization. Previous results had indicated that DNA polymerase α was unable to bind 3′-phosphoryl primer termini (Fisher & Korn, 1979a). The catalytic behavior of 2′-deoxyribosyl- vs. ribsoyl-terminated primers was examined by comparing the three primer–templates $(dA)_{100}$-$(dT)_{25}$-$(U)_2$, $(dT)_{100}$-$(dA)_{25}$-$(A)_2$, and (dA, dG, dC, dT)$_{57}$-(A, G, C, U)$_2$ with their deoxy-primed precursors with respect to the apparent K_m (primer–template) and V_{max} of dNMP incorporation. With all three systems, the values of both kinetic parameters were found to be essentially identical. The products synthesized by the polymerase on the diribonucleotide-terminated primers were covalently linked to primer–template, but they could be separated completely by gentle alkaline hydrolysis (0.25 N KOH, 40 min, 37 °C), a treatment that had no effect on products synthesized on deoxy primers. The same treatment could be shown to result in the quantitative cleavage of terminal ribonucleotides from diribonucleotidyl-terminated primer molecules, as demonstrated by release of radioactive label from such primer molecules that had been prepared with [^{3}H]NTPs. These results thus confirmed the 2′,3′-OH structure of the original primer termini. When the diribosyl-terminated primers were treated with alkali under the same conditions *prior* to the polymerase reaction, to create a mixture of 2′-OH, 3′-PO_4 and 2′-PO_4, 3′-OH monoribosyl-terminated primers, the velocity of dNMP incorporation under conditions of limiting polymer was reduced by 50%. Together with our previous evidence that polymerase α does not recognize 3′-PO_4 termini, this result indicated that the enzyme could use 2′-PO_4, 3′-OH primers. While 2′,3′-dideoxy primers were, of course, unusable as substrates, the results in the accompanying paper demonstrated that in the absence of secondary, dNTP-dependent effects, DNA polymerase α bound such termini with essentially the same affinity as usable 2′-H, 3′-OH, and 2′,3′-OH primers. From these observations, we conclude that while a terminal 3′-PO_4 substituent can block the polymerase–primer reaction, a 3′-OH residue is not essential but, like a 3′-H, is permissive for binding to the primer stem. Moreover, it appears that the primer-binding interaction is not affected by the presence of H, OH, or PO_4 in the 2′-terminal position.

Single Terminal Mismatched Deoxynucleotide Prevents Correct Primer Binding by DNA Polymerase α. We had previously noted that an incompletely purified fraction of KB cell DNA polymerase α appeared capable of using a mismatched primer terminus (Sedwick et al., 1975), although extremely poorly and at a rate only ≤10% of that exhibited by a homogeneous preparation of KB cell DNA polymerase β (Wang et al., 1974). Similar studies with the calf thymus enzymes had also demonstrated effective utilization of mismatched primer termini by polymerase β but had suggested that polymerase α was unable to use such primer molecules (Chang, 1973). [In those earlier studies, we addressed explicitly some of the limitations of the nearest-neighbor methodology employed (Wang et al., 1974), particularly with respect to quantitative interpretations.] The mechanistic insights provided by our characterization of the binding of dideoxy termini by polymerase α (Fisher & Korn, 1981) suggested a novel and more rigorous approach to this question of mismatched primer-terminus recognition.

We reacted the hook polymer $(dC)_{100}$-$(dG)_{25}$ with TdT and ddATP, ddTTP, and ddCTP (see Materials and Methods) to produce a population of molecules in which essentially 100% of the primer termini were blocked with a single mismatched dideoxynucleotide. We then compared the mismatched hook polymers with $(dC)_{100}$-$(dG)_{25}$ and $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$ with respect to their capacity to inhibit the polymerization reaction with $(dA)_{100}$-$(dT)_{25}$ plus dTTP, and for their ability to induce substrate (dNTP) inhibition by inhibitor-complementary dGTP. The results are presented in Figure 1. In the *absence* of all three nonsubstrate dNTPs (Figure 1A), or in the presence of the *inhibitor-noncomplementary* dNTPs, dATP (Figure 1C) or dCTP (Figure 1D), the inhibitory potency of the mismatched polymer was clearly less than that of either of its two homologues; estimates of the apparent K_i (nucleotide) from the data in panel 1A were the following: for $(dC)_{100}$-$(dG)_{25}$, 41 nM; for $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, 45 nM; for $(dC)_{100}$-$(dG)_{25}$-(ddA, ddT, ddC)$_1$, 70 nM. Indeed, this last value of the apparent K_i was similar to that measured for $(dC)_{100}$ under the same reaction conditions, suggesting that there was minimal if any contribution from the terminally mispaired primer moiety to the kinetically determined binding affinity. The addition specifically of dGTP to the system (Figure 1B) led to the expected (Fisher & Korn, 1981) effects on the inhibition produced by $(dC)_{100}$-$(dG)_{25}$ (apparent K_i *increased* to ~210 nM) and $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$ (apparent K_i *decreased* to ~5 nM) but was without significant effect on the inhibitory potency of the mismatched polymer (apparent $K_i \approx 76$ nM). Finally, in Figure 1E, we demonstrate the inhibition by these three hook polymers as a function of dGTP concentration. While concentrations of the inhibitor-complementary dNTP of ≥20 μM led to complete inhibition of

