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Structure–Function Analysis of Mononucleotides and Short Oligonucleotides in the Priming of Enzymatic DNA Synthesis

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ABSTRACT: The reversed-phase chromatography technique was employed in the measurement of DNA synthesis at the primers $d(pT)_n$, $r(pU)_n$, $d(pA)_n$, and $r(pA)_n$ (n = 1–16) in the presence of template poly(dA) or poly(dT). DNA synthesis was catalyzed by *Escherichia coli* DNA polymerase I Klenow fragment, *Physarum polycephalum* DNA polymerase β-like, *P. polycephalum* DNA polymerase α, and human placenta DNA polymerase α. Values of K_m and V_{max} were measured as functions of the primer chain lengths. It was found that all mononucleotides and small oligonucleotides served as primers of DNA synthesis. Values of the logarithm of both K_m and V_{max} increased linearly until primers had attained a chain length of 9–12 nucleotides, where a break was observed. The incremental as well as the absolute values of K_m were interpreted in terms of free binding energies. These together with other data indicate that the 3′-ultimate nucleotide of the primer contributes a decisive amount of free energy of binding to DNA polymerase both from the nucleoside and from the phosphate moiety. The incremental increase is due to a complementary interaction between bases of primer and template buried in the binding cleft of the polymerase. It is also the ultimate nucleotide that determines whether the ribonucleotide or the deoxyribonucleotide is an efficient primer. It is of interest that the major results seem preserved for all four DNA polymerases. An energetic model for the binding of the template-primer was proposed and compared with available crystallographic data.

DNA polymerases catalyze the elongation of oligo- or polynucleotides at their 3′-terminus by covalent addition of nucleoside 5′-monophosphates from the triphosphates complementary to the base-pairing polynucleotide template strand (Kornberg, 1980). The length of these nucleotides serving as primers of DNA polymerization and their ribose or deoxyribose nature determine the success at which elongation occurs. The types of DNA polymerases that function in chromosome replication easily use RNA as primers while DNA repairing polymerases strongly prefer DNA. The length can be imagined to be associated with the number of noncovalent complementary interactions with a given template and probably with the polymerase binding cleft and thus with the efficiency holding the 3′-terminus in the position that is optimal for the catalytic addition of mononucleotides. This efficiency has been measured previously in experiments employing synthetic hook polymers (Fisher & Korn, 1979) and small primers with natural templates (Grosse & Krauss, 1984). The success of priming has been examined after routine acid precipitation of product DNA, usually after extensive primer elongation. The minimal number of nucleotides in these primers has been suggested to be three to five and four, respectively.

The crystal structure has been elucidated for *Escherichia coli* DNA polymerase I Klenow fragment (Ollis et al., 1985; Joyce & Steitz, 1987). From the structural coordinates as well as from model fitting, the binding site for DNA has been localized and also a site that binds nucleoside 5′-monophosphates. This site is very likely to be involved in the 3′–5′ exonuclease (proofreading activity; Kornberg, 1980) located on a small structural domain while the activity to synthesize DNA resides on the large domain of Klenow fragment (Freemont et al., 1986). The exact location of the primer 3′-terminus and of nucleoside 5′-triphosphates has remained mainly unsolved.

We have investigated the structure–function relation of synthetic ribo and deoxyribo primers for four representative DNA polymerases employing Michaelis–Menten kinetic analysis and measurement of products by reversed-phase chromatography. We find that all these polymerases catalyze DNA synthesis in the presence of primers as short as nucleoside 5′-monophosphates. From the results of a systematic structure–energy investigation, we have developed an energetic model of the template-primer binding site of DNA polymerases.

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Materials and Methods

Enzyme Preparations. Two preparations of human placenta DNA polymerase α were used. The first one (preparation I) has been obtained according to Nevinsky et al. (1986) and Lavrik et al., 1989) and was free of DNA primase activity. Purification was (2×10^3)-fold and specific activity 8.5×10^3 units/mg. The preparations contained proteins or protein complexes having molecular masses of 460, 230, 170, and 150 kDa as analyzed by the method of electrophoresis under nondenaturing conditions (Holler et al., 1985). All these species were active in catalyzing DNA polymerization. According to analysis by polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate, preparation I contained proteins having molecular masses of 140, 104, 98, and 72 kDa, which were suggested to be polymerase subunits (Lavrik et al., 1989). On the basis of this analysis, the preparations did not contain DNA binding proteins C_1 and C_2 (Vishwanatha et al., 1986) in accordance with our purification, which included chromatography on phosphocellulose. Preparation I was employed in most experiments unless mentioned otherwise.

Highly purified human placenta DNA polymerase α (preparation II) with a specific activity of 3×10^4 units/mg was obtained after immunochromatography on immobilized monoclonal antibody SJK 287-38 against human DNA polymerase α (Podust et al., 1989). Preparation II contained DNA primase activity. It contained proteins having molecular masses of 180, 160, 145, and 140 kDa (all immunologically positive), a protein of 73 kDa (function unknown), and proteins of 59 and 52 kDa (DNA primase). Preparation II has been employed in DNA priming experiments with [α-^{32}P]dATP and poly(dT) (in the absence of any other nucleotide).

Highly purified DNA polymerases type α and type β-like from *Physarum polycephalum* with specific activities of 1×10^4 and 2×10^3 units/mg, respectively, had been purified as described (Weber et al., 1988; Holler et al., 1987). The preparation of DNA polymerase α contained the 135-kDa catalytic subunit and was devoid of primase activity. The preparation of type β-like DNA polymerase contained the 135-kDa polymerizing subunit and the associated 60-kDa protein (function unknown).

The Klenow fragment of *Escherichia coli* DNA polymerase I was homogeneous according to polyacrylamide gel electrophoresis and had a specific activity of 6×10^4 units/mg (Nevinsky et al., 1987; Levina et al., 1985). One unit of DNA polymerase corresponds to the activity incorporating 1 nmol of nucleotides into acid-insoluble material during 1 h at 37 °C under the conditions of the standard assays.

