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Ordered Sequential Mechanism of Substrate Recognition and Binding by KB Cell DNA Polymerase α[†]

Paul A. Fisher and David Korn*

ABSTRACT: We have used a steady-state kinetic approach in conjunction with direct velocity gradient sedimentation binding studies to examine the detailed steps that are involved in the recognition of DNA primer-template and dNTPs by near-homogeneous human DNA polymerase α. We demonstrate that the interaction of the polymerase with its substrates obeys a rigidly ordered sequential terreactant mechanism, with template as the first substrate, followed by primer as the second substrate and dNTP as the third. Although the binding of primer is prerequisite to the kinetically significant binding of dNTP, specification of which of the four dNTPs can then add to the enzyme is absolutely determined by the base sequence of the template (the first substrate). The critical element in the proof of the ordered mechanism is the demonstration of the phenomenon of induced substrate inhibition; the presence of a dideoxy-terminated primer (dead-end inhibitor) induces substrate inhibition by dNTP which is absolutely restricted to the dNTP complementary to the template to which the blocked primer is annealed. This inhibition is kinetically competitive with 3'-hydroxyl-terminated (unblocked) primer and approaches 100% at saturating levels of the complementary dNTP. Direct binding studies document the specific and exclusive ability of complementary dNTPs to drive the polymerase into a stable dead-end complex with the proposed structure, enzyme·template·dideoxy primer·dNTP, thus corroborating the kinetic observations. Attempts to elucidate the order of product release from the enzyme by product inhibition studies have shown the polymerization reaction to be essentially irreversible and have thus been unsuccessful. On the basis of the known processivity of KB cell DNA polymerase α, a preliminary model involving initial release of pyrophosphate is reasonable; however, the relationship between product release and the process of polymerase translocation remains obscure. All of the kinetic and sedimentation binding studies were performed on a variety of homopolymeric and natural heteropolymeric DNA substrates, and the consistency of the results establishes absolutely the qualitative identity of the general mechanism by which human DNA polymerase α recognizes and replicates polydeoxynucleotide primer-templates, regardless of their precise physicochemical nature.

Previous studies from this laboratory (Fisher et al., 1979, 1981; Fisher & Korn, 1979a,b; Korn et al., 1981) have established the moderately processive mechanism of polymerization by a near-homogeneous preparation of KB cell DNA polymerase α (Fisher & Korn, 1977) and have provided an initial description of some of the molecular signals that appear to govern the interactions of this enzyme with a variety of defined nucleic acid substrates. From the results of these earlier studies (Fisher & Korn, 1979a,b), we concluded that the initial step in nucleic acid recognition involved the binding of single-stranded DNA at a template-binding site and that only subsequent to this event was the polymerase capable of recognizing and binding a (potentially) base-pairable primer terminus. We have further demonstrated (Fisher et al., 1981) that the template-binding step appears to be strongly regulated by the template base composition (or sequence), and we have suggested from these and other observations that a number of the kinetic properties of KB cell DNA polymerase α are compatible with the behavior of a conformationally active protein.

In this report, we describe the results of a detailed steady-state kinetic study, performed in conjunction with direct sedimentation binding analyses, that was undertaken to explore the possible relationship of these several nucleic acid binding

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0006-2960/81/0420-4560$01.25/0

events to the important mechanistic problem of appropriate dNTP recognition and insertion. We demonstrate, first, that substrate addition to DNA polymerase α obeys a rigidly ordered sequential terreactant mechanism, such that kinetically significant dNTP binding (third substrate) is absolutely dependent on antecedent binding to the polymerase of both template (first substrate) and primer (second substrate). Second, we demonstrate that specification of which dNTP can be bound is rigorously dictated by the template base sequence. These data thus provide strong evidence of conformational activity within the active site of DNA polymerase α, and they offer not only substantial new insights into the intrinsic mechanism of catalysis by the enzyme but also a novel approach to the problem of DNA polymerase fidelity.

Materials and Methods

The sources of most materials, and many of the methods, were as previously noted (Fisher & Korn, 1977, 1979a,b; Fisher et al., 1979, 1981). Unlabeled dNTPs were from Boehringer, and unlabeled dideoxynucleoside triphosphates (ddNTPs) were from P-L Biochemicals; all triphosphates were used without further purification. Bacteriophage T7 DNA was prepared as described by Hinkle & Chamberlin (1972). Terminal deoxynucleotidyl transferase (TdT), purified from calf thymus, was the generous gift of Dr. R. L. Ratliff (Los Alamos Scientific Laboratory). All other defined nucleic acid molecules were prepared and characterized as described under Materials and Methods.

Preparation of 2′,3′-Dideoxy-Terminated Heteropolymers.[1] Dideoxynucleotide-terminated synthetic heteropolymers—(dA, dG, dC)$_{51}$-(ddA, ddG, ddC)$_1$, (dT, dG, dC)$_{77}$-(ddT, ddG, ddC)$_1$, and (dA, dG, dC, dT)$_{57}$-(ddA, ddG, ddC, ddT)$_1$—were synthesized from the respective 3′-hydroxyl-terminated heteropolymer substrates that had been prepared and characterized as previously described (Fisher et al., 1981). For the addition of a single ddNMP by TdT, reactions (1 mL) contained 2 mM (nucleotide) heteropolymer, 50 μM each of the appropriate ddNTPs, 40 mM sodium cacodylate, pH 7.2, 8 mM $MgCl_2$, 1 mM 2-mercaptoethanol, and 6000 units of TdT and were incubated at 37 °C for 6 h. Blockage of 3′ termini was assessed both by addition of labeled dNTPs plus a fresh aliquot of TdT to small portions of the preparative incubation and by ablation of the capacity of the synthetic polymers to support the single-strand incorporation reaction with DNA polymerase α (Fisher & Korn, 1979b; Fisher et al., 1981). By both criteria, 3′-terminal blockage was essentially complete. The blocked polymer products were recovered as previously described (Fisher & Korn, 1979b; Fisher et al., 1981).

Preparation of 2′,3′-Dideoxy-Terminated Hook Polymers. Hook polymer (dA)$_{100}$-(dT)$_{25}$-(ddT)$_1$ was prepared in a reaction (400 μL) containing 100 mM KPO_4, pH 6.8, 100 mM sodium cacodylate, pH 7.2, 2 mM $CoCl_2$, 1 mM 2-mercaptoethanol, 1 mM (nucleotide) (dA)$_{100}$-(dT)$_{25}$ [prepared as earlier described (Fisher et al., 1981)], 50 μM ddTTP, and 6000 units of TdT. Incubation was for 6 h at 37 °C; progress of the reaction to completion was monitored as described above for the heteropolymers. Hook polymer (dC)$_{100}$-(dG)$_{25}$-(ddG)$_1$ was prepared in a reaction similar to that for (dA)$_{100}$-(dT)$_{25}$ except that the divalent cation was 8 mM $MgCl_2$, 50 μM ddGTP was used as monomer, and 1 mM (nucleotide) (dC)$_{100}$-(dG)$_{25}$ (Fisher et al., 1981) served as polymer.

Preparation of Gapped T7 DNA Containing Base-Paired 2′,3′-Dideoxy Termini. Phage T7 [^{3}H]DNA was reacted with pancreatic DNase I under conditions previously determined to introduce about 1 nick per 5000 nucleotides (Fisher & Korn, 1979a) and was then resected with *Escherichia coli* exonuclease III as earlier described (Fisher et al., 1979) to generate a population of single-stranded gaps with a (Poisson) mean length of 100 nucleotides. Release of [^{3}H]dTMP from the nicked T7 DNA was used to monitor exonucleolytic digestion and control the extent of gap formation. The gapped [^{3}H]DNA was then reacted to extent with excess *E. coli* DNA polymerase I in the presence of three dNTPs (no dTTP), essentially as described by Fisher et al. (1979), in order to create gaps in which the next nucleotide to be incorporated was defined. The product was characterized as previously described for "specific competitor DNA" (Fisher & Korn, 1979a). A portion of the specifically gapped (i.e., partially replicated) T7 [^{3}H]DNA was then incubated with TdT plus ddTTP under conditions identical with those detailed above for the preparation of (dA)$_{100}$-(dT)$_{25}$-(ddT)$_1$. Completeness of the dideoxy block was monitored by measuring the loss of capacity of the gapped DNA to serve as a substrate for the incorporation of [^{32}P]dTMP by TdT or DNA polymerase α. [Note that attempts to take advantage of the ability of *E. coli* DNA polymerase I to incorporate dideoxynucleotides (Atkinson et al., 1969) were unsuccessful in producing a completely blocked product. Rather, at extent (equilibrium), a significant fraction of the 3′ termini appeared to retain hydroxyl groups.]