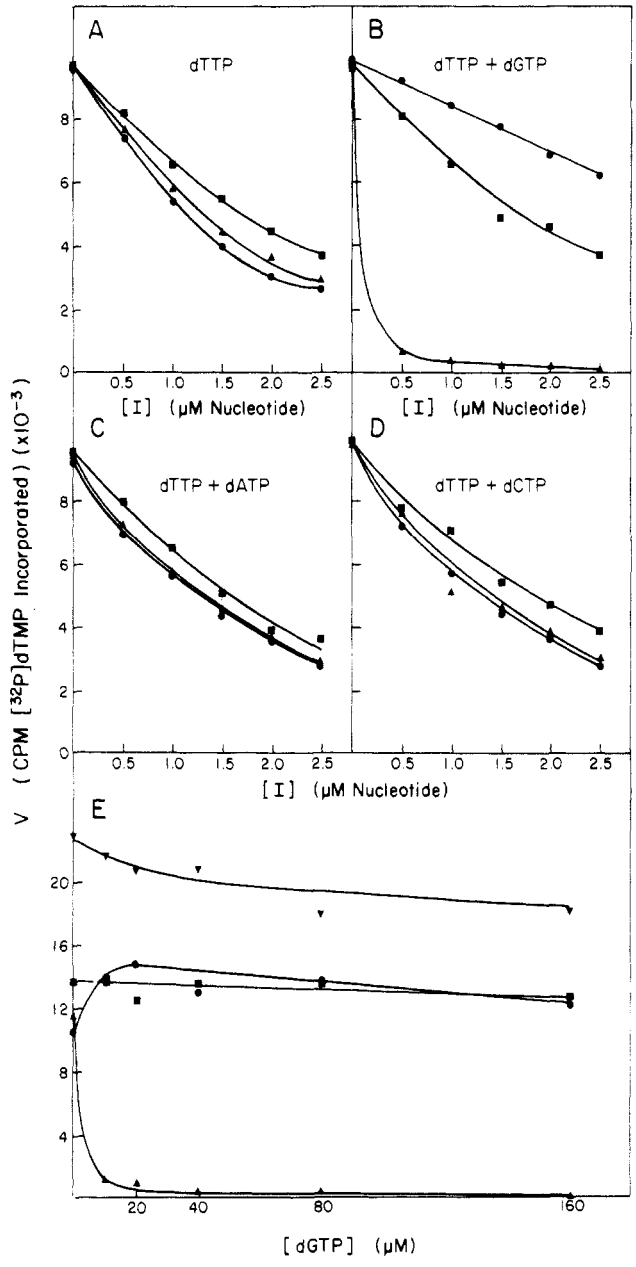

FIGURE 1: Effect of complementary and noncomplementary dNTPs on inhibition of DNA polymerase α by $(dC)_{100}$-$(dG)_{25}$, $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, and $(dC)_{100}$-$(dG)_{25}$-(ddA, ddT, ddC)$_1$. (A–D) Plots of initial velocity (v) vs. nucleic acid inhibitor concentration ([I]) were generated under conditions as described under Materials and Methods and as follows: In all incubations, substrate $(dA)_{100}$-$(dT)_{25}$ was at 40 μM (nucleotide), and [α-^{32}P]dTTP, 790 cpm/pmol, was at 40 μM. Additional unlabeled dNTPs were added as indicated on the individual panels, each at 40 μM. Concentrations of inhibitor nucleic acids were as shown on the abscissae. (●) $(dC)_{100}$-$(dG)_{25}$; (▲) $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$; (■) $(dC)_{100}$-$(dG)_{25}$-(ddA, ddT, ddC)$_1$. (E) Activity (v) vs. dGTP concentration. Substrate $(dA)_{100}$-$(dT)_{25}$ was at 40 μM; [α-^{32}P]dTTP, 1080 cpm/pmol, was at 40 μM. dGTP was added as shown on the abscissa. (▼) No nucleic acid inhibitor; the remaining symbols are as defined above.

the polymerization reaction in the presence of $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, and to the expected decrease in the inhibition by $(dC)_{100}$-$(dG)_{25}$, dGTP levels as high as 160 μM were without effect on the inhibitory capacity of the mismatched polymer homologue, which thus behaved identically with the unprimed template, $(dC)_{100}$.

DNA Polymerase α Requires an Octanucleotide Primer To Catalyze DNA Synthesis. The effect of oligonucleotide length on its ability to serve as primer for DNA polymerase α was examined by using oligo(thymidylate) molecules with $(dA)_{100}$

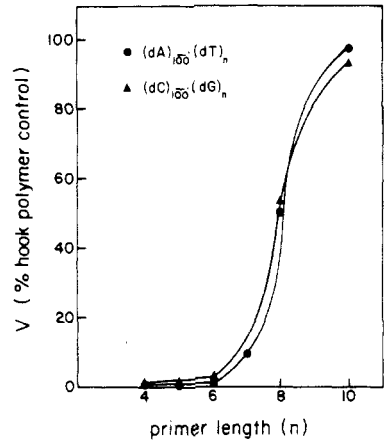

FIGURE 2: Effect of oligodeoxynucleotide primer length on activity of DNA polymerase α. Incubations were formulated as described under Materials and Methods and processed by spotting on DE-81 paper as described (Wang et al., 1977). Template was $(dA)_{100}$ or $(dC)_{100}$ and was present at 50 μM (nucleotide); primer was present at 1 μM (3′-OH termini). The appropriate dNTPs were at 40 μM each. Reactions were for 10 min, 37 °C. Hook polymer control incubations were performed under identical conditions with $(dA)_{100}$-$(dT)_{25}$ and $(dC)_{100}$-$(dG)_{25}$ at 50 μM (total nucleotide).

as template, and oligo(deoxyguanylate) molecules with $(dC)_{100}$ as template (Figure 2). Consistent with earlier studies with calf thymus DNA polymerases α and β (Chang et al., 1972; Chang, 1973), the minimum length for effective priming by either oligo(dT) or oligo(dG) was observed to be eight nucleotides; moreover, the results were identical whether the incubations were performed at 37 or at 23 °C (not shown), in agreement with our earlier interpretation (Fisher & Korn, 1979b) that preformed, stable primer–template complexes were not required for catalysis and consistent with our model of two-step binding of polymerase α to template followed by primer stem (Fisher & Korn, 1979a, 1981). The primer length requirement was also independent of the presence of the terminal 5′-PO_4 residue; i.e., the results were not altered by prior phosphatase treatment of the primer molecules.

Mg^{2+} Is an Effector of the Template-Binding Reaction of DNA Polymerase α with Pyrimidine Polydeoxynucleotides and Natural Heteropolymeric Single-Stranded DNA, but Not with Purine Polydeoxynucleotides. It is well established that a divalent metal, generally Mg^{2+} or Mn^{2+}, is a required cofactor for DNA polymerases. Although it is clear that one essential role of the metal is in the binding, and perhaps the alignment and conformational alteration (Slater et al., 1972; Sloan et al., 1975) of incoming dNTPs in the active center of the polymerase molecule, there are numerous observations pertaining to the effects of these cations on primer–template utilization [e.g., see Wang et al. (1977)] which suggest their important participation at other steps in polymerization catalysis. In the results that follow, we shall present evidence from kinetic and direct binding assays that Mg^{2+} is a potent effector of the binding interactions of KB cell DNA polymerase α with both templates and primers.