It was verified by reversed-phase chromatography (see Methods) that the polymerase preparations were not contaminated by any nucleotides or nucleic acids, nor did they contain phosphatase activity.

Materials. DNA from calf thymus, poly(dA), poly(dT), dNMP, NMP, and dNTP were from NIKTI BAV (USSR), bovine serum albumin was from Koch-Light (England), alkaline phosphatase and DNase I were from Serva (Heidelberg, FRG), $MgCl_2$ and $MnCl_2$ were from Merck (FRG), and [^{3}H]dTMP, [^{3}H]dAMP, [^{3}H]dTTP, [^{3}H]dATP, and [^{32}P]dATP were from IZOTOP (USSR). These nucleotides were purified to homogeneity by reversed-phase chromatography on Lichrosorb RP-18 with a methanol gradient [0–8% (v/v)].

Unlabeled oligonucleotides were homogeneous according to results from ion exchange and reversed-phase chromatography. Their synthesis and characterization have been reported (Nevinsky et al., 1986, 1987a,b; Levina et al., 1985). Oligonucleotides of the type $d(pA)_n$ for $n = 50 \pm 10$, 100 ± 20, 200 ± 30, and 300 ± 30 were prepared by hydrolysis of poly(dA) and subsequent electrophoresis of the digest. Commercial preparations of poly(dA), poly(dC), and poly(dT) were purified by electrophoresis and were devoid of oligonucleotides with chain lengths of less than 50 nucleotides as verified by reversed-phase chromatography.

^{32}P-Labeled oligonucleotides were prepared as follows. [^{32}P]$d(pT)_n$ and [^{32}P]$d(pA)_{n+1}$ were obtained after polymerization employing the complementary polynucleotides as templates and the tetranucleotides as primers in the presence of Klenow fragment. The products were subjected to limited digestion with DNase I and separated into defined oligonucleotides by reversed-phase chromatography (see below, defined unlabeled oligonucleotides as standards). [^{32}P]$d(Ap)_nA$ was prepared from [^{32}P]$d(pA)_{n+1}$ in the presence of alkaline phosphatase.

Methods. The kinetic parameters of DNA synthesis for all DNA polymerases were determined at 30 °C. The reaction mixture (100 μL) for *P. polycephalum* DNA polymerases contained 50 mM Tris-HCl, buffer (pH 7.5), 2.5 mM $MgCl_2$, 1 mM EDTA, 2 mM KCl, 0.3 mg/mL bovine serum albumin, 30 μM [^{3}H]dTTP or [^{3}H]dATP [$(1–2) \times 10^{14}$ Bq/mol], 0.65 A_{260} unit/mL poly(dA), and various concentrations of primers (for these and the other DNA polymerases, the concentrations were between 0.5 and 15 times the K_m values). The reactions were started by the addition of 0.01–0.1 unit of enzymes.

The reaction mixture (100 μL) for human placenta DNA polymerase α (Nevinsky et al., 1986, 1987a,b,d) contained 50 mM Tris-HCl buffer (pH 7.5), 0.15 mM $MnCl_2$, 10 μM EDTA, 1 mM 2-mercaptoethanol, 0.5 mg/mL bovine serum albumin, 50 μM poly(dT) or poly(dA) (mononucleotide concentration), 40 μM [^{3}H]dATP or [^{3}H]dTTP [$(0.2–2) \times 10^{14}$ Bq/mol], and primers at various concentrations. The reactions were started by the addition of 0.01–2 units of the enzyme. In the presence of 5 mM $MgCl_2$ instead of $MnCl_2$, the initial rates of polymerization were about 3–4 times lower than those for Mn^{2+}. However, K_m values for $d(pA)_{10}$ were not measurably affected.

The reaction mixture (100 μL) for Klenow fragment contained either 50 mM HEPES/NaOH buffer (pH 8.0), 10 mM $MgCl_2$, 10 mM KCl, 10 μM EDTA, 0.1 mg/mL bovine serum albumin, and the constituents below (reaction mixture I) or 50 mM Tris-HCl buffer (pH 7.5), 2.5 mM $MgCl_2$, 3 mM KCl, 5 mM NaF, 50 μM EDTA (reaction mixture II) and the following constituents: 0.32 A_{260} unit/mL poly(dA) [or 0.4 A_{270} unit/mL poly(dT)], 30 μM [^{3}H]dTTP (or 30 μM [^{3}H]dATP) [$(0.4–4) \times 10^{14}$ Bq/mol], and primer nucleotides. The reaction was started by the addition of 0.01–2 units of the enzyme.

The reaction conditions for human placenta DNA polymerase α and Klenow fragment had been previously optimized with regard to DNA polymerization rates (Nevinsky et al., 1987a; Levina et al., 1985). The 3′–5′ exonuclease activity of Klenow fragment was not completely negligible under the conditions employed in reaction mixture I though high concentrations of substrates were used, and the nuclease rates were relatively low (1–2% of the rates of DNA synthesis). In order to minimize the exonuclease activity, reaction mixture II contained NaF. This salt (5 mM NaF) inhibited (80–95% of the 3′–5′ exonuclease activity only in the presence of Mg^{2+} (and not Mn^{2+}) (Mikhailov et al., 1989; Volchkova et al., 1989).

When priming of DNA synthesis by mononucleotides was measured, the mononucleotides used were radioactively labeled (Nevinsky et al., 1987a,d; Venyaminova et al., 1987). The

reaction mixture (100 μL) contained 10–300 μM [^{3}H]dNMP or [^{3}H]NMP [(0.5–2) × 10^{14} Bq/mol] and 30–40 μM dNTP.

In the presence of $d(pN)_2$ or $d(pN)_3$ as primers, [^{3}H]dNTP [(0.5–2) × 10^{14} Bq/mol] was used. In this case and for priming with mononucleotides, the reaction mixtures contained 0.5–2 units of DNA polymerases.