Assay of DNA Polymerase α. Unless otherwise indicated, all kinetic assays of DNA polymerase α were performed in reactions (100 μL) that contained 20 mM Tris-HCl, pH 8.2, 2 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 200 μg/mL gelatin, 0.05 unit (~50–100 pM) of fraction VIII DNA polymerase α, and concentrations of nucleic acid and dNTPs as specified in the individual figures. All incubations were for 5 min at 37 °C; incorporation was linear for at least 20 min under these conditions. Straight lines for kinetics plots were drawn by the method of least squares.

Sedimentation Binding Analyses. Sedimentation binding studies were performed in linear 20–40% (v/v) glycerol gradients that contained 20 mM Tris-HCl, pH 8.2, 1 mM 2-mercaptoethanol, 4 mM $MgCl_2$, 50 μg/mL bovine serum albumin, 0.1 mM EDTA, and additional components as indicated in the individual figures. The loaded samples (100 μL) were formulated to be consistent with the gradients in composition, and they contained in addition 2 units of fraction VIII DNA polymerase α and additional components as indicated. Sedimentation was in the SW 60 rotor at 59000 rpm at −2 °C for the times specified. Gradient fractions (150 μL) were collected from the bottom of the tube directly into the appropriate reaction mixture for polymerase assay. All incubations contained activated DNA at a final concentration of 15 mM (nucleotide) in order to compete out differential effects due to nucleic acid inhibitors that were present in the gradients, 20 mM Tris-HCl, pH 8.2, 10 mM $MgCl_2$, 2 mM 2-mercaptoethanol, 200 μg/mL bovine serum albumin, and dNTPs as indicated. The incubations were for 30 min at 37 °C in a final volume of 300 μL. The recoveries of loaded polymerase activity varied from 35 to 50% and did not correlate with any particulars of the sedimentation conditions. Values of incorporated radioactivity for individual gradient fractions were corrected for differential glycerol inhibition of polymerase activity in different regions of the gradient according to an experimentally constructed standard curve. At the extremes, a given amount of added polymerase activity in the top fraction of a typical gradient assayed at 70% of that value in the bottom fraction.

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

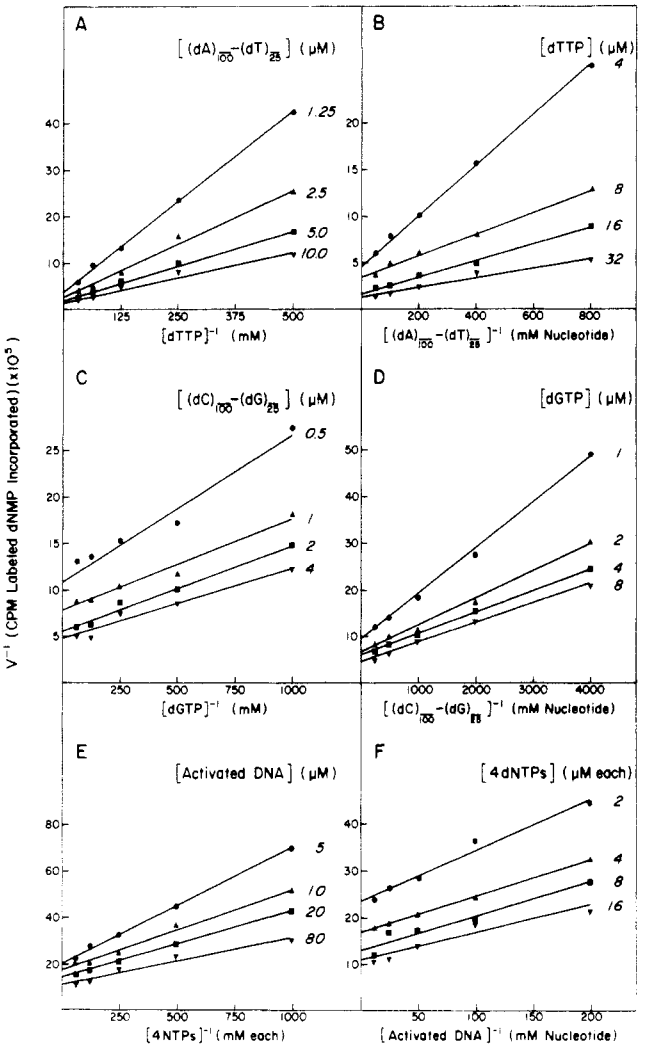


FIGURE 1: Initial velocity patterns for DNA polymerase α with three different primer–template/dNTP systems. The Lineweaver–Burk analyses were performed as indicated; concentrations of the various substrates are shown in the individual panels. (A and B) dTTP at a specific activity of 15 000 cpm/pmol; (C and D) dGTP at a specific activity of 3000 cpm/pmol; (E and F) each dNTP at 2000 cpm/pmol.

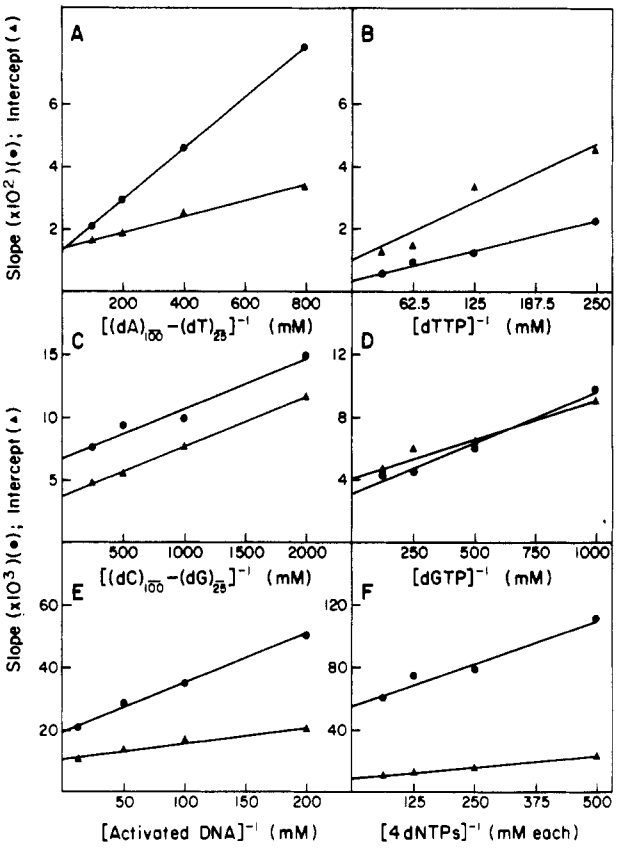


FIGURE 2: Slope and intercept replots of the data in Figure 1. Slopes and intercepts were derived from Figure 1 and plotted as indicated. Values of kinetic constants were computed from these replots according to Cleland (1970) and are as follows: For the dNTPs, dTTP, K_m = 10 μM, K_{ia} = 30 μM; dGTP, K_m = 2 μM, K_{ia} = 1 μM; four dNTPs, K_m = 2 μM (each), K_{ia} = 3 μM (each). For the nucleic acids (in terms of nucleotide concentrations), $(dA)_{100}$-$(dT)_{25}$, K_m = 4 μM, K_{ia} = 5 μM; $(dC)_{100}$-$(dG)_{25}$, K_m = 1 μM, K_{ia} = 1 μM; activated DNA, K_m = 6 μM, K_{ia} = 4 μM. Maximal velocities of incorporation (picomoles of total nucleotide/5 min) were for dTMP, 7; dGMP, 10; four dNMPs, 7.