Kinetic assessment of the effect of Mg^{2+} on the polymerase α–template interaction is presented in Figure 3. Values of the apparent K_i for unprimed single-stranded polydeoxynucleotide (template) inhibitors were determined at varying concentrations of Mg^{2+}, with activated DNA as substrate (Figure 3A) and then with a set of hook primer–templates (Figure 3B). Variation of the divalent metal concentration between 2 and 8 mM was without significant effect on the apparent K_i values for the purine polydeoxynucleotides $(dA)_{100}$ and $(dG)_n$ but led to dramatic enhancement (decreased ap-

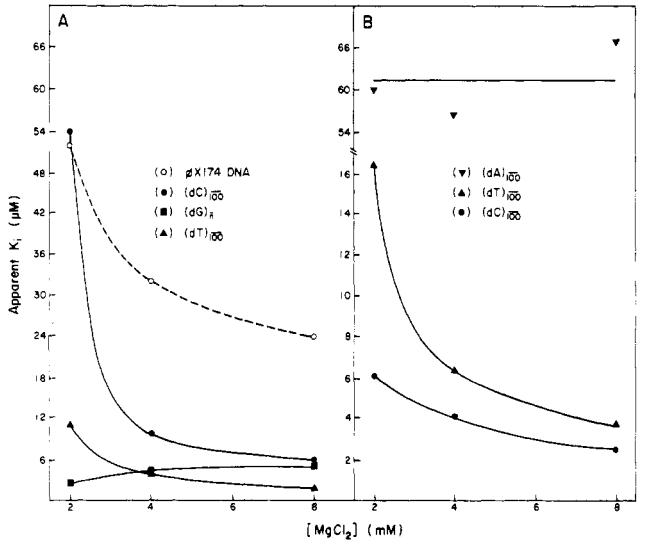

FIGURE 3: Effect of $MgCl_2$ concentration on values of the apparent K_i for various single-stranded polydeoxynucleotide inhibitors. Values of the apparent K_i were determined by Lineweaver-Burk analysis of inhibition of DNA polymerase α by single-stranded deoxypolymers at the indicated concentrations of $MgCl_2$. A single level of single-stranded inhibitor was used for each determination, and values of K_i were computed from changes in slope compared to the minus inhibitor line. (A) The single-stranded inhibitors were as shown. Incubations were performed as described under Materials and Methods, with activated salmon sperm DNA as the variable substrate in all determinations, and in the presence of four dNTPs at 40 μM each. (B) The single-stranded inhibitors were as shown. The variable substrates were (▼) $(dA)_{100}$-$(dT)_{25}$, (▲) $(dT)_{100}$-$(dA)_{25}$, and (●) $(dC)_{100}$-$(dG)_{25}$. The complementary dNTP was present at 40 μM. Note the discontinuity in the ordinate scale.

parent K_i) of the apparent affinity of the polymerase for pyrimidine deoxynucleotide polymers and to moderate enhancement of affinity for natural heteropolymeric single-stranded (φX174) DNA. The results with a pyrimidine copolymer (dC, $dT)_{100}$ were identical with those displayed for the pyrimidine homopolymers (not shown). Note that these responses to increased Mg^{2+} concentration are opposite to those that would be expected on the basis of nonspecific salt effects.

Sedimentation experiments were carried out to determine whether the kinetic effects of Mg^{2+} concentration on the apparent affinity of polymerase α for templates could be correlated directly with changes in the binding of polymerase to single-stranded DNA. In Figure 4, we demonstrate the effect of $MgCl_2$ concentration on the cosedimentation of the polymerase with φX174 single-stranded DNA. As $MgCl_2$ concentration was increased (panels A–C), the amount of polymerase α that was bound to the DNA (i.e., shifted away from the position of free enzyme) increased progressively. (Note that the relative peak heights in Figure 4 are somewhat deceptive due to the increased sedimentation velocity of φX174 DNA with increasing $MgCl_2$ concentration. This increase, which by itself would lead to a *decrease* in the amount of bound enzyme due to the greater disparity between the *s* values of free DNA and free enzyme, also serves to spread the peak of bound enzyme, as observed.) In Figure 4D, we compare the change in the fraction of bound (i.e., shifted) polymerase activity (demarcated by the broken lines in panels A–C), performed in duplicate, with the change in the kinetically determined values of the apparent K_i, derived from Figure 3, both as a function of $MgCl_2$ concentration. It is evident that

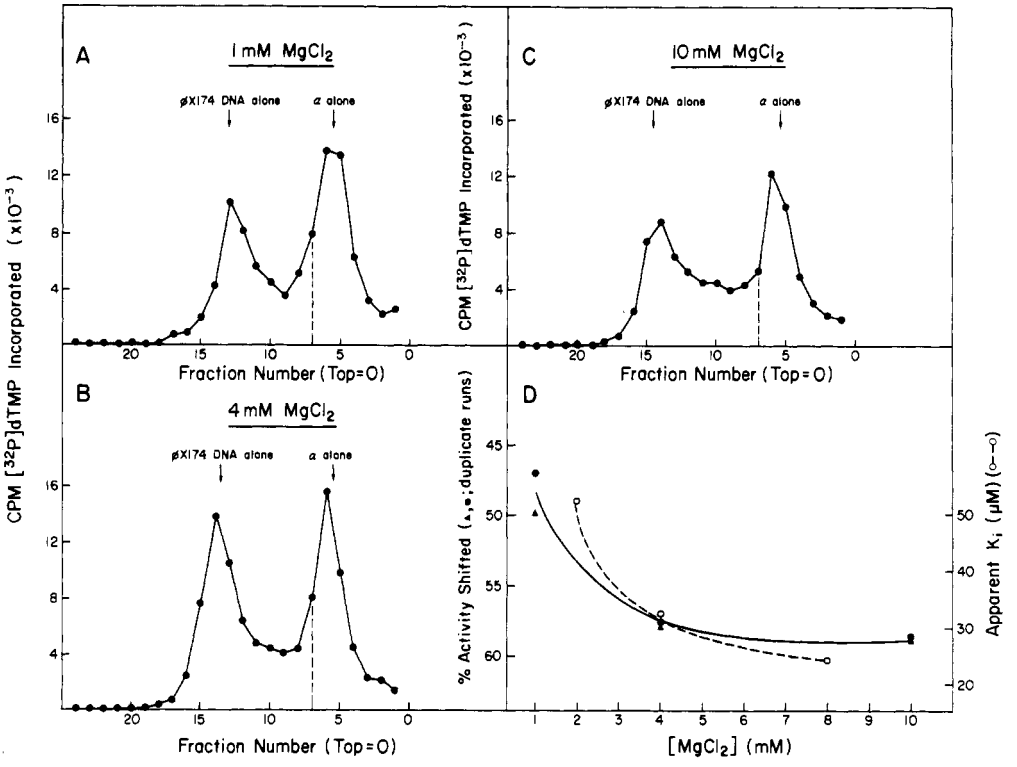

FIGURE 4: Sedimentation binding analysis of the effect of $MgCl_2$ concentration on the interaction of polymerase α with φX174 circular single-stranded DNA. Gradients were formulated as described under Materials and Methods, with concentrations of $MgCl_2$ as indicated in the individual panels. Centrifugation was in the SW 60 rotor for 4.5 h, 59 000 rpm, −2 °C, and assays were performed as described under Materials and Methods, with each dNTP at 20 μM and [α-^{32}P]dTTP at 170 cpm/pmol. The broken line in each profile (panels A–C) indicates the boundary that was used to calculate the "percent of polymerase activity shifted". (Raw recovered activity was corrected for position in the gradient as detailed in the accompanying paper.) Gradient loads were formulated as described under Materials and Methods and contained, in addition, 1 mM (nucleotide) φX174 single-stranded circular DNA and the concentration of $MgCl_2$ consistent with the particular gradient. (D Plot of percent polymerase activity shifted (bound) vs. $MgCl_2$ concentration (duplicate determinations), with superimposed values of the kinetically determined apparent K_i (over a similar range of $MgCl_2$ concentrations; obtained from Figure 3). Note that the left-hand ordinate scale is decreasing toward the top.