For DNA synthesis primed with oligonucleotides of chains of 5–16 nucleotides, the radioactivity of samples was measured after routine trichloroacetic acid precipitation (Nevinsky et al., 1986, 1987a; Holler et al., 1987; Levina et al., 1985). Aliquots (5–20 μL) of the reaction mixture were applied to filter paper (pieces 2.5 cm in diameter), which had been washed with 5% trichloroacetic acid and dried. The filter disks were then washed by immersing them seven times in a series of beakers each containing 300 mL of 5% trichloroacetic acid at 1–2 °C and finally once in ethanol (200 mL). The filter disks were then dried and counted for radioactivity.

After DNA synthesis was primed with mononucleotides or short oligonucleotides (one to six nucleotides long), reversed-phase chromatograpy on Lichrosorb RP-18 was used to separate products from starting materials (Nevinsky et al., 1987a,d; Venyaminova et al., 1987). Aliquots (10–50 μL) of the reaction mixture were added to 50 μL of 0.5 M EDTA, and then the mixture was applied to the reversed-phase column (2 × 60 mm). Mononucleotides were eluted first with 2 mL of a solution containing 0.02 M Tris–acetate buffer (pH 6.5) and 4% (v/v) methanol. Dinucleotides and oligonucleotides were then eluted with 80% methanol/water. Portions of the eluates were spotted on filter paper, dried, and counted for radioactivity.

The completeness of precipitation of the acid-insoluble product on trichloroacetic acid treated filter papers after DNA synthesis in the presence of short oligonucleotides [$d(pN)_5$ through $d(pN)_{10}$] as primers was measured in comparison to the amount of product determined by the reversed-phase method. The reaction mixture (70 μL) for human placenta DNA polymerase α contained primer in a concentration equal to its K_m value. The reaction was stopped after 1-min incubation time by the addition of 100 μL of 0.5 M EDTA. One portion (75 μL) was spread on the paper disk, and the other portion (75 μL) was loaded onto the Lichrosorb RP-18 column. The paper disk and the sample from the reversed-phase column were prepared for radioactivity counting as described above. The ratio of radioactivity obtained after acid precipitation in comparison to that after reversed-phase chromatography was 34%, 66%, 82%, 89%, 95%, and 100% for $d(pN)_5$, $d(pN)_6$, $d(pN)_7$, $d(pN)_8$, $d(pN)_9$, and $d(pN)_{10}$, respectively. Presented data were corrected in order to account for incomplete acid precipitation.

In control experiments templates poly(dA) and poly(dT) were replaced by noncomplementary poly(dC) (0.5–1.0 A_{270} unit/mL).

For mononucleotides as primers the distribution of products among synthesized oligonucleotides was measured as follows. Aliquots (20–50 μL) of reaction mixtures were stopped with 100 μL of 0.5 M EDTA. Unlabeled marker oligonucleotides [(5–8) × 10^{-3} A_{260} unit) of known composition and chain length were added, and the mixture was loaded onto the reversed-phase column. Chromatography was carried out with a 0–40% (v/v) gradient of methanol/water (2 mL) containing 0.02 M Tris–acetate buffer (pH 6.5) at a rate of 100 μL/min. The absorption of the eluate was followed with a microspectrophotometer (Millichrom, USSR). Fractions of 30–40 μL of the eluate were collected on paper filters, dried, and counted for radioactivity.

When products of the priming reaction were analyzed by gel electrophoresis, poly(dT) was used as template and 0.1 mM [α-^{32}P]dATP (6 × 10^{14} Bq/mol) as the priming agent in the DNA polymerization reaction mixture (no other oligonucleotides present) catalyzed by either 1–2 units of human placenta DNA polymerase α or 0.2 unit of Klenow fragment. After incubation for variable times (0.5–30 min), the reaction was terminated by the addition of 0.5 mL of acetone containing 2% (by weight) $NaClO_4$. Precipitation was completed during 30 min at −50 °C. After centrifugation, the pellet was washed twice with 0.4 mL of cold acetone and dried. The material was then dissolved in 50 μL of a solution containing 20 mM Tris-HCl buffer (pH 8.2) and 2 units of alkaline phosphatase. After incubation of the mixture for 5 h at 37 °C, the above precipitation was repeated and the resulting pellet was dissolved in 20 μL of 8 M urea. Aliquots of 5 μL were subjected to standard polyacrylamide (20%) gel electrophoresis in the presence of 8 M urea according to Maxam and Gilbert (1977). Oligonucleotides [^{32}P]$d(Ap)_ndA$ and [^{32}P]$d(pT)_n$ of known chain length were employed as standards. Oligonucleotides $d(Ap)_ndA$ migrated slower than their corresponding $d(Ap)_n$ forms. Products were positioned after autoradiography.

Initial rates have been measured in kinetic experiments. Samples were drawn between 10 s and 2 min, for slower reactions after intervals up to 10 min, and rates were determined from the tangents to the data curves at zero time. The concentration of oligonucleotides as primers was changed from 0.5 to 20 times the K_m values (six to seven data points). The level of radioactivity incorporated into DNA product was under all conditions equal or higher than 2.5 × 10^3 counts per minute, whereas the background incorporation was 1–3% of that radioactivity.

Michaelis–Menten parameters K_m and V_{max} were determined according to Eisenthal and Cornish-Bowden (1974). We found this method more useful than the conventional linearization methods of Lineweaver and Burk (1934) or Eadie (1942) due to simple error calculations instead of the more tedious least-squares method. Experiments were performed at least in duplicate. Errors in K_m and V_{max} values are of the order of ±20%. It was recently shown that for primers the values of Michaelis–Menten constants, K_m, and of dissociation constants of the enzyme–template–primer complexes, K_d, were the same within a factor of 2 (Nevinsky et al., 1986). We assume that the values of K_m are equal to those of the corresponding dissociation constants and that the observed variations between K_m and K_d values are merely due to different experimental techniques. On these grounds we calculated the free energies of primer binding as $\Delta G = RT \ln K_d = RT \ln K_m$ and the differences in free energies of binding as $\Delta\Delta G = RT \ln [K_m(n+1)/K_m(n)]$ when a primer with a length of n nucleotides was compared with a primer of the same series with a length of $n + 1$ nucleotides. For determination of K_m values, true molar concentrations were employed for the oligonucleotides. In studies using polynucleotides as primers ($n \geq 50$), this was not possible because of the inhomogeneity in chain lengths. In these cases (Figure 3), concentrations (and thus K_m values) are given in terms of moles of total bases that could be measured by UV absorbance.