Results

DNA Polymerase α Exhibits a Sequential Pattern of Substrate Addition. In previous reports (Fisher & Korn, 1979a,b), we have established that template (single-stranded polydeoxynucleotide) and primer behave as mechanistically distinct substrates for KB cell DNA polymerase α and that their order of binding to the enzyme is template first, followed by primer stem. In the present study, the use of unimolecular primer–templates permitted these two components to be treated as a single substrate with which "primer" and "template" concentrations could be varied together at a constant fixed ratio while the effects of varying the concentration of the third substrate, dNTP, were being examined. [This is a generally accepted approach for simplifying the steady-state kinetic analysis of terreactant systems, i.e., the variation of the concentration of one substrate in the presence of different levels of the other two substrates held in a fixed ratio (Cleland, 1970; Rudolph & Fromm, 1979).] Figure 1 displays the results obtained when primer–template and dNTP were each treated as the variable substrate in the presence of changing fixed concentrations of the other. The experiments were performed with two hook polymer substrates, $(dA)_{100}$-$(dT)_{25}$ and dTTP (panels A and B) and $(dC)_{100}$-$(dG)_{25}$ and dGTP (panels C and D), as well as with activated DNA and the full complement of four dNTPs (panels E and F). In each instance, qualitatively identical linear converging patterns were obtained that intersected to the left of the ordinate, and in all cases (Figure 2), replots of the slopes and intercepts derived from the double-reciprocal plots were also linear. Values of apparent kinetic constants computed from the replots were consistent with those previously reported (Fisher et al., 1979, 1981) on the basis of less thorough analyses. Values of K_{ia}(DNA) and K_{ia}(dNTP) (Cleland, 1970) were generally similar to or identical with the respective K_m values. These initial velocity patterns are characteristic of a classical sequential reaction mechanism and exclude a ping-pong mechanism with respect to the addition of primer–template and dNTP, respectively, to DNA polymerase α. Because of the imprecise physicochemical nature of the primer–template substrates, i.e., the potential for nonproductive binding of polymerase to stretches of template that are distant from primer termini, the values of the kinetic constants obtained from the replots must be regarded as upper limits. However, the qualitative mechanistic significance of the linear replots is not diminished by this quantitative uncertainty. It is important to emphasize that there is no evidence of complex (i.e., nonlinear) behavior in the polymerase–substrate interaction, and both the patterns of the double-reciprocal plots and the fact that none of the slope replots goes through the origin rule out any kind of equilibrium-ordered mechanism (Cleland, 1970) of substrate addition to the polymerase.

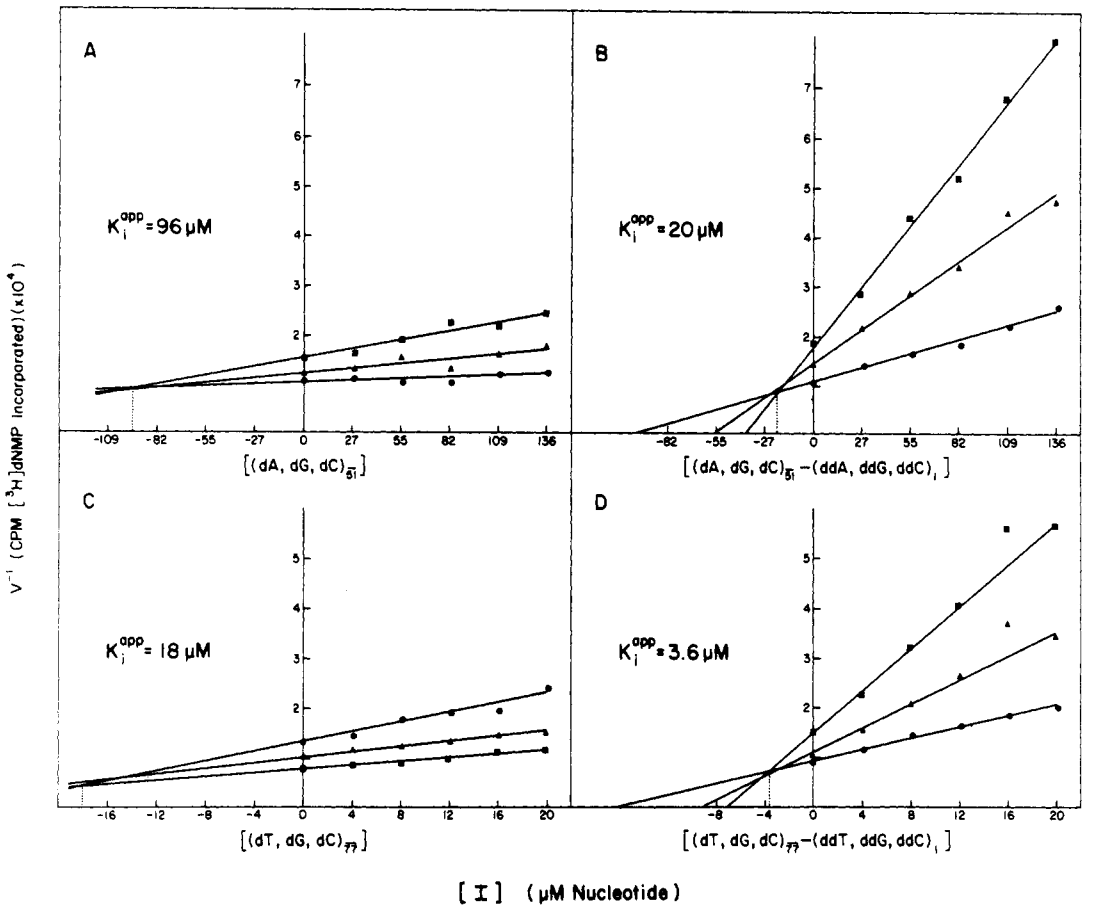


FIGURE 3: Comparison of inhibitory potency of synthetic heterotripolymers containing 3′-hydroxyl or 3′-dideoxy termini. Heteropolymers and their dideoxy-terminated homologues, prepared as described under Materials and Methods, were compared by Dixon plot analysis with respect to their ability to inhibit the polymerization reaction with activated DNA. Concentrations of inhibitor polymers and values of the apparent K_i are as indicated. (A and B) Activated DNA was at 20 (■), 40 (▲), or 80 μM (●); four dNTPs were present at 40 μM each; [^{3}H]dATP was at 1060 cpm/pmol. (C and D) Activated DNA and dNTP concentrations were as in (A) and (B); [^{3}H]dTTP was at 1340 cpm/pmol.

Polymerization Reaction Is Kinetically Irreversible. The evidence that substrate addition to *E. coli* DNA polymerase I (McClure & Jovin, 1975) and to mouse myeloma DNA polymerase β (Tanabe et al., 1979) is ordered and not random was derived entirely from product inhibition studies with PP_i, the results of which supported the interpretation that the order of substrate binding to both enzymes was DNA followed by dNTP. In the case of the *E. coli* polymerase, with poly(dA-dT) as substrate, a $K_i(PP_i)$ of 400 μM was reported, while with murine polymerase β, with DNA as substrate, significant inhibition was observed at PP_i concentrations of 20–80 μM. Our attempts to examine the kinetics of $MgPP_i$ (product) inhibition of DNA polymerase α were unsuccessful. At concentrations of DNA or dNTP of about $0.2K_m$ (i.e., well below saturation), we observed only minimal inhibition of the polymerization reaction even at $MgPP_i$ concentrations as high as 1.2 mM, while at lower concentrations, $MgPP_i$ was actually stimulatory. When the different hook polymer substrates were used, the effects of $MgPP_i$ were complex and variable and seemed most reasonably to be secondary to changes in free Mg^{2+} concentration, assuming that chelation of Mg^{2+} by PP_i was less than complete. (In the accompanying paper, we describe in some detail the effects of varying Mg^{2+} levels on these several polymerase reactions.) Attempts to use Na_2PP_i as inhibitor were similarly unsatisfactory. The polymerization reaction performed by the purified KB enzyme (under these reaction conditions) thus appears to be kinetically irreversible at workable levels of PP_i (≤1 mM), and what effects of PP_i were observed were complex, probably indirect, and kinetically uninterpretable. PP_i was therefore of no value for probing the order of substrate binding to polymerase α.

2′,3′-Dideoxy-Terminated Synthetic Polymers Are More Potent Inhibitors of DNA Polymerase α Than Their 3′-Hydroxyl-Terminated Homologues. We previously reported (Fisher & Korn, 1979a) that the inhibitory potency of 3′-hydroxyl-terminated single-stranded fragments of heteropolymeric DNA was significantly greater than that of intact single-stranded circular DNA molecules, while the inhibitory potency of 3′-phosphoryl-terminated fragments was comparable to that of the intact circles. In striking contrast, 3′-dideoxy-terminated synthetic heterotripolymers were found to be considerably *more potent* than their 3′-hydroxyl-terminated homologues as inhibitors of activated DNA replication by polymerase α. The results are presented as Dixon plots in Figure 3 for the blocked and unblocked heterotripolymer pairs $(dA, dG, dC)_{51}$ vs. $(dA, dG, dC)_{51}$-$(ddA, ddG, ddC)_1$ (panels A and B) and $(dT, dG, dC)_{77}$ vs. $(dT, dG, dC)_{77}$-$(ddT, ddG, ddC)_1$ (panels C and D). In each instance, as described previously (Fisher et al., 1981), the inhibition produced by the heterotripolymeric fragment was linearly competitive with the activated DNA substrate (as confirmed by Lineweaver–Burk analysis), but in both of the cases illustrated, the apparent K_i for the dideoxy-blocked polymer was ~5-fold lower than that of the unblocked homologue. Results qualitatively identical with those in Figure 3 were obtained from comparisons of $(dA)_{100}$-$(dT)_{25}$ with $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$, and $(dC)_{100}$-$(dG)_{25}$ with $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, but with the hook polymers, where all dideoxy termini would be expected to be stably base paired with template, the decreases in the apparent K_i were ~10–20-fold.