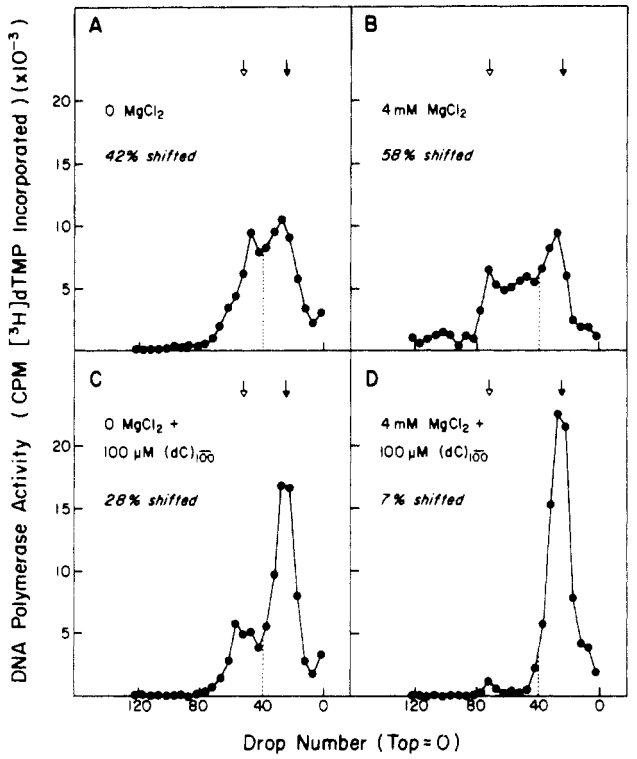

FIGURE 5: Sedimentation binding analysis of the interaction of polymerase α with ϕX174 circular single-stranded DNA and $(dC)_{100}$ in the presence and absence of $MgCl_2$. Gradients were formulated as described under Materials and Methods; centrifugation was in the SW 60 rotor, 4.5 h, 59000 rpm, -2 °C. Concentrations of $MgCl_2$ in gradients and loads were as shown on the figure. All loads contained 1 mM (nucleotide) ϕX174 DNA ± $(dC)_{100}$ as indicated. The broken line on each profile defines the boundary that was used for computation of the fraction of shifted polymerase activity, as in Figure 4. Assays were performed as described under Materials and Methods, with four dNTPs at 10 μM each and [^{3}H]dTTP at 370 cpm/pmol. The solid arrow on each panel indicates the position of DNA polymerase α when sedimented alone; the open arrow indicates the position of ϕX174 single-stranded circular DNA sedimented alone.

the increase in bound enzyme parallels almost exactly the decrease in the measured apparent K_i. Similar experiments were performed with the homopolymers $(dA)_{4000}$ and $(dT)_{3000}$ (data not shown); in both cases, the sedimentation data corroborated the kinetic results in Figure 3. Thus, the binding of the polymerase to $(dT)_{3000}$ was markedly enhanced, while binding to $(dA)_{4000}$ was unaffected, by increasing Mg^{2+} concentrations between 1 and 10 mM.

The experiments displayed in Figure 5 were carried out, first, to confirm the effect of Mg^{2+} concentration on polymerase binding to $(dC)_{100}$ by a competition binding assay (Fisher et al., 1981; Fisher & Korn, 1981) and, second, to examine the binding of enzyme to single-stranded DNA in the complete absence of added Mg^{2+}. As shown in panels 5A,B, the binding of polymerase α to ϕX174 DNA alone was significantly reduced, but certainly not abolished, in the absence of added Mg^{2+}. (Note that direct visual comparison of the profiles in panels A and B is complicated because the sedimentation velocity of ϕX174 DNA is reduced in the absence of Mg^{2+}, an effect that tends to counter the decreased affinity of the polymerase for the DNA in this circumstance.) In the presence of 4 mM Mg^{2+}, the enhanced affinity of polymerase α for $(dC)_{100}$ was clearly evidenced by the dramatic reduction in the fraction of polymerase that remained bound to the ϕX174 DNA (panels 5C,D). However, as above, the ability of the enzyme to bind $(dC)_{100}$ (i.e., the ability of $(dC)_{100}$ to compete with ϕX174 DNA for polymerase α) was readily demonstrable even in the absence of added Mg^{2+} (compare

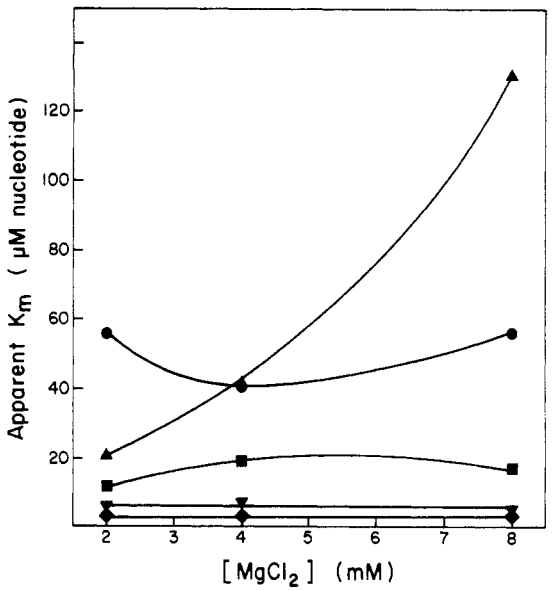

FIGURE 6: Effect of $MgCl_2$ concentration on the apparent affinity of DNA polymerase α for natural and synthetic primer–templates. Reactions were formulated as described under Materials and Methods, at varying levels of Mg^{2+} as indicated, with the primer–templates (●) activated DNA, (■) (dA, dG, dC, dT)$_{57}$, (♦) $(dT)_{100}$-$(dA)_{25}$, (▼) $(dC)_{100}$-$(dG)_{25}$, and (▲) $(dA)_{100}$-$(dT)_{25}$. Values of the apparent K_m for primer–template were obtained from Lineweaver–Burk analyses.

panels 5A,C). From the results of these kinetic and sedimentation experiments, we conclude that although (exogenous) Mg^{2+} enhances the binding of DNA polymerase α to pyrimidine polydeoxynucleotides and single-stranded DNA, it does not appear to be required for the binding of either.