Results

Priming of DNA synthesis by mononucleotides was suggested by the time-dependent formation of oligonucleotides catalyzed by human placenta DNA polymerase α, with poly(dT) as template and [^{3}H]dAMP as primer. The observation of progressively larger amounts of radioactivity in the elution position of oligonucleotides was compatible with

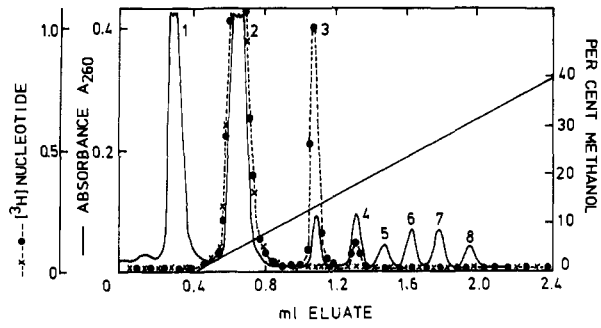

FIGURE 1: Analysis of ^{3}H-containing nucleotides by reversed-phase chromatography after DNA synthesis in the presence of primer [^{3}H]dAMP. Poly(dT) was the template, dATP was the elongating substrate, and DNA polymerase α from human placenta was used. The reaction time was 5 min. Concentrations were 100 μM [^{3}H]-dAMP and 40 μM dATP. One unit of ^{3}H-labeled nucleotide of the ordinate equals 5×10^3 cpm; for other details, see Methods. (●) Complete reaction mixture; (×) that without poly(dT) or the enzyme. (1) dATP; (2) dAMP; (3) $d(pA)_2$; (4) $d(pA)_3$; (5) $d(pA)_5$; (6) $d(pA)_6$; (7) $d(pA)_8$; (8) $d(pA)_{10}$. Oligonucleotides 3–8 were added after termination of the reaction before reversed-phase chromatography.

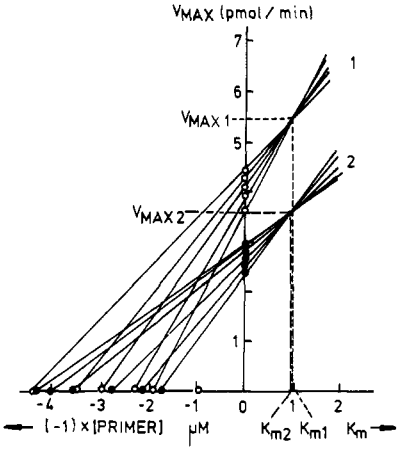

FIGURE 2: Presentation of initial rates as a function of initial primer concentrations and determination of K_m and V_{max} according to the method of Eisenthal and Cornish-Bowden (1974). For experimental details, see Methods. Primers were $d(pT)_7$ (1) and $r(pU)_7$ (2). One picomole corresponds to 1×10^4 cpm contained in a 20-μL sample.

the assumption of a direct reaction between the radioactivity labeled mononucleotide and the substrate dATP. The reaction strictly depended on the presence of enzyme, a polynucleotide template, and the complementary nature of the nucleobases of this polynucleotide as demonstrated by controls in the absence of poly(dT), after replacement of poly(dT) with poly-(dC), in the absence of dATP, or in the absence of DNA polymerase. The possibility that radioactively labeled nucleotides were covalently linked to the complementary template poly(dT) could not be ruled out, however.

The reaction products of the described priming reaction above were analyzed by reversed-phase chromatography (Figure 1). Indeed, the radioactively labeled mononucleotide [^{3}H]dAMP was the only kind of primer as evidenced by the coincidence of radioactivity and absorbance in the column eluate and ruled out a covalent attachment to template poly(dT). The main product was [^{3}H]$d(pA)_2$ besides little [^{3}H]$d(pA)_3$ after a reaction time of 5 min. Note that the results did not exclude the possibility that the nucleoside 5′-triphosphate might also function as primer. In order to clarify this possibility, experiments were carried out with prepurified poly(dT) as template and 0.1 mM [α-^{32}P]dATP as primer/elongating substrate (freshly purified by chromatography). Products were analyzed by urea gel electrophoresis (see Methods). The results (not shown) were as follows. (i) A mixture of di- and trinucleotides was formed in the presence of human placenta DNA polymerase α preparation I or preparation II and either Mg^{2+} or Mn^{2+} after 15 min. Higher amounts of nucleotides were found in the presence of Mn^{2+} ions. (ii) Klenow fragment (Mg^{2+}) synthesized a mixture of di- to pentanucleotides during an incubation time of 1 min. (iii) Controls were performed (60-min incubation time) that did not contain the DNA polymerases or that contained poly(dC) [arbitrarily poly(dA)] instead of poly(dT) together with each of the DNA polymerases. All controls were devoid of oligo- or polynucleotide synthesis. Thus, we may conclude that dAMP and dATP can serve as primers of enzymatic DNA synthesis.

The question of product distribution was approached in a further experiment. In this case the dinucleotide $d(pA)_2$ (60 μM) was used as primer [with poly(dT) as template], and the radioactive incorporation of nucleotide from [^{3}H]dATP (40 μM) was followed in the presence of human DNA polymerase α. The amounts of oligonucleotides were measured by the technique described for Figure 1. As in the case of [^{3}H]dAMP as primer, the synthesis of the next two higher oligomers, $d(pA)_3$ and $d(pA)_4$, was favored by factors of 10–40 over that of the higher oligomers $d(pA)_5$ and $d(pA)_6$ at short reaction times (0.5–1.0 min); however, at longer times the short oligonucleotides served themselves as primers for the synthesis of higher nucleotides.