Enhanced Inhibitory Potency of a 2',3'-Dideoxy Polymer Is Dependent on the Presence of the dNTP That Is Complementary to the Template to Which the Dideoxy Primer Is Annealed. Two mechanistic explanations seemed plausible for the enhanced inhibitory potency of dideoxy- vs. 3'-hydroxyl-terminated inhibitor polymers. The first was that in the presence of a full complement of dNTPs (as in Figure 3), the 3'-hydroxyl polymers (I) were themselves capable of serving as alternate substrates, and thus as alternate product inhibitors, while the dideoxy polymers (ddI) functioned as true dead-end inhibitors regardless of the complement of dNTPs in the incubation. Thus, in the presence of dNTP complementary to the inhibitor polymer template (I)

$$E + I + dNTP \underset{k_2}{\overset{k_1}{\rightleftharpoons}} E{\cdot}I{\cdot}dNTP \xrightarrow{k_3} E + P'$$

where P' = alternate product. If this forward pathway to E + P' were fast relative to the backward dissociation of the E·I complex formed in the absence of dNTP, then the availability of the appropriate complementary dNTP would open an additional fast pathway to free E and lead to a decreased inhibitory potency of I relative to that of ddI. Although this explanation was compatible with the known substrate capacity of the hook homopolymer and the 3'-hydroxyl-terminated heterotripolymer inhibitors, it did not per se distinguish between random and ordered sequential mechanisms of substrate addition to the polymerase.

In contrast, the second mechanistic possibility *required* that the inhibitor polymers, I and ddI, enter the reaction pathway *prior* to the addition of dNTP and that both E·I and E·ddI be capable of binding a complementary dNTP and thus proceeding one step further down the reaction pathway to E·I·dNTP and E·ddI·dNTP, respectively. According to this scheme, the former complex could proceed as before to the formation of the alternate product and free E, while the latter would continue to be a dead-end complex, but one from which the backward dissociation to free E was now that much more difficult. This second scheme thus leads to the prediction of the phenomenon of induced substrate inhibition, which is "highly diagnostic for ordered mechanisms" (Cleland, 1979).

These two hypotheses were tested by using a variety of synthetic polymers of defined base composition as both substrates and inhibitors. With $(dA)_{100}$-$(dT)_{25}$ as substrate in the presence of dTTP alone, we first examined the inhibitory potency of $(dC)_{100}$-$(dG)_{25}$ (Figure 4A). In this case, no forward dissociation of the E·I complex is possible because of the absence of dGTP. The addition of either dCTP or dATP to the reaction was without effect on the apparent K_i, but upon addition of dGTP to the system, there was an approximately 5-fold *decrease* in the inhibitory potency of $(dC)_{100}$-$(dG)_{25}$, consistent with the first hypothesis, the opening of a rapid "alternate product" pathway to free enzyme. As previously reported (Fisher et al., 1981), in the *absence* of $(dC)_{100}$-$(dG)_{25}$, the noncomplementary dNTPs (dATP, dCTP, and dGTP) had no effect on the replication of $(dA)_{100}$-$(dT)_{25}$. In contrast to the data in Figure 4A, the results obtained with the blocked-polymer inhibitor, $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$ (Figure 4B), were strikingly different. In the absence of dGTP, whether with dTTP alone or in the presence of dATP or dCTP, the inhibitory potency of the dideoxy polymer was very similar to that of the 3'-hydroxyl-terminated homologue. Upon addition of dGTP, however, there was a 5–10-fold *increase* in the inhibitory potency of the blocked polymer. This result is clearly consistent with the second hypothesis of an ordered sequential mechanism of substrate addition to polymerase α. Thus, by the combined effects of both proposed mechanisms, in the presence specifically and exclusively of the complementary triphosphate, dGTP, the inhibitory efficacy of the blocked polymer compared to that of its unblocked homologue was enhanced by 20–50-fold.

The results of a comparable experiment (Figure 4C,D) with the polymer pairs $(dT, dG, dC)_{77}$ (a poor polymerase substrate) and $(dT, dG, dC)_{77}$-$(ddT, ddG, ddC)_1$ demonstrated a qualitatively similar but quantitatively far less dramatic effect of the dideoxy block that could be largely attributed to the greatly diminished contribution of the alternate product pathway (Figure 4C) with this particular heteropolymer. [Note that both the poor substrate capacity and the decreased magnitude of the induced substrate inhibition observed with the $(dT, dG, dC)_n$ polymers are very likely due to the limited ability of those polymers to form suitable base-paired primer termini.] Finally, similar experiments were carried out with $(dA)_{100}$-$(dT)_{25}$ and $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$ as inhibitors of the reaction with $(dC)_{100}$-$(dG)_{25}$. Entirely consistent with the results in Figure 4B, while dCTP and dATP had no effect on the apparent K_i for either inhibitor, the addition of dTTP increased the inhibitory potency of the dideoxy polymer by about 10–20-fold (data not shown).[2]

Presence of dNTPs Has No Effect on the Inhibiton of DNA Polymerase α by Unprimed Templates. When experiments analogous to those displayed in Figure 4 were carried out with the unprimed templates $(dC)_{100}$, $(dA)_{100}$, and ϕX174 DNA, it was found that the inhibition of DNA polymerase α produced by these single-stranded polydeoxynucleotides was completely independent of which dNTPs were present in the incubations (data not shown). Thus, Dixon plot analysis (v^{-1} vs. [I]) in the presence of the substrate dNTP alone, or of substrate dNTP plus each of the nonsubstrate triphosphates, resulted in four coincident straight lines.

Demonstration of Induced Substrate (dNTP) Inhibition of DNA Polymerase α in the Presence of Dideoxy Polymer (Blocked Primer–Template) Inhibitors. In order to establish rigorously the induction of classical substrate (dNTP) inhibition by a dideoxy-blocked primer, the kinetics of dNTP utilization were compared in polymerization reactions that contained either substrate polydeoxynucleotide alone or a mixture of substrate polydeoxynucleotide and a fixed quantity of the homologous dideoxy-blocked primer–template inhibitor

[2] In the experiments described here, i.e., with $(dC)_{100}$-$(dG)_{25}$ and dGTP as substrates and $(dA)_{100}$-$(dT)_{25}$ as the inhibitor, the addition of dTTP was without discernible effect on the apparent K_i of the unblocked polymer; however, in the absence of dTTP, inhibition of this reaction by either $(dA)_{100}$-$(dT)_{25}$ or $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$ was so minimal (Fisher et al., 1981) as to make comparisons difficult. Subsequent experiments, performed under conditions similar to those in Figure 9D, allowed definition of a dTTP-mediated effect in this system which was contrary to those illustrated in Figure 4; i.e., the addition of increasing concentrations of dTTP significantly *increased* the inhibitory potency of $(dA)_{100}$-$(dT)_{25}$. [The effect followed a simple saturation profile which exactly paralleled the incorporation of [α-^{32}P]dTMP (monitored along with the inhibition of incorporation of [^{3}H]dGMP).] The maximally inhibited reaction (at 320 μM dTTP) was ~60% of that seen in the absence of dTTP. We have previously described in detail (Fisher et al., 1981) the extremely low affinity of polymerase α for poly(dA), particularly in the presence of competing heterologous ligands, e.g., $(dC)_n$-$(dG)_m$. Although we did not know whether this was due to a very low on-rate or a very high off-rate, the present results indicate that in the presence of dTTP, the opening of the alternate product pathway actually serves to stabilize the interaction of polymerase α with the $(dA)_n$-$(dT)_m$ hook polymer and thus modestly to increase the inhibitory potency of this polymer. This in turn suggests, in terms of the kinetic formalism depicted in Figure 10, that for this specific hook polymer system $k_2 + k_4 > k_6 + k_7$ and that the low apparent affinity of polymerase α for poly(dA) templates is most likely due to a very high off-rate.