Mg^{2+} Is Also a Potent Effector of the Primer-Binding Reaction of DNA Polymerase α. From the observations described in the previous section, it was reasonable to predict that as a consequence of enhanced nonproductive binding of the polymerase at template sites that were distant from primer termini, increasing Mg^{2+} concentrations in the range of 2–8 mM would prove to be inhibitory to the replication of heteropolymeric and pyrimidine polydeoxynucleotide templates, but not of exclusively purine-containing templates; that the patterns of inhibition would be essentially noncompetitive with respect to primer–template; and that the enhanced affinity of the enzyme for pyrimidine-containing templates would be manifested by corresponding decreases in values of the apparent K_m for the primed homologous templates. Direct test of these predictions led to the results presented in Figures 6 and 7. It is apparent (Figure 6) that variation of Mg^{2+} over the concentration range of interest was in fact without systematic effect on the values of the apparent K_m measured with activated DNA, (dA, dG, dC, dT)$_{57}$, $(dT)_{100}$-$(dA)_{25}$, and $(dC)_{100}$-$(dG)_{25}$ but did lead to an unanticipated and dramatic *decrease* in the apparent affinity of the polymerase for the substrate $(dA)_{100}$-$(dT)_{25}$; recall that over this same concentration range, Mg^{2+} is without effect on the affinity of the enzyme for poly(dA) template. These results would seem to require that the divalent cation, in addition to its effect on template binding, have an opposite and partially compensating effect of decreasing the affinity of polymerase α for primer. Accordingly, we carried out the detailed kinetic analysis of the inhibition of the replication of $(dA)_{100}$-$(dT)_{25}$ by Mg^{2+} that is presented in Figure 7.

From the Lineweaver–Burk analysis in panels 7A,B, it was evident that the Mg^{2+} inhibition appeared to be purely competitive with respect to primer–template and noncompetitive with respect to dTTP. Slope replots of the data in Figure 7A,

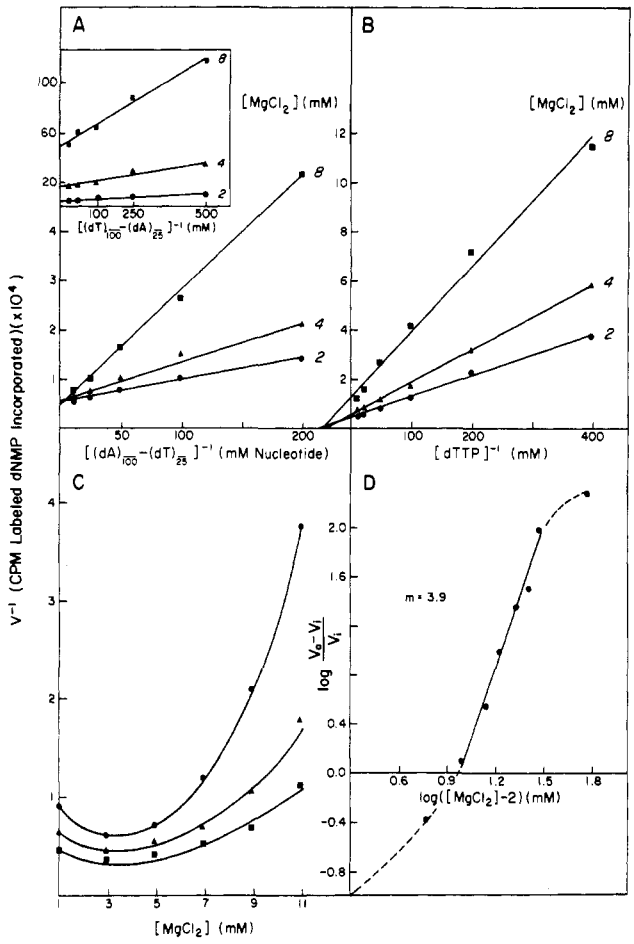

FIGURE 7: Effect of $MgCl_2$ concentration on substrate kinetics of DNA polymerase α with $(dA)_{100}$-$(dT)_{25}$. Reactions were formulated as described under Materials and Methods with variations as specified below. (A) Lineweaver–Burk analysis, v^{-1} vs. $[(dA)_{100}\text{-}(dT)_{25}]^{-1}$, at various levels of $MgCl_2$. The concentrations of $(dA)_{100}$-$(dT)_{25}$ and Mg^{2+} were as indicated on the figure; [α-^{32}P]dTTP, 690 cpm/pmol, was at 40 μM; DNA polymerase α was at 0.1 unit/100 μL. The inset shows a Lineweaver–Burk plot, v^{-1} vs. $[(dT)_{100}\text{-}(dA)_{25}]^{-1}$, at various levels of $MgCl_2$. [^{3}H]dATP, 760 cpm/pmol, was at 40 μM; DNA polymerase α was at 0.1 unit/100 μL. The pattern of inhibition is noncompetitive. (B) Lineweaver–Burk analysis, v^{-1} vs. $[dTTP]^{-1}$, at various levels of $MgCl_2$. Concentrations of $MgCl_2$ and of Mg:[α-^{32}P]dTTP (1:1), 830 cpm/pmol, were as shown on the figure; $(dA)_{100}$-$(dT)_{25}$ was at 5 μM (nucleotide). Incubations in panels A and B were for 6 min at 37 °C. (C) Dixon plot analysis, v^{-1} vs. $[MgCl_2]$, at various concentrations of $(dA)_{100}$-$(dT)_{25}$: (●) 10 μM; (▲) 20 μM; and (■) 40 μM (nucleotide). [α-^{32}P]dTTP, 450 cpm/pmol, was at 40 μM; DNA polymerase α was at 0.1 unit/100 μL. (D) Hill plot analysis of inhibition of DNA polymerase α by $MgCl_2$. V_o and V_i are as previously defined (Fisher & Korn, 1979a). Concentrations of Mg^{2+} were as indicated; $(dA)_{100}$-$(dT)_{25}$ was at 40 μM; [α-^{32}P] dTTP, 1930 cpm/pmol, was at 40 μM. The slope of the Hill plot (*m*) indicated on the panel was derived from the linear portion of the graph, as indicated by the *solid line*.

and slope and intercept replots of the data in Figure 7B, were markedly nonlinear (not shown), and at three different levels of $(dA)_{100}$-$(dT)_{25}$, Dixon plots (Figure 7C) displayed dramatic concavity consistent with multisite binding. Hill plot analysis (Figure 7D) of a similar experiment using a wider range of $MgCl_2$ concentrations generated a plot with an extended linear component with a slope (Hill coefficient) of 3.9. With primer–templates other than $(dA)_{100}$-$(dT)_{25}$, e.g., with $(dT)_{100}$-$(dA)_{25}$, $(dC)_{100}$-$(dG)_{25}$, and activated DNA, the patterns of Mg^{2+} inhibition were clearly different from that in Figure 7A and appeared to be distinctly noncompetitive (as originally expected) with respect to both DNA and dNTP substrates. With respect to primer–template, the most dramatic intercept