So far, priming has been concerned with the adenosine nucleotide family. Priming was also shown in the presence of dTMP (60 μM) with poly(dA) as the template, [^{3}H]dTTP (60 μM) as the chain elongating substrate, and human DNA polymerase α. Again, the nucleoside monophosphate served as primer. In the absence of both dTMP and poly(dA) or of poly(dA) alone, synthesis of oligonucleotides was not observed. Here conditions were such that self-priming of the chain-elongating nucleotide was also observed though at one-tenth of the rate for dTMP. Indeed, in the absence of dTMP and the presence of only [^{3}H]dTTP, (chromatographically pre-purified) poly(dA), and enzyme, the products of priming were a mixture of di- to pentanucleotides according to reversed-phase chromatography. The results for adenine and thymidine nucleotides might be generalized in that both dNMP and dNTP could function as primers. Priming seemed more frequent for the monophosphate, however.

In the following experiments we anticipated that the efficiency of a primer is a function of its composition and chain length. By varying its concentration, we measured the Michaelis–Menten parameters K_m and V_{max} and established correlations with primer chain lengths. Of the many structural possibilities, we chose the thymidylate (adenylate) nucleotides in the deoxyribose series and the uridylate (adenylate) nucleotides in the ribose series. It seemed important to see whether any observed kinetic or thermodynamic patterns were of more general validity or just pecularities of each DNA polymerase studied.

The dependence of initial velocities as a function of primer concentration is shown in Figure 2 with the graphical presentation introduced by Eisenthal and Cornish-Bowden (1974). The example is set for the primers $d(pT)_7$ and $r(pU)_7$. The accuracy of the set of kinetic data is immediately realized by the common point of intersection of all lines. These lines are obtained by plotting reaction velocities along the V axis and corresponding substrate concentrations along the abscissa to the left (Eisenthal & Cornis-Bowden, 1974). The coordinates of the common intersection are V_{max}/K_m.

Michaelis–Menten parameters have been collected for

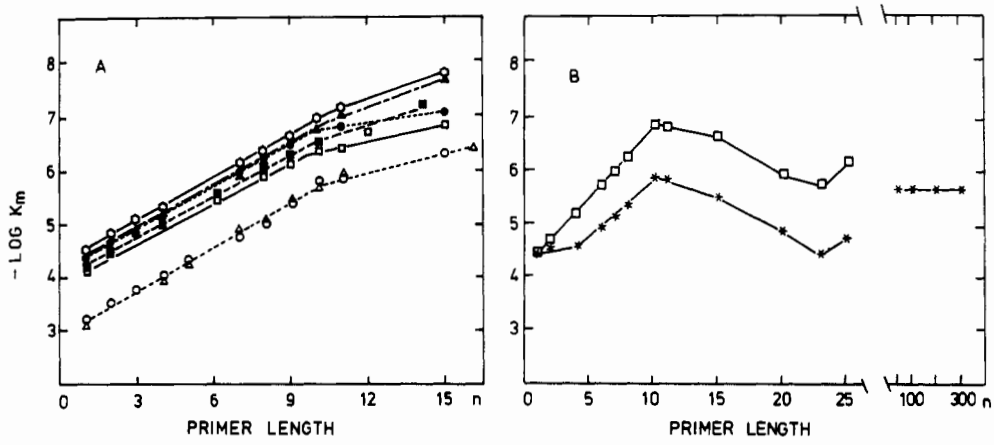

FIGURE 3: Dependence of the logarithm of K_m on the length of the primers for DNA synthesis catalyzed by various DNA polymerases. Poly(dA) was the template and $d(pT)_n$ the primer (n = the number of nucleotide residues). Concentrations of primers were 0.5–15 times K_m values; other conditions were those of Figure 4. (Panel A) (●) Human placenta DNA polymerase α; (○) *P. polycephalum* DNA polymerase α; (▲) *P. polycephalum* DNA polymerase β-like; (□) Klenow fragment of *E. coli* DNA polymerase I. With poly(dT) and human placenta DNA polymerase α the following primers were used: (○) $r(pU)_n$; (■) $d(pA)_n$; (△) $r(pA)_n$. (Panel B) Klenow fragment of *E. coli* DNA polymerase I and reaction mixture II containing NaF. (□) Calculation of K_m on the basis of the molar concentrations of primers; (*) calculation of K_m on the basis of the molar concentrations of bases contained in the primers.

primers as a function of chain length from 1 to 15 nucleotides and for Klenow fragment even at higher lengths. The log K_m values are shown in Figure 3, which essentially represents a free energy plot of the primer chain length. It contains the results for four different DNA polymerases: human placenta DNA polymerase α, *P. polycephalum* DNA polymerase α, *P. polycephalum* DNA polymerase β-like, and *E. coli* DNA polymerase I Klenow fragment. Despite the different sources and functions (repair versus replication polymerases), the following common features are evident from Figure 3: (1) The values are aligned in two groups. That for deoxyribose corresponds to higher affinities than that for ribose primers in the enzyme–template-primer complex. (2) The values follow a linear dependence of primer chain length that, given the high experimental accuracy at low chain lengths, appears to have a break at a length of 9–12 nucleotides. While that for human DNA polymerase and for Klenow fragment is quite obvious, that for the *Physarum* DNA polymerases is marginal but receives support from the chain length dependence of V_{max} in Figure 4. (3) The slopes at shorter lengths are the same for all dependencies shown. Its value corresponds to an incremental factor of 1.8 between K_m values when the primer length differs by one nucleotide. (4) The break and the slope in the chain length dependence remain the same with reaction mixture II (see Methods) for Klenow fragment. (5) The affinity for Klenow fragment and reaction mixture II decreases at higher chain lengths and passes through a minimum at n = 20–23. At very high numbers of n, the affinity becomes length independent.