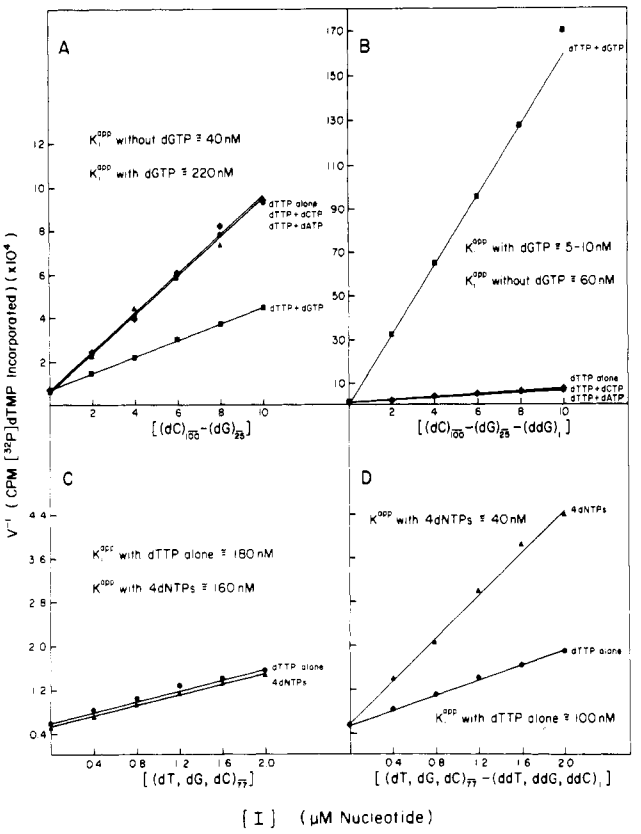


FIGURE 4: Effect of complementary and noncomplementary dNTPs on the inhibitory potency of 3′-hydroxyl-terminated and 3′-dideoxy-terminated synthetic polymers. Reaction mixtures were formulated as described under Materials and Methods. The data are displayed as Dixon plots; the concentrations of inhibitor polymers are indicated on the abscissae. (A and B) The *substrates* were $(dA)_{100}$-$(dT)_{25}$ at 40 μM and [α-^{32}P]dTTP at 540 cpm/pmol, 40 μM. *Additional dNTPs* were added to the various incubations as follows: (●) none; (■) 40 μM dGTP; (▲) 40 μM dATP; (♦) 40 μM dCTP. Note the difference in ordinate scales in panels A and B. (C and D) The *substrates* were $(dA)_{100}$-$(dT)_{25}$ at 40 μM and [α-^{32}P]dTTP at 710 cpm/pmol, 40 μM. *Additional dNTPs* were added as follows: (●) none; (▲) 40 μM each of dATP, dGTP, and dCTP.

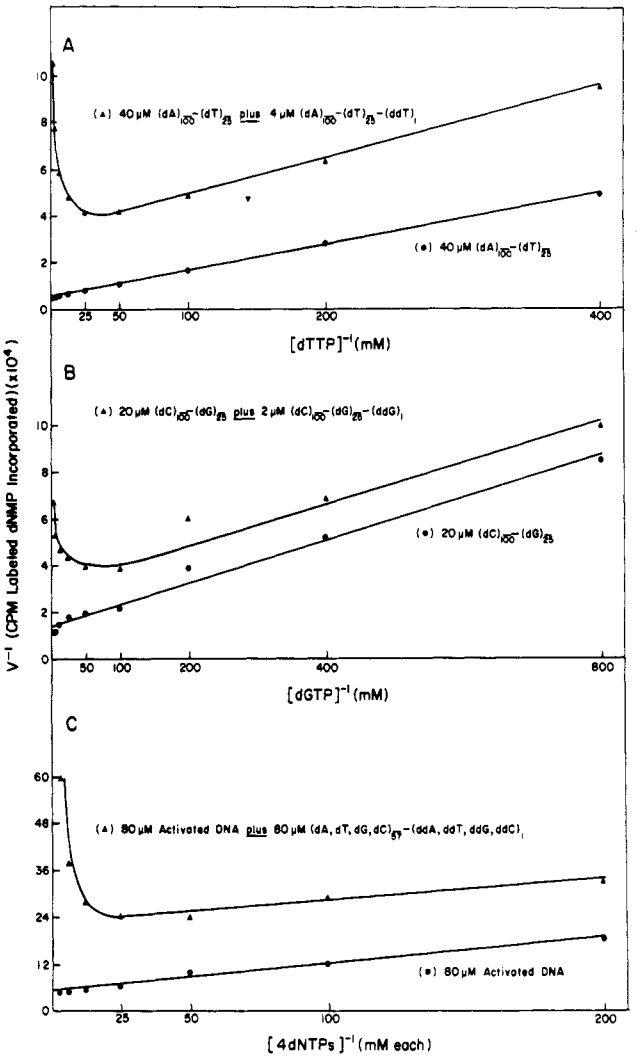


FIGURE 5: Induced substrate (dNTP) inhibition of DNA polymerase α activity. Reaction mixtures were formulated as described under Materials and Methods and as further specified in each panel. (A) [α-^{32}P]dTTP was at 620 cpm/pmol. (B) [^{3}H]dGTP was at 420 cpm/pmol. (C) [α-^{32}P]dTTP was at 620 cpm/pmol.

(Figure 5). Reciprocal plots (velocity^{-1} vs. [dNTP]$^{-1}$) are shown for $(dA)_{100}$-$(dT)_{25}$ ± $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$ (Figure 5A), for $(dC)_{100}$-$(dG)_{25}$ ± $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$ (Figure 5B), and for activated DNA ± (dA, dT, dG, dC)$_{57}$-(ddA, ddT, ddG, ddC)$_1$ (Figure 5C). All three systems demonstrated classical induced substrate (dNTP) inhibition that was dependent upon the presence of the dideoxy polymers. In contrast, and most important, when *unprimed* single-stranded template molecules [$(dA)_{100}$, $(dC)_{100}$, or φX174 DNA] were mixed with the corresponding primer–templates, there was no substrate-induced inhibition at increasing levels of dNTP, and the pattern of inhibition produced by the unprimed polydeoxynucleotides was linearly noncompetitive with respect to dNTP (data not shown).

Corroboration of Kinetic Interpretations by Direct Sedimentation Binding Experiments. The effect of dNTPs on the binding of polymerase α to gapped T7 DNA bearing either 3′-hydroxyl or 2′,3′-dideoxy base-paired termini is shown in Figure 6. As illustrated in panels A, C, and E, binding to the 3′-hydroxyl DNA molecules decreased progressively in the presence of increasing concentrations of dNTPs, consistent with the kinetically derived conclusions drawn from Figure 4A. Similarly consistent with the kinetic interpretations from Figures 3–5 was the even more dramatic effect of dNTPs on the binding of polymerase α to the 2′,3′-dideoxy DNA (panels B, D, and F). In the absence of dNTPs, the binding of the polymerase to the 2′,3′-dideoxy DNA (panel B) was very similar to that observed with the 3′-hydroxyl DNA molecules (panel A). In the presence of dNTPs, however, binding to the dideoxy DNA was remarkably enhanced, and it was further evident (panels D and F) that there was both a dNTP-dependent shift of the [^{3}H]DNA to a higher average *s* value as well as a significant amount of enzyme that sedimented more rapidly than the main peak of T7 DNA toward the bottom of the gradient. We have previously presented kinetic evidence in support of the interpretation that KB polymerase α possesses at least two interactive DNA binding sites (Fisher & Korn, 1979a; Fisher et al., 1981); the gradient profiles in panels D and F strongly suggest polymerase-mediated cross-linking of DNA that, if confirmed, would require that there be at least two active sites on each enzyme molecule. As is indicated in the legend to Figure 6, recoveries of polymerase activity were very similar in all gradients, and all have been normalized to a single arbitrary value to facilitate direct visual comparison of the several panels.