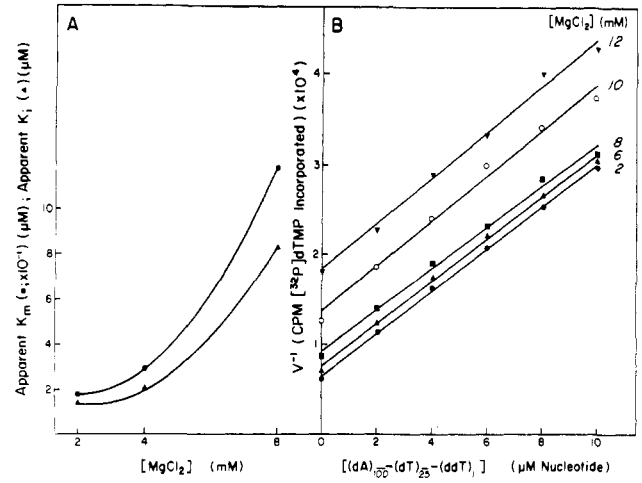

FIGURE 8: Effect of $MgCl_2$ concentration on the interaction between DNA polymerase α and $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$. Incubations were formulated essentially as described under Materials and Methods. (A) Values of the apparent K_m for $(dA)_{100}$-$(dT)_{25}$ were derived from Figure 7A; values of the apparent K_i for $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$ were determined by adding 10 μM (nucleotide) of the blocked polymer to reactions as described in the legend to Figure 7A and were computed from the resulting changes in slopes. $MgCl_2$ concentrations were as indicated. (B) Dixon plot analysis (Cleland, 1970) of the effect of $MgCl_2$ concentration on polymerase inhibition produced by $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$. Concentrations of blocked polymer and of $MgCl_2$ were as shown on the panel; $(dA)_{100}$-$(dT)_{25}$ (primer–template) was present at 80 μM (nucleotide) in each incubation; [α-^{32}P]dTTP, 640 cpm/pmol, was at 100 μM.[2]

effects were seen with $(dT)_{100}$-$(dA)_{25}$; these data are presented in the *inset* of Figure 7A.

Mg^{2+} Competes with Primer for the Primer-Binding Site on DNA Polymerase α. The combined results in Figures 3, 6, and 7, and, in particular, the lack of a detectable effect of Mg^{2+} concentration on the binding of polymerase α to $(dA)_{100}$ (template) and the behavior of Mg^{2+} as a purely competitive inhibitor with respect to the primer–template $(dA)_{100}$-$(dT)_{25}$, were compatible with the interpretation that the divalent cation competed directly with the primer moiety for the primer-binding site on the enzyme. However, at least two alternative possibilities had to be considered. The first was that the effect of Mg^{2+} concentration on primer binding was modulated *indirectly* through an effector site on the enzyme and thus that the polymerase could bind both primer stem and Mg^{2+} simultaneously. The second was that by virtue of the ordered sequential mechanism of substrate addition (Fisher & Korn, 1981), the Mg^{2+} effect was exerted somewhere downstream in the reaction sequence, analogously, for example, to dideoxy primer induced substrate inhibition by dNTPs. By examining the kinetics of the interaction between the two competitive inhibitors, Mg^{2+} and the blocked polymer $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$, and from sedimentation binding studies, we were able to rule out the latter possibility completely and show the former to be extremely unlikely.

In Figure 8A, the effect of Mg^{2+} concentration on the apparent K_m for $(dA)_{100}$-$(dT)_{25}$ is compared to its effect on the apparent K_i for $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$. It is clear that the effect of the cation on the apparent affinity of polymerase α for the blocked polymer was the same as that for the $(dA)_{100}$-$(dT)_{25}$ substrate; thus, the possibility that Mg^{2+} acted downstream of dNTP addition (i.e., in the product-generation steps) to affect primer binding could be excluded. To determine whether Mg^{2+} competed directly with primer or acted indirectly through an effector site, we performed the experiment depicted in Figure 8B. At a fixed level of all substrates, we analyzed the effect of Mg^{2+} concentration on the inhibition

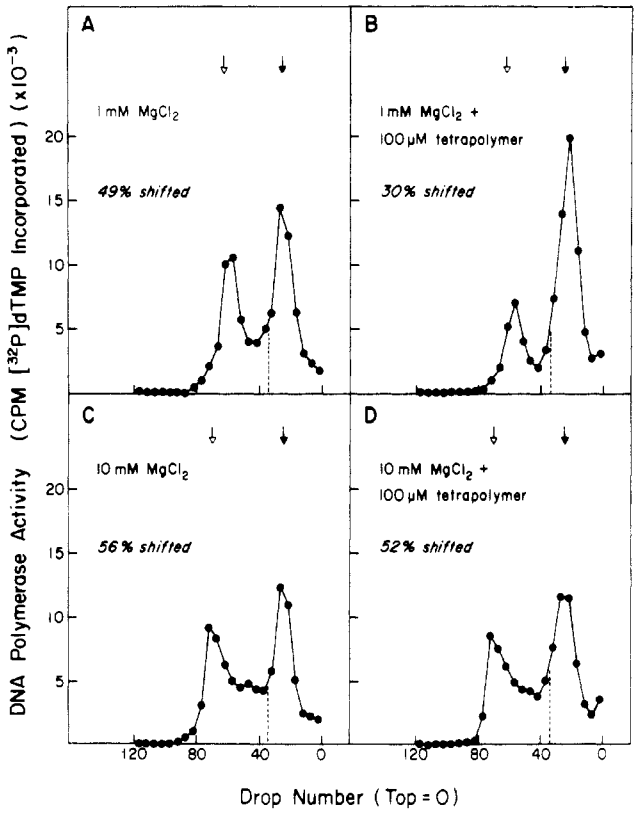

FIGURE 9: Sedimentation binding analysis of the effect of $MgCl_2$ concentration on primer binding to DNA polymerase α. Gradients were formulated essentially as described under Materials and Methods. Centrifugation was in the SW 60 rotor, 4.5 h, 59 000 rpm, −2 °C. The concentrations of $MgCl_2$ in the loads and gradients, and of (dA, dT, dG, dC)$_{57}$-(ddA, ddT, ddG, ddC)$_1$ (tetrapolymer) in the loads, were as indicated on the individual panels. Each loaded sample contained 1 mM (nucleotide) ϕX174 circular single-stranded DNA. The broken lines on each panel are as in Figure 4. Assays were performed as described under Materials and Methods; four dNTPs were each at 20 μM; [α-^{32}P]dTTP was at 160 cpm/pmol; raw recovery data were treated as in Figure 4. The arrows in each panel are as in Figure 5.