Maximal velocities (V_{max}) as a function of primer chain length are shown in Figure 4. The diversity obtained for different sources of DNA polymerases contrasts the uniformity in the case of K_m values in Figure 3. Again, log V_{max} depends linearly on the primer chain length and has a break at the position of decanucleotide, close to the position in Figure 3 for the dependence of log K_m. Klenow fragment, which has been studied also at higher chain lengths, has a minimum at position n = 20–23, where also a minimum of the binding affinity is observed. The slopes for short primers in Figure 4 correspond to an incremental increase by a factor of 1.36 in the case of human placenta DNA polymerase α and by a factor of 1.09 in the case of the *P. polycephalum* DNA polymerases. The initial slope for the *Physarum* DNA polymerases resembles more closely that for Klenow fragment than

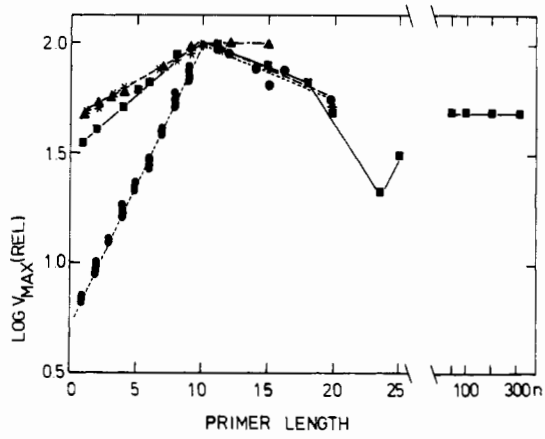

FIGURE 4: Dependence of the logarithm of V_{max} on the length of primers for DNA synthesis catalyzed by various DNA polymerases. (▲) *P. polycephalum* DNA polymerase α; (×) *P. polycephalum* DNA polymerase β-like; (■) Klenow fragment and the reaction mixture II containing NaF. For these three enzymes, all reaction mixtures contained template poly(dA) and primer $(d(pT)_n$. (●) Human placenta DNA polymerase α and either template poly(dA) and primers $d(pT)_n$ and $r(pU)_n$ or template poly(dT) and primers $d(pA)_n$ and $r(pA)_n$. Velocities are given in percent with reference to that of decanucleotide primers. For example, V_{max} for dTMP and $d(pT)_{10}$ in the case of *Physarum* DNA polymerase α was equal to 2.7×10^3 and 5.3×10^3 cpm/min, respectively (20-μL aliquots of reaction mixtures, five time points during the first 6 min of the reaction).

that for human DNA polymerase α. Their priming efficiency as a function of primer length is not so much a matter of the maximal velocity as of the primer binding affinity (comparison of Figures 3 and 4). The V_{max} values in Figure 4 are given in relation to those for the decanucleotide primers, and it does not show the dependence on the deoxyribose/ribose status of the primer. This has been measured separately for human placenta DNA polymerase α: V_{max} for oligo(rA) is higher by a factor of 15–20 than the V_{max} for oligo(dA), and the V_{max} for oligo(rU) is higher by a factor of 4–5 than the V_{max} for oligo(dT). A comparison of V_{max} and K_m values is also shown in Table I for other examples.

Discussion

The "natural" function of DNA polymerases is catalytic elongation of so-called "primers", which either are oligo- and polydeoxribonucleotides or oligoribonucleotides (Kornberg, 1980). Here we have obtained results showing that their ability

Table I: Kinetics Parameters K_m and Relative V_{max} of Ribo and Deoxyribo Primers of Various DNA Polymerases

oligonucleotide	human DNA polymerase α		*Physarum* DNA polymerase α		*Physarum* DNA polymerase β	
	K_m (μM)	V_r/V_d[a]	K_m (μM)	V_r/V_d[a]	K_m (μM)	V_r/V_d[a]
$r(pU)_7$	22	4.8	0.8	0.75	12	1.5
$d(pT)_7$	1.4		0.8		1.4	
$r(pU)_9$	3.9	4.6	0.24	0.77	3.7	1.6
$d(pT)_9$	0.45		0.25		0.4	
$d(pT)_8$-r(pU)	3.6	4.9[b]	0.24	0.9[b]	2.2	1.7[b]

[a] Relative values of maximum velocities, V_r, in the presence of ribo primers with reference to maximum velocities, V_d, in the presence of deoxyribo primers of the same length and base composition. [b] Relative values of maximum velocity for primer $d(pT)_8r(pU)$ with reference to that for primer $d(pT)_9$.

to serve as primers is not limited by size and that they can be as small as a mononucleotide. As the chain length increases, the efficiency of a primer seems to become optimal at a length of 9–12 nucleotides. We have selected four DNA polymerases of different functions, complex compositions, and sources: *E. coli* DNA polymerase I (Klenow fragment) is a prokaryotic repair enzyme (Kornberg, 1980). *P. polycephalum* DNA polymerase β-like is a supposed repair enzyme of lower eukaryotes (Holler et al., 1987). By its molecular mass, complex structure, and certain enzymatic properties, it is different from DNA polymerase β of higher eukaryotes, however. *P. polycephalum* DNA polymerase α and human placenta DNA polymerase α (preparations I and II) are "replicative" DNA polymerases of a lower and a higher eukaryote, respectively, and are of different subunit composition. Despite the difference in subunit composition, function, and origin, the obtained results share properties that seem to be independent of the individual preparations of DNA polymerases and thus could be of general importance for the different DNA polymerases. Details on the structural, kinetical, and thermodynamical aspects are discussed in the following sections.

(1) The efficiency of an oligonucleotide in functioning as a primer is manifested in both K_m and V_{max} values (Figures 3 and 4) and is a function of the molecule length. The data points for K_m suggest a common break or bend at a primer chain length of 9–12 nucleotides, and this is clearly borne out together with the dependence for V_{max}. The findings are consistent with the assumption of a cleft on the DNA polymerases with a size limited to the uptake of a chain 9–12 nucleotides long. DNA footprinting indicated that Klenow fragment, when bound at a primer terminus, covered about 8 bases of the primer and 19–20 bases of the template strand (Joyce et al., 1986). A minimal length of eight nucleotides necessary for optimal priming has been reported by Fisher and Korn (1981). It is interesting to note that the overall size of the cleft is similar if not identical for all DNA polymerases we have investigated.