Having established that a stable, dNTP-dependent enzyme-dideoxy primer-template complex could be identified and quantitated by direct physical means, it was essential to demonstrate that the formation of that complex was complementary dNTP specific in the manner predicted from the kinetic results in Figure 4. To examine this point, we made

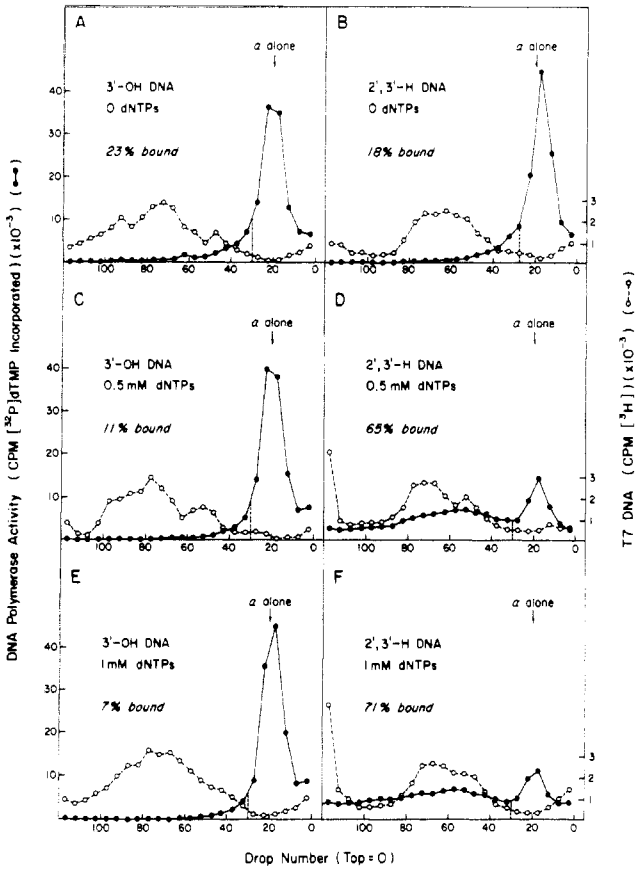


FIGURE 6: Direct sedimentation binding analysis of the interaction of DNA polymerase α with gapped T7 DNA. Gradients, loads, and preparation of 3'-hydroxyl-terminated (3'-OH DNA) and 2',3'-dideoxy-terminated (2',3'-H DNA) gapped T7 DNA were as described under Materials and Methods. In addition, loaded samples contained 80 μM (nucleotide) of the indicated T7 [^{3}H]DNA and dNTPs as follows: (A and B) No added dNTPs; (C and D) 0.5 mM each of dATP, dGTP, and dCTP; 20 μM dTTP; (E and F) 1 mM each of dATP, dGTP, and dCTP; 40 μM dTTP. The above concentrations of dNTPs were also present throughout the gradient; dTTP concentrations were low so as to facilitate subsequent polymerase assay. Sedimentation was for 4 h. Assays were as described under Materials and Methods, with final dNTP concentrations adjusted to 500 μM each of dATP, dGTP, and dCTP; 20 μM [α-^{32}P]dTTP at 150 cpm/pmol. Recoveries of polymerase activity varied between 35 and 50%; for ease of graphical presentation, all counts have been normalized to a single overall recovery. The fraction of bound polymerase in each panel is defined as the ratio of that to the left of the dashed vertical line divided by total recovered activity.

use of competition binding assays (Fisher et al., 1981), the results of which are depicted in Figure 7 and are once again entirely corroborative of the kinetic data. The principle of these experiments was to examine the effects of specific complementary and noncomplementary dNTPs on the ability of two homologous hook polymers, $(dC)_{100}$-$(dG)_{25}$ and $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, to compete with single-stranded circular ϕX174 DNA as ligands for polymerase α. Panels A–C demonstrate that the binding of the polymerase to unprimed template (ϕX174 DNA) alone is unaffected by the presence of one or four dNTPs, thus confirming the fact that primer is required in order for dNTPs to have any effect on the polymerase–nucleic acid interaction. (The profiles in panels A–C also serve to illustrate the extremely good reproducibility of this simple binding assay.) The addition of $(dC)_{100}$-$(dG)_{25}$ to the system led to the results shown in panels D–F. In the absence of dNTPs (panel D), or in the presence of three *noncomplementary* dNTPs (panel F), the slowly sedimenting (3–4S) hook polymer present in the load at 12.5 μM (nucleotide) competed very effectively with ϕX174 DNA [present at 1 mM (nucleotide)] such that only about 25% of the polymerase originally sedimenting with the circular DNA molecules continued to do so. Note particularly that the presence of the noncomplementary dNTPs was totally without effect. In contrast, in the presence specifically of dGTP (panel E), the ability of the hook polymer to compete with ϕX174 DNA was substantially reduced, leading to an approximately 2-fold increase in the amount of polymerase that sedimented with the circular DNA. The results in panels D–F thus confirm exactly the conclusions drawn from the kinetic experiments in Figure 4A.

In the final set of competition experiments, we examined the effects of adding the blocked polymer $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$ to this system (panels G–I). Once again, in the absence of dNTPs (panel G), or in the presence of three *noncomplementary* dNTPs (panel I), the blocked polymer was essentially identical with its unblocked homologue in its capacity to compete with ϕX174 DNA for the binding of polymerase α; again note that the noncomplementary dNTPs were totally without effect on the binding interactions. However, in the presence of dGTP, as predicted from Figure 4B, the binding of the dideoxy polymer by DNA polymerase α was enhanced dramatically, as indicated by the almost complete elimination of binding to ϕX174 DNA.

Dideoxy Primer Induced Substrate (dNTP) Inhibition Is Competitive with DNA Primer–Template. An important prediction of the ordered sequential mechanism of substrate binding that we are proposing is that the substrate inhibition that is induced by dideoxy polymers must be competitive with DNA primer–template and not, for example, with dNTP (Cleland, 1979). This can be tested most easily with DNA polymerase α by using different hook homopolymers as substrates and inhibitors, and thus taking advantage of the four independent template/dNTP pairs. The results of the experiments are shown in Figure 8. In the presence of $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, and with $(dA)_{100}$-$(dT)_{25}$ and dTTP as substrates, dGTP inhibits the polymerization reaction in a manner that is classically competitive with $(dA)_{100}$-$(dT)_{25}$ (panel A); in the absence of $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, these concentrations of dGTP have no effect whatever. The reciprocal experiment is shown in panel B, with $(dC)_{100}$-$(dG)_{25}$ and dGTP as substrates, $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$ as the inducing agent, and dTTP as the induced inhibitor. Once again, the inhibition produced by dTTP is classically competitive with the polymer primer–template. In both experiments in Figure 8, the patterns of induced inhibition were noncompetitive with respect to the substrate dNTPs, as predicted (data not shown).

Induced Substrate Inhibition Can Be Driven to Completion by Saturating Levels of Inhibitor dNTP. The final question to be addressed is whether the ordered sequence of substration addition is quantitatively the only available reaction pathway (i.e., a "rigidly ordered" mechanism) or simply a relatively preferred route with less favored alternatives also likely (i.e., a random mechanism with a preferred binding order) (Cleland, 1979). Induced substrate inhibition provides one approach to evaluating these two possibilities by determining whether the induced inhibition becomes essentially total or remains only partial at truly saturating levels of the induced substrate inhibitor. For the rigidly ordered mechanism, plots of (reaction velocity)$^{-1}$ vs. concentration of induced inhibitor [I], would be expected to be linear, while for the random, preferred mechanism, the plots would be hyperbolic. Two types of experiments were performed to examine this question (Figure 9). In the first (panels A and B), pairs of blocked and unblocked polymer homologues were studied in the presence of