produced by $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$ to discern whether or not the binding of these two competitive inhibitors was mutually exclusive (Cleland, 1970). The pattern of parallel lines that was obtained supports the interpretation of mutually exclusive binding and the conclusion that the two inhibitors compete with one another for a common binding site on DNA polymerase α.[2]

Although the experiments presented in Figure 8A ruled out an effect of Mg^{2+} downstream of dNTP addition, it was necessary to perform a final set of sedimentation binding assays to exclude definitively any *necessary* contribution of dNTPs

[2] It should be noted that the level of discrimination of parallelism of the lines in Figure 8B is limited by the fact that $(dA)_{100}$ alone, whose binding to polymerase α is not affected at these levels of Mg^{2+} (Figure 3B), is itself a modestly potent competitive inhibitor of $(dA)_{100}$-$(dT)_{25}$ replication (Fisher et al., 1981). In an experiment like that in Figure 8B, i.e., v^{-1} vs. [$(dA)_{100}$] at varying levels of Mg^{2+}, one obtains a patterns of lines that converge on or just above the abscissa, consistent with simultaneous and essentially independent binding of the two inhibitor species. Thus, any contribution of the $(dA)_{100}$ template to the measured inhibition by $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$ would be expected to lead to ultimate convergence of the lines in Figure 8B. To maximize the primer-dependent component of the inhibition, we therefore ran these reactions at 2.5 times the normal concentration of dTTP to take advantage of the ordered mechanism of substrate addition. At these levels of dTTP, and over the range of Mg^{2+} concentrations used, the inhibition by the dideoxy-blocked polymer is 96–99% primer dependent; a convergence of the lines at this level would be within the error of the determnation.

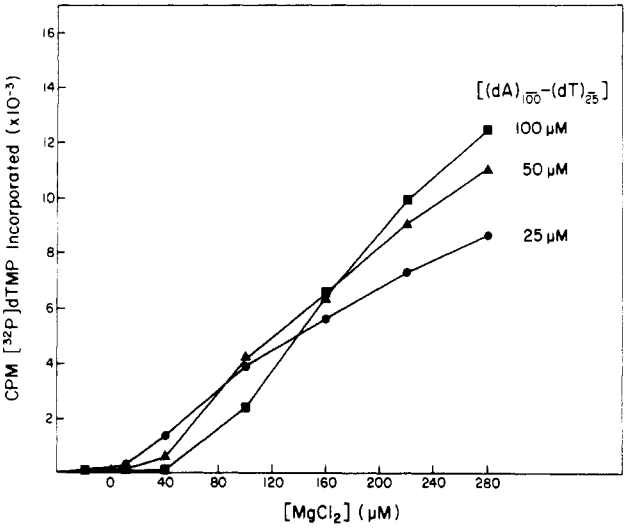

FIGURE 10: Effect of primer–template (nucleotide) concentration on the minimum level of $MgCl_2$ required for detection of DNA polymerase α activity. Incubations were formulated as described under Materials and Methods, with concentrations of $(dA)_{100}$-$(dT)_{25}$ as indicated; [α-^{32}P]dTTP, 1450 cpm/pmol, was at 40 μM; DNA polymerase α was presented at 0.1 unit/100 μL. $MgCl_2$ concentrations are shown on the abscissa; *zero* is defined as the point at which [$MgCl_2$] = [dTTP].

to the inhibition of primer binding caused by Mg^{2+}. The ability of the small polydeoxynucleotide (dA, dG, dC, dT)$_{57}$-(ddA, ddG, ddC, ddT)$_1$ to compete with ϕX174 single-stranded DNA was measured at 1 and 10 mM $MgCl_2$ in the absence of dNTPs (Figure 9). It can be seen in panels 9A,B that at the lower level of Mg^{2+} concentration, the addition of a small quantity of the dideoxy-terminated tetrapolymer resulted in a substantial decrease in the amount of polymerase α that cosedimented with the ϕX174 DNA. In contrast, at the higher level of divalent cation (panels 9C,D), the same quantity of added tetrapolymer caused only minimal displacement of the ϕX174 DNA-bound polymerase. Identical results were obtained in a parallel experiment in which (dA, dG, dC, dT)$_{57}$ was substituted for its dideoxy-blocked homologue (not shown). Since Mg^{2+} would be expected to have a comparable, positive effect on the (template) binding of *ϕX174* single-stranded DNA and the synthetic tetrapolymers, and given the significant contribution of base-pairable 3′ termini to the affinity of polymerase α for single-stranded DNA fragments (Fisher & Korn, 1979a), we interpret this experiment to indicate directly that primer binding was decreased by the higher level of Mg^{2+} concentration in the complete absence of dNTPs.

Minimum Level of Mg^{2+} Concentration Required for Catalysis Is Directly Related to DNA Concentration. The data presented thus far support the reasonable hypothesis that the catalytically productive binding of primer stem by DNA polymerase α occurs through a Mg^{2+} chelate of the phosphodiester backbone and that free Mg^{2+} competes at that binding site with the Mg^{2+}–primer complex. It is a prediction of this model that the concentration of Mg^{2+} required for catalytic activity should be dependent on the amount of DNA that is present as primer–template and requires chelation, as well as on the presence of competing cations. That the former is true is demonstrated by the data in Figure 10. As primer–template concentration was increased, the minimum level of Mg^{2+} required for detectable catalysis increased in parallel, such that at 40 μM $MgCl_2$, incorporation with 25 μM $(dA)_{100}$-$(dT)_{25}$ was greater than with 50 μM primer–template, and no activity could be measured with 100 μM polymer. By 220 μM $MgCl_2$,

the three curves had crossed, and the more typical reponse to increasing primer–template concentration was observed. The curves plateau between 0.5 and 1.5 mM $MgCl_2$ (not shown), beyond which activity decreases as seen in reciprocal form in Figure 7A,C. The addition of KCl to reactions like those in Figure 10 displaced the entire Mg^{2+} response curve to the right, again consistent with the model. Finally, at threshold concentrations of Mg^{2+}, i.e., the maximum levels of Mg^{2+} from Figure 10 that did *not* result in detectable catalysis, polymerase activity could not be elicited by adding K^+ or spermidine (Fisher & Korn, 1979a) to the reactions.