(2) Our interpretation of K_m values in terms of binding affinity is based on the assumption that they follow within experimental accuracy the particular values of dissociation constants. Examples of dissociation constants have been measured indirectly in protection experiments against active site directed chemical inactivation of DNA polymerase (Nevinsky et al., 1986). Here, the polymerases was inactivated by coordination with $(pT)_2pC[Pt(NH_3)_2OH](pT)_7$ by affinity reaction with the template binding site. The polymerase was protected in the presence of synthetic single-stranded oligonucleotides (templates). By varying concentrations of the oligonucleotides, their dissociation constants were measured from the protection effects. The addition of small complementary oligonucleotides (primers) to this mixture increased protection, and this increase was used to measure the dissociation constant for the primer from the DNA polymerase–template complex. Finally, the K_d values were compared with K_m values measured for the same template-primers and found to resemble them within experimental accuracy. Our assumption seems also validated by the observation that the binding affinity for the primer increases linearly with nucleotide length independent of the type and source of the investigated DNA polymerases (Figure 3). The incremental value is the same for all four DNA polymerases and thus likely to be due to a common type of interaction in all of these enzymes. The factor of 1.8 per added nucleotide residue equals a change in free binding energy of –0.35 kcal/mol, which among possible molecular interactions ranks in the order of hydrogen bonds. Together these arguments suggest that the free energy contribution is due to the interaction between complementary bases (dA-dT, dA-rU, dT-rA) of the primer and the template strand in the complex.

(3) The effect of the 2′-OH in the ribose family of primers is to increase the K_m values by factors of 8–12 relative to those of deoxy primers for human placenta DNA polyerase α (Figure 3). A small effect is also seen for *P. polycephalum* DNA polymerase β-like in Table I [the comparison of $r(pU)_9$ and $d(pT)_9$] but not for *P. polycephalum* DNA polymerase α. Thus, the effect of the ribose 2′-OH varies with different polymerases. A comparison of primers $r(pU)_9$ and $d(pT)_8r(pU)$ (Table I) reveals that it is the 3′-terminal ribose moiety that causes the specific effect. An interpretation of the results on a molecular interaction basis cannot be given at present because the exact position of the primer 3′-end in the crystal structure is still not known (Ollis et al., 1985). However, the increase in V_{max} that parallels the decrease in affinity (human placenta DNA polymerase α and, to some extent, *P. polycephalum* DNA polymerase β-like) suggests that for these enzymes the higher affinity of the 2′-deoxyribose primer may involve unproductive binding at the primer terminus and that the presence of the 2′-OH in the ribo primer diminishes this mode of binding due to some steric hindrance inferred by this group.

Ribo primers are accepted by type α DNA polymerases but not by type β DNA polymerases (Fry & Loeb, 1986). Inspection of Table I reveals that among type α DNA polymerases of different sources the acceptance is borne out by affecting the Michaelis–Menten parameters in different ways. While *Physarum* DNA polymerase α accepts deoxyribo and ribo primers equally well, the human enzyme has less preference for the ribose primer at the level of binding but has a higher maximal rate than for the deoxy primer. The "nonacceptance" of ribo primers by *Physarum* DNA polymerase β-like is a matter of K_m and is expressed only at low, subsaturating concentrations of primer. This may be a specialty of the *Physarum* enzyme and be different from typical β-type DNA polymerases of higher eukaryotes, which could be more stringent even at high primer concentrations.

(4) The significance of the 3′-terminus is further illustrated by the previous finding that the absence sof the 3′-OH in the oligonucleotide $d(pT)_9p(ddT)$ is accompanied by a loss of binding affinity (Veniaminova et al., 1987). Furthermore, the 3′-ultimate phosphodiester (or, respectively, the 5′-phosphate of the mononucleotide) moiety is also of importance. This has been shown in binding experiments with primers that were ethylated at their phosphate groups. Only ethylation of the 3′-ultimate phosphodiester group was inhibitory (Nevinsky et al., 1987a,c; Levina et al., 1985). The contribution to binding

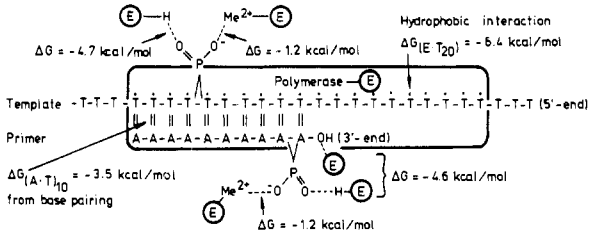

FIGURE 5: Proposed free energy model for binding of template-primer to DNA polymerases (described in the text).

affinity by the charge on the phosphate was estimated to be −1.2 kcal/mol. Thus, the overall affinity for binding of the primer dTMP [poly(dA) as template] may be disected as follows: Under the premise that K_m = 45 μM reflects the dissociation constant, the overall free energy of binding is −6 kcal/mol. After subtraction of the contributions from the phosphate moiety (−1.2 kcal/mol) and from the complementary interaction of thymine with adenine (−0.35 kcal/mol) the remaining amount of −4.5 kcal/mol is tentatively assigned to the interaction of both the 3′-OH group and the phosphate oxygen with the enzyme binding site (Figure 5).

The exact position of the 3′-priming site in the crystal structure of Klenow fragment is not known. Originally, Huberman and Kornberg (1970) derived from results of equilibrium dialysis that dAMP and other nucleoside 5′-monophosphates bound to the active site of *E. coli* DNA polymerase I at a locus that was different from that binding the nucleoside 5′-triphosphate substrates. The structural feature essential for binding involved two groups: the 3′-OH and the 5′-phosphate. The hydroxyl was thought to interact via H-bonding and the phosphate through coordination of Zn^{2+} ion with the enzyme. Crystallographic (Joyce & Steitz, 1987), genetic (Freemont et al., 1986), and kinetic data (Que et al., 1987, 1979) suggested that the active sites for the polymerase and exonuclease activities were separate and that the primer terminus could bind to either of these active sites. The above mononucleotide binding site was part of the exonuclease active site. For the polymerase active site, it has been shown that the interaction of oligonucleotides with the primer site depends on the complex formation of the enzyme with the template [Fisher & Korn, 1981; Fry and Loeb (1986) and references cited therein; Nevinsky et al., 1986, 1987a,b]. The criteria that we have worked out for the binding of primers to the polymerizing active site extend to the three eukaryotic DNA polymerases, which do not contain the 3′–5′ exonuclease activity.