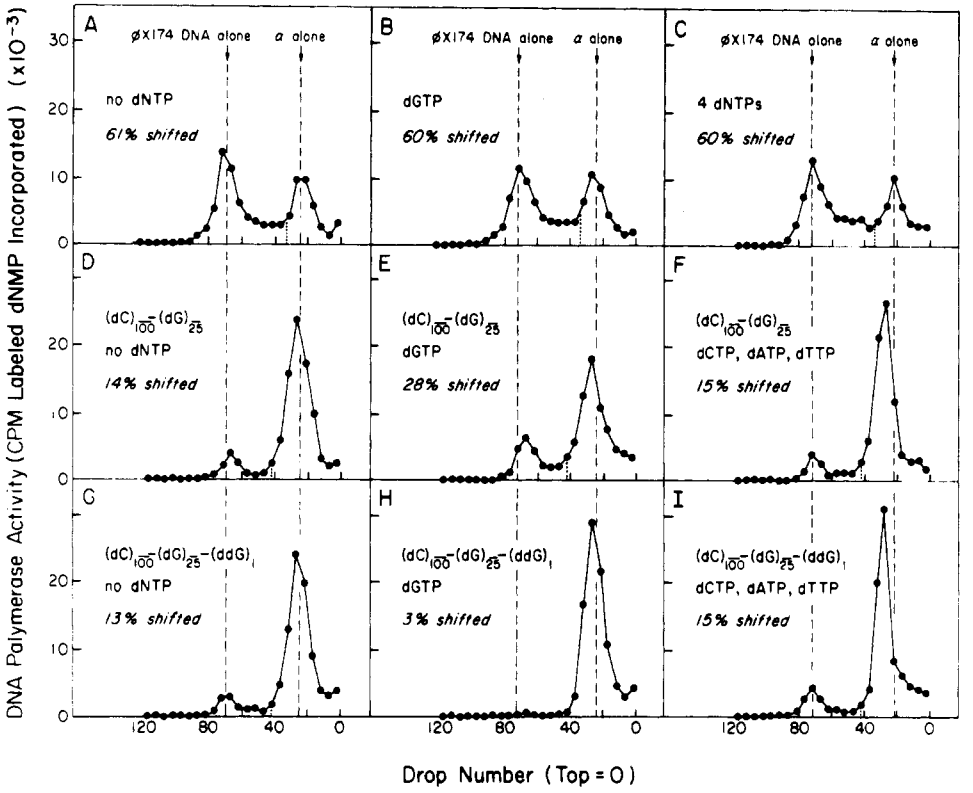


FIGURE 7: Competitive sedimentation binding analysis of the interaction of polymerase α with φX174 DNA vs. 3′-hydroxyl and 2′,3′-dideoxy hook homopolymers. Gradients and loads were formulated as described under Materials and Methods. All loaded samples contained 1 mM (nucleotide) φX174 single-stranded circular DNA and hook polymer competitors as indicated on the panels. Both loads and gradients contained the specified dNTPs, each at a final concentration of 100 μM. "% shifted" refers to the percent of total recovered DNA polymerase activity that had shifted down the gradient from the position of polymerase alone (7 S). Assays were performed as described under Materials and Methods. Final concentrations of unlabeled dNTPs were 55 μM of each; labeled dNTPs were as follows: (A, D, and G) [^{3}H]dTTP, 10 μM, 280 cpm/pmol; (B, E, and H) [^{3}H]dTTP, 10 μM, 310 cpm/pmol; (C) [^{3}H]dTTP, 40 μM, 180 cpm/pmol; (F and I) [^{3}H]dGTP, 10 μM, 300 cpm/pmol. Recoveries of polymerase activity ranged from 40 to 50%; for ease of graphical presentation, all counts were normalized to a single overall recovery.

varying concentrations of the (single) complementary dNTP; the insets in panels A and B (v^{-1} vs. [I]) are reasonably linear and consistent with a rigidly ordered mechanism. However, a maximum level of inhibition of only ~80% (panel B) was reached in these analyses. The use of a single dNTP both as substrate and as inhibitor requires that extremely large quantities of labeled dNTP be expended; in addition to the economic cost, this raises technically insurmountable problems with respect to very high and variable radioactivity backgrounds. To circumvent this difficulty, we made use of independent primer–template/dNTP pairs to achieve nearly complete induced substrate inhibition by unlabeled dNTPs. These results are displayed in panels C and D and demonstrate induced substrate inhibition by dTTP of >95% (panel C) and by dGTP of >98% (panel D) with no evidence of deviation from linear patterns. In the absence of the appropriate inducing dideoxy polymer, as shown, the effect of the inhibitory dNTP on the polymerization reaction is minimal and may be due either to small amounts of contaminating triphosphates in these commercial dNTPs or to nonspecific effects on the polymerase of high levels of noncomplementary dNTPS, as have previously been described (McClure & Jovin, 1975) and attributed to possible interactions of the dNTPs at the template-binding site.

Discussion

Previous studies of the mechanism of catalysis of a near-homogeneous preparation of KB cell DNA polymerase α have focused on some of the detailed properties of the interaction of the purified enzyme protein with a variety of defined nucleic acid substrates (Fisher et al., 1979, 1981; Fisher & Korn, 1979a,b). These investigations have provided considerable evidence in support of a model with the following salient features: (1) The polymerase–nucleic acid interaction takes place by a two-step mechanism that requires initial binding of single-stranded polydeoxynucleotide (template), followed by binding of primer; (2) primer recognition requires that there be at least a minimal degree of terminal base pairing between primer and template, tentatively involving approximately three to five nucleotides (Fisher & Korn, 1979b); (3) a terminal 3′-phosphate group blocks primer binding completely; and (4) the kinetic features of the interaction of the polymerase with single-stranded DNA support the interpretation that each enzyme molecule must possess at least two positively cooperative single-stranded DNA binding sites and have led us to propose (Fisher & Korn, 1979a; Fisher et al., 1981) that DNA polymerase α is capable of exhibiting catalytically significant conformational responses to template base composition (or sequence). Although these earlier studies did not reach the question of polymerase–dNTP interaction, the admittedly provocative postulate of base composition regulated conformational responsivity suggested the possibility of a relationship between these observations and the important and poorly understood mechanisms that are involved in dNTP recognition and binding and in polymerase fidelity.

The experiments described in this paper demonstrate clearly that unlike a terminal 3′-PO_4 group, a terminal 3′-H residue allows normal primer binding by DNA polymerase α. By exploiting this observation and the consequent phenomenon of induced substrate inhibition, we have been able to establish that the interaction of KB cell DNA polymerase α with its substrates obeys the rigidly ordered sequential terreactant

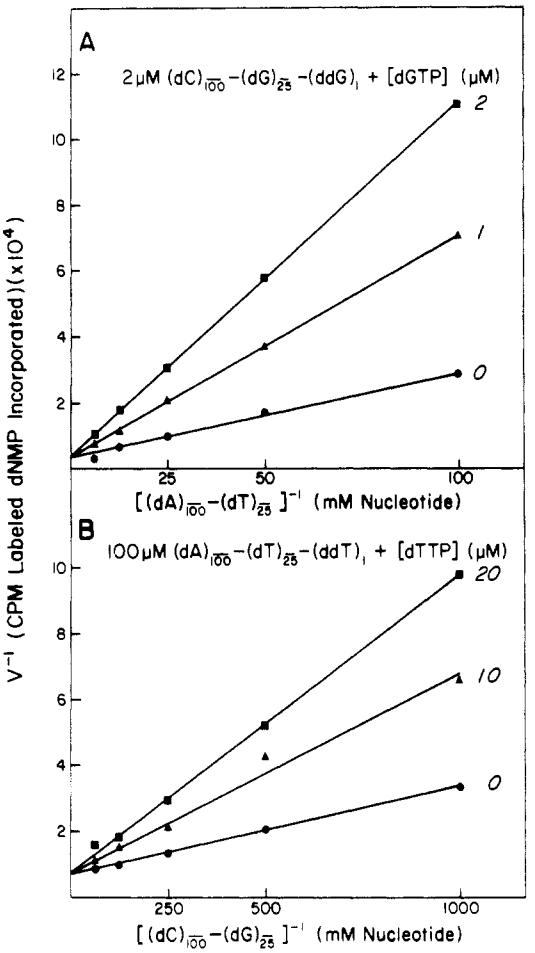


FIGURE 8: Lineweaver–Burk analysis of the dideoxy primer induced dNTP inhibition of DNA polymerase α. Reactions were formulated as described under Materials and Methods. (A) Each incubation contained [α-^{32}P]dTTP (40 μM, 1710 cpm/pmol), 2 μM $(dC)_{100}$-$(dG)_{25}$-$(ddG)_1$, and concentrations of dGTP and $(dA)_{100}$-$(dT)_{25}$ as indicated. (B) Each incubation contained [^{3}H]dGTP (40 μM, 730 cpm/pmol), 100 μM $(dA)_{100}$-$(dT)_{25}$-$(ddT)_1$, and concentrations of dTTP and $(dC)_{100}$-$(dG)_{25}$ as indicated.