Discussion

Recent reports from this laboratory have demonstrated the capacity of combined classical kinetic analyses and direct sedimentation binding studies to provide substantial elucidation of many of the detailed features of the interactions of KB cell DNA polymerase α with dNTPs and nucleic acid templates (Fisher et al., 1979, 1981; Fisher & Korn, 1979a,b, 1981). The application of these same general strategies to the important problem of the polymerase–primer stem interaction has proved to be similarly successful and has now yielded significant new insights into a number of the specific properties of the enzyme's primer-binding site. From the data described in this and our earlier papers (Fisher & Korn, 1979a,b, 1981), it is possible to identify the following major features of the polymerase α–primer-binding reaction.

(1) Catalytically significant primer recognition requires an octanucleotide moiety, the terminal three to five nucleotides of which must be template complementary, and this minimum total length requirement appears to be independent of base composition, i.e., A-T vs. G-C, and of temperature. This result is in agreement with previous suggestions by us (Fisher & Korn, 1979b) and by Chang et al. (1972) that polymerase α does not require stably preformed primer–template complexes, and with the ordered sequence of substrate addition which specifies that template binding precedes primer binding (Fisher & Korn, 1979a, 1981).

(2) The primer-binding site is somewhat permissive with respect to the structure of the 3′-terminal sugar moiety. Thus, 3′-terminal ribonucleotidyl and deoxyribonucleotidyl residues appear to be utilized with equal catalytic facility, a finding in accord with earlier reports of the ability of partially purified DNA polymerases α from various sources to use oligo- and polyribonucleotide primers (Chang & Bollum, 1972; De Recondo et al., 1973; Spadari & Weissbach, 1975). Although a 3′-PO_4 substituent blocks primer binding, the recognition of termini bearing 3′-OH or 3′-H substituents, and 2′-H, 2′-OH, or 2′-PO_4 functions, appears to be indistinguishable.

(3) In sharp contrast to the relative lack of stringency for the terminal sugar, correct primer binding demands absolutely the proper pairing of the terminal primer nucleotide with template. It is clear from the experiments in Figure 1 that a single mismatched terminal base prevents correct primer binding and therefore the subsequent step of binding of complementary dNTP. The latter result, although again in accord with the ordered sequence of substrate addition, was unexpected, and it identifies an additional step in the polymerase reaction mechanism that must certainly contribute to the fidelity of replication. In the previous paper, we provide compelling evidence that the binding of complementary dNTP is template-sequence directed and primer requiring. The present observations further specify that the primer-binding site must itself exhibit remarkable discrimination for correct pairing of (at least) the terminal base, in the absence of which the conformational events that are presumably required for creation of the kinetically significant triphosphate binding site are prevented or impaired.

(4) Mg^{2+} plays an important role in both primer binding and template binding by DNA polymerase α. With respect to the latter reaction, we have demonstrated in this paper (Figures 3–5) that within a defined concentration range (2–10 mM) the divalent cation markedly enhances the binding of the enzyme to pyrimidine polydeoxynucleotides and heteropolymeric single-stranded DNAs (templates) but is without effect on polymerase binding to purine polydeoxynucleotides. With respect to the former reaction, we have documented for the first time, both by kinetic analyses and by physical binding experiments (Figures 6–9), that Mg^{2+} is directly inhibitory to the primer-binding step of the polymerase–nucleic acid interaction and that the binding of primer and of free Mg^{2+} appears to be mutually exclusive and thus competitive for an identical site on the enzyme protein. The kinetic analysis of the inhibition produced by Mg^{2+} (Figure 7) supports the interpretation that each polymerase α molecule possesses at least four Mg^{2+}-binding sites (or subsites) that are implicated in each inhibitory event. The kinetic evidence for four Mg^{2+}-binding sites is intriguing in light of previous evidence obtained from EPR studies of Mn^{2+} binding to *Escherichia coli* DNA polymerase I (Slater et al., 1972) that each enzyme molecule contained 1 tight and 4 ± 1 intermediate Mn^{2+}-binding sites that could be competed for by Mg^{2+} and were proposed to function in the polymerization reaction. However, what possible relationship there may be between the divalent cation binding sites suggested in our kinetic experiments and those earlier identifed by EPR can only be speculated at this time.

Given the purely competitive pattern of the Mg^{2+} inhibition of $(dA)_{100}$-$(dT)_{25}$ replication, the demonstrated lack of effect of the metal on polymerase binding to poly(dA), the requirement of the enzyme for a primer stem of eight nucleotides in length, the documented relationship (Figure 10) between the minimum amount of Mg^{2+} required for catalysis and the amount of nucleic acid substrate that is present, and the lack of any obvious structural relationship between Mg^{2+} and the DNA primer stem with which it competes, it is reasonable to propose that the normal binding of primer by polymerase α occurs through a Mg^{2+}–primer complex that involves the coordination of four Mg^{2+} ions with seven or eight phosphate residues in a phosphodiester linkage and thus that the primer-binding site contains four Mg^{2+}–primer-binding subsites at which free Mg^{2+} is able effectively to compete. In the context of this model, it is of interest to recall that phosphatase treatment of the octanucleotide primer is without effect, suggesting that only the seven internal phosphate groups in the primer backbone may be involved and that one of the four Mg^{2+}-binding subsites might thus be available to participate in polymerase translocation subsequent to the insertion of the next dNMP. The model also supports the attractive corollary hypothesis that given the relatively low intracellular concentrations of Mg^{2+} in vivo, as well as the relatively low affinity of DNA (or "chromatin") for this cation, Mg^{2+} may be brought to the polymerase·template·primer complex by dNTPs (which have an extremely high affinity for Mg^{2+}) and may then be cycled through the polymerization reaction, univectorially and processively to each of the four postulated Mg^{2+}–primer-binding subsites, thereby facilitating not only phosphodiester bond formation (catalysis) but also polymerase translocation. Clearly, a critical test of these hypotheses demands the application of more sophisticated technology and the expenditure of far more enzyme protein than is currently available.

As a final comment, we note that a good deal of information has now been reported concerning the deleterious effects of a number of different divalent metals, some of them known mutagens or carcinogens, on the fidelity of a variety of prokaryotic and eukaryotic DNA polymerases [(see, for example, Sirover & Loeb (1976), Sirover et al. (1979), and Kunkel & Loeb (1979)]. Although a convincing model that encompasses this substantial body of experimental data has not yet been forthcoming, it is of interest that with most or all of these metals the misinsertion frequency of noncomplementary dNMPs tends to increase progressively and dramatically under conditions of divalent cation concentration that are increasingly inhibitory to the polymerization reaction per se. In light of the evidence we have presented in this paper that (1) the divalent cation Mg^{2+} is a powerful effector of the primer-binding reaction and (2) the primer-binding step is of critical importance to the subsequent step of complementary dNTP recognition and binding by the polymerase, it is attractive to suggest that divalent cation–induced polymerase infidelity may be largely, if not entirely, the consequence of perturbation of the polymerase–primer interaction.

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