(5) The maximal velocity, V_{max}, increases concomitantly with the larger sizes of primers (Figure 4). From a naive point of view one would not expect such a dependence, if a primer is fixed correctly with its 3′-terminus in its binding site. The dependences on the primer length for the *Physarum* DNA polymerases are close to that expection. However, the dependence for human placenta DNA polymerase α exhibits a rather steep decrease in activation energy as the primer binding cleft is thought to be filled with more and more nucleotides (Figure 4). We interprete this by assuming that the position of the primer 3′-end is not optimal for catalysis because of unfavorable orientations or binding to unproductive subsites in the cleft. Increasing the primer length diminishes these unfavorable modes by an increasingly tighter binding and sterically less ambiguous positioning and orientation in the polymerase cleft. The minimum in both primer affinity and maximum velocity at positions n = 20–23 for Klenow fragment (Figures 3 and 4) is surprising. It may reflect structural properties of the poly(dA)-oligo(dT) template-primer or of the topography of the DNA binding cleft (19–20 nucleotides long; Joyce et al., 1986; Kolocheva et al., 1989) or be connected with processivity of DNA synthesis (average chain lengths of 20–25 nucleotides; Kmiec & Worcel, 1985), though molecular mechanisms for any of these aspects are not known yet.

(6) In an attempt to establish an energetic model of the complex between template-primer and the polymerase, we take also into account previous results from the investigation of the template–DNA polymerase interaction (Nevinsky et al., 1986, 1987b,c; Lavrik et al., 1987; Knorre et al., 1988; Volchkova et al., 1989; Kolocheva et al., 1989). Similarly as with primers, only a single phosphodiester group has been found to interact with the enzyme active cleft in the position shown in Figure 5. The free energy contribution is −1.2 kcal/mol and depends on the presence of Mg^{2+} ions. The same phosphate group contributes an additional −4.5 kcal/mol by direct interaction with the binding cleft. The other phosphodiester groups of the template did not interact measurably with the DNA polymerase. An increase in the length of the oligonucleotide templates ($n \leq$ 19–20; Kolocheva et al., 1989) by one nucleotide was followed by an incremental value for the free energy of binding [−0.32 kcal/mol for $d(pT)_n$], assumed to arise mainly from hydrophobic interactions. The affinity of the templates increased in the order $d(pC)_n < d(pT)_n < d(pG)_n \leq d(pA)_n$.

It is interesting to compare the model in Figure 5 with the model for the Klenow fragment that has been derived from crystallographic data, model building, electrostatic field calculation, and genetics [Ollis et al., 1985; Joyce et al. (1987) and references cited therein]: (1) In both models, a DNA binding cleft of similar length is proposed that can enclose DNA templates and primers, the 3′-end of the primer being located approximately half-way between the ends of the cleft. A similar model has been derived for KB DNA polymerase α (Fisher & Korn, 1979, 1981). (2) About half of the free binding energy for the template DNA is thought to arise from incremental hydrophibic interaction per base in the model (Figure 5), whereas in the crystallographic model a more or less incremental free energy per phosphate moiety is inferred by the calculated positive electrostatic field potential within the cleft. (3) A metal ion aided interaction together with another type of interaction is exerted by a single template phosphate group in the model (Figure 5). Whereas a metal binding site in or near the cleft has not been reported by the crystallographic analysis, an arginine residue has been identified as part of helix K in that region. Genetic work has provided evidence that this side chain is involved in DNA binding. By its relative position within the cleft together with the magnitude of the free energy reported here for the template–phosphate interaction with the enzyme, the arginine residue could well be the enzymic group that contributes the −4.7 kcal/mol by electrostatic interaction. (4) As already indicated, the exact location of the primer 3′-end in the crystal structure is not known. The 3′-terminal phosphate group in the model in Figure 5 is bound to a metal ion that coordinates with the enzyme. However, a metal ion as part of the cleft has not yet been observed by crystallographic analysis. The only two metal ions so far identified were constituents of the presumed 3′–5′ exonuclease center of the enzyme that was supposed to be not identical with the 3′-priming center of the polymerase active site.

Magnesium ions are reported to play important roles in the interaction of KB DNA polymerase α with both template and primer (Fisher & Korn, 1981). They propose that the "normal" binding of primer occurs through a complex that

involves the coordination of four Mg^{2+} ions simultaneously to the primer and to the enzyme. It is not unlikely that cofactor metal ions are introduced on the occasion of substrate binding and are thus not found in the DNA-free crystals. Another possibility is that a conformational change during binding brings the enzyme-bound metal ion and the DNA phosphate group together. This is to be considered in the case of the mentioned template DNA phosphate group, which would be located in the crystal model of the Klenow fragment close to a region that is highly mobile and supposed to enclose the bound DNA duplex from the otherwise open side of the cleft (Joyce et al., 1987).

Our results support a model that seems applicable for the description of a variety of DNA polymerases. These may not include DNA polymerase β of higher eukaryotes, which differ on the basis of their low molecular mass (40 kDa) and catalytic properties from those enzymes investigated here (Fry & Loeb, 1986). Also since the model is not a dynamic one, it will not provide molecular understanding of the problem of processivity in enzymatic DNA synthesis.

Registry No. $r(pU)_7$, 96923-44-3; $d(pT)_7$, 2537-41-9; $r(pU)_9$, 96923-43-2; $d(pT)_9$, 54284-62-7; $d(pT)_8r(pU)$, 112241-87-9; DNA polymerase, 9012-90-2.

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