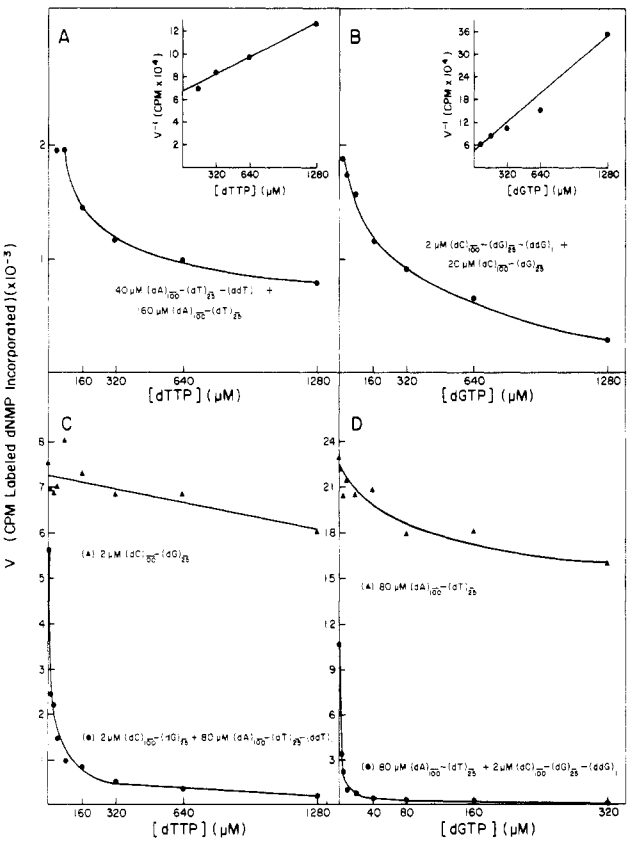
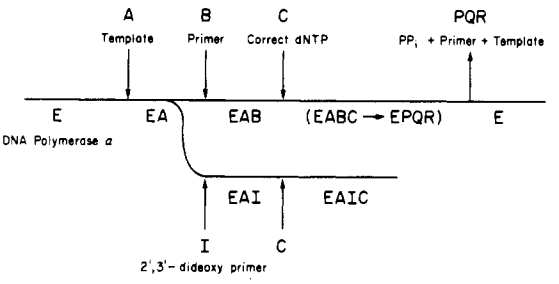


FIGURE 9: Extent of induced substrate inhibition of DNA polymerase α. Reactions were formulated essentially as described under Materials and Methods, with additional reagent concentrations as specified in the panels. (A) [α-^{32}P]dTTP was at 150 cpm/pmol; incubations were for 10 min, 37 °C. (B) [^{3}H]dGTP was at 120 cpm/pmol; incubations were for 10 min, 37 °C. The insets in (A) and (B) show Dixon plots of the data in the corresponding panels. (C) [^{3}H]dGTP, 40 μM, 640 cpm/pmol. (D) [α-^{32}P]dTTP, 40 μM, 960 cpm/pmol.



$$E \underset{k_2}{\overset{k_1 A}{\rightleftharpoons}} E\cdot\text{Template} \underset{k_4}{\overset{k_3 B}{\rightleftharpoons}} E\cdot\text{Template}\cdot\text{Primer} \underset{k_6}{\overset{k_5 C}{\rightleftharpoons}} E\cdot\text{Template}\cdot\text{Primer}\cdot\text{dNTP} \xrightarrow{k_7} E + \text{Products}$$

FIGURE 10: Partial steady-state kinetic model of KB cell DNA polymerase α. The diagram has been constructed according to the convention of Cleland (1970). Products are grouped together due to lack of information regarding the order of product release and incomplete understanding of polymerase processivity. In the kinetic formulation beneath the diagram, k_7 represents a combination of the several rate constants that characterize the individual steps in product generation and release. Note that for the second step (E·template $\rightleftharpoons$ E·template·primer), the concentration of primer, B, is not that of the primer in the total volume of solution but rather the concentration of primer within the partial specific volume of the template to which it is annealed.

mechanism that is schematized in Figure 10. As indicated, only the correct triphosphate that is dictated by the template sequence can participate in the dNTP-binding step. In a general way, this mechanism, the first to be elucidated for a putative replicative DNA polymerase, is in good agreement with the ordered bi–bi mechanism of catalysis that had previously been deduced from steady-state kinetic analyses of *E. coli* DNA polymerase I (McClure & Jovin, 1975) and murine myeloma DNA polymerase β (Tanabe et al., 1979). In both of those studies, primer–template (DNA) was treated exclusively as a single substrate, and thus no information was provided about the detailed relationship between the primer-binding and the template-binding steps. Further, neither of those studies approached the problem of dNTP selection since they both relied on the use of product inhibition experiments with PP_i to establish the order of substrate addition (DNA followed by dNTP). In all of these studies, including the present one, it must be emphasized that particularly for processive DNA polymerases, detailed understanding of the steps of product release and polymerase translocation continues to be lacking (McClure & Chow, 1980).

The mechanistic scheme depicted in Figure 10 prescribes that DNA polymerase α must first bind single-stranded polydeoxynucleotide (template), followed by primer stem and then dNTP in a rigidly ordered sequence. As previous investigators have stressed (McClure & Jovin, 1975; Tanabe et al., 1979), the critical implication of the ordered mechanism of reactant addition is that the binding of each substrate creates, presumably by induction of conformational alterations, the kinetically significant binding site for the substrate that follows. These results thus provide substantial support for our earlier proposal (Fisher et al., 1981) that KB cell DNA po-

lymerase α is a conformationally active protein that is capable of recognizing and responding to template base sequence through its interactions with single-stranded polydeoxynucleotides in its template binding site(s). More specifically, the phenomenon of dideoxy primer induced substrate (dNTP) inhibition strongly argues that the binding of dNTP by polymerase α is both primer dependent and absolutely directed by the template sequence. It should be recalled that the implication that DNA polymerase–template binding might substantially affect the subsequent steps of primer binding and dNTP recognition, possibly by conformational changes in the polymerase protein, had earlier been suggested from the results of studies with *E. coli* DNA polymerase I and bacteriophage T4 DNA polymerase (Huberman & Kornberg, 1970; Englund et al., 1969; Gillin & Nossal, 1975; Travaglini et al., 1975; McClure & Jovin, 1975). However, the data described in this paper present for the first time of which we are aware direct proof, both kinetic and physicochemical, of template-base specification and primer dependence of dNTP binding. The requirement of antecedent primer binding in addition to template binding is attractive and intuitively reasonable since with heteropolymeric DNA templates, it is only the template base immediately adjacent to the 3′-primer terminus that must be recognized and copied, and it is only in the presence of a bound primer stem that the "next" template nucleotide can be specified.

Although the complexity of the DNA polymerase reaction is recognized to pose significant difficulties with respect to the design and execution of rigorous mechanistic studies, there are, nonetheless, a number of features of the reaction that offer unique advantages. First, because of the fact that two of the polymerase substrates are macromolecular (template and primer), we have been able to use sedimentation analyses to study the binding of polymerase α to these substrates directly (Fisher & Korn, 1979a; Fisher et al., 1981; present report) and in the absence of catalysis. In every instance, the results of these binding studies have been completely corroborative of the kinetic interpretations, thus obviating in convincing fashion many of the general concerns that are appropriately voiced about the indirect nature of kinetic investigations. Second, the qualitatively identical mechanism by which polymerase α replicates both heteropolymeric and homopolymeric DNA (Fisher et al., 1981), and the fact that binding of dNTP is template-sequence directed, provides four apparently independent substrate systems for such mechanistic studies. Thus, one can readily examine the interactions of the polymerase with one primer–template in the presence or absence of the correct dNTP (unlabeled) simply by using it as an inhibitor of the reaction measured with a second primer–template/dNTP (labeled) set. In this manner, it should be possible to obtain a good deal of information about the individual rate constants that describe each step of the polymerase–substrate interaction. Finally, the phenomenon of dideoxy primer induced substrate (dNTP) inhibition provides a powerful and novel approach to the further exploration of a number of fundamental aspects of the polymerase α mechanism. The fact that essentially 100% of the polymerase protein can be driven into a stable, dead-end primer·template·dNTP complex at saturating levels of the (specific) complementary dNTP provides a highly specific affinity probe that should offer attractive and readily exploitable experimental systems for examining the mechanisms of polymerase fidelity (Hopfield, 1974, 1980; Galas & Branscomb, 1978), for testing directly the postulate of multiple single-stranded DNA (template) binding sites on each polymerase α molecule (note that the velocity sedimentation patterns in Figure 7B,D,F already provide a strong suggestion of the validity of this postulate), and for pursuing further detailed study of a number of the specific physicochemical features of the binding interactions of DNA polymerase α with primer–template [e.g., by "footprinting" techniques (Galas & Schmitz, 1978; Johnson et al., 1979)] and with complementary dNTPs, notwithstanding the availability of only extremely small quantities of the purified polymerase protein.

Acknowledgments

We thank Adriana Johnson for expert technical assistance with cell cultures.

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