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Oligothymidylate Analogues Having Stereoregular, Alternating Methylphosphonate/Phosphodiester Backbones as Primers for DNA Polymerase†

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ABSTRACT: Oligothymidylate analogues having stereoregular, alternating methylphosphonate/phosphodiester backbones, d-T*p*(TpT*p*)$_4$T isomers I and II and d-T*p*(TpT*p*)$_3$T(pT)$_{1-5}$ isomers I and II, were prepared by methods analogous to the phosphotriester synthetic technique. The designations isomer I and isomer II refer to the configuration of the methylphosphonate linkage, which is the same throughout each isomer. Analogues with the type I methylphosphonate configuration form very stable duplexes with poly(dA) while those with the type II configuration form either 2T:1A triplexes or 1T:1A duplexes with poly(dA) of considerably lower stabilities. The oligothymidylate analogues were tested for their ability to initiate polymerizations catalyzed by *Escherichia coli* DNA polymerase I or calf thymus DNA polymerase α on a poly(dA) template. Neither d-T*p*(TpT*p*)$_4$T nor d-T*p*(TpT*p*)$_3$TpT served as initiators of polymerization while d-T*p*(TpT*p*)$_3$T(pT)$_{2-5}$ showed increasing priming ability as the length of the 3′-oligothymidylate tail increased. Analogues with type I methylphosphonate configuration were more effective initiators than the type II analogues at 37 °C. The apparent activation energies of polymerizations initiated by d-T*p*(TpT*p*)$_3$T-(pT)$_{4\text{ and }5}$ isomer I were greater than those for reactions initiated by isomer II or d-(Tp)$_{11}$T. The results suggest that DNA polymerase interacts with the charged phosphodiester groups of the primer molecule and may help stabilize primer/template interaction. At least two contiguous phosphodiester groups are required at the 3′ end of the analogue primers in order for polymerization to occur. Interactions between the polymerase and primer also appear to occur with phosphodiester groups located at sites remote from the 3′-OH polymerization site and may be influenced by the configuration of the methylphosphonate group.

Oligonucleotide analogues having nonionic alkyl phosphotriester or methylphosphonate backbones have served as models for studying the influence of backbone structure on the conformation and interactions of nucleic acids (Miller et al., 1971; Pless & Ts'o, 1977; Miller et al., 1979; Kan et al., 1980). As a result of their ability to form complexes with complementary nucleic acid sequences, these noncharged analogues have been used to probe and regulate cellular nucleic acid function both in the test tube and in living cells (Miller et al., 1974, 1977, 1981; Barrett et al., 1974; Jayaraman et al., 1981). In a recent publication we described the synthesis and physical properties of novel oligothymidylate analogues having methylphosphonate groups of fixed configuration arranged in an alternating manner with negatively charged phosphodiester groups throughout the backbone of the oligonucleotide (Miller et al., 1980a). The structure of the analogues may be written as d-T*p*(TpT*p*)$_4$T[1] where *p* denotes 3′–5′ methylphosphonate linkages with either *R* or *S* configuration throughout and p denotes 3′–5′ phosphodiester linkages.

These oligothymidylate analogues are able to form complexes with both poly(dA) and poly(rA). The stoichiometries and stabilities of the complexes are dependent upon the configuration of the methylphosphonate group. Although the

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[1] Abbreviations: d-N*p*N, an oligonucleotide having a 3′–5′ internucleoside methylphosphonate linkage; d-N*p*N, an oligonucleotide having a 3′–5′ *p*-chlorophenyl phosphotriester linkage; MST, (mesitylenesulfonyl)tetrazole; TSNI, (*p*-toluenesulfonyl)-4-nitroimidazole; DEAE, diethylaminoethyl; Tris, tris(hydroxymethyl)aminomethane; NMR, nuclear magnetic resonance; CD, circular dichroism. The symbols used to represent protected nucleosides and oligonucleotides follow the IUPAC–IUB Commission on Biochemical Nomenclature (1970) recommendations.

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methylphosphonate linkages of the analogues are completely resistant to nuclease hydrolysis, the phosphodiester linkages either are resistant or are slowly hydrolyzed (Miller et al., 1980a). We have also found that these analogues inhibit nuclease-catalyzed hydrolysis of DNA.

The presence of negatively charged phosphodiester groups in these analogues provides potential recognition sites for various nucleic acid enzymes. This and the ability of the analogues to form complexes with complementary polynucleotides suggested that they might serve as primers or substrates for nucleic acid replication enzymes such as DNA polymerase or DNA ligase. In this paper we explore the ability of d-T*p*(TpT*p*)$_4$T and oligothymidylate derivatives of this analogue, d-T*p*(TpT*p*)$_3$T(pT)$_{1-5}$, to initiate template-directed polymerizations catalyzed by DNA polymerases from *Escherichia coli* and calf thymus. The results of these studies provide some new insights into possible recognition features between the polymerases and their oligonucleotide initiators.

Experimental Procedures

Thymidine, d-(Tp)$_{11}$T, poly(dA), *E. coli* DNA polymerase I, and calf thymus DNA polymerase α were purchased from P-L Biochemicals. Tritium-labeled thymidine triphosphate was obtained from New England Nuclear. Anhydrous pyridine (Miller et al., 1980b), (mesitylenesulfonyl)tetrazole (Stawinski et al., 1977), (*p*-toluenesulfonyl)-4-nitroimidazole (Gough et al., 1979), d-[(MeO)$_2$Tr]T*p*(TṗT*p*)$_3$TṗCNET isomers I and II, and d-T*p*(TpT*p*)$_4$T isomers I and II (Miller et al., 1980a) were prepared as previously described. Preparative thick-layer silica gel chromatography (PTLC) was performed on PLK 5 F plates (20 cm × 20 cm × 1 mm) (Whatman, Inc.) with mixtures of methanol and chloroform as solvents. Preparative high-performance liquid chromatography (HPLC) was performed on a Partisil M-9 silica gel column (9.0 mm × 50 cm) (Whatman, Inc.). The column was eluted at a flow rate of 4.0 mL/min with a linear gradient of 0–20% methanol in chloroform (total volume 240 mL). Analytical and preparative ion-exchange HPLC were carried out on a Pellionex AL WAX column (4.6 × 50 cm). The column was eluted at a flow rate of 1.0 mL/min with a linear gradient of 0.001–0.50 M or 0.001–1.0 M ammonium acetate in 60% ethanol/water (total volume 40 mL). Analytical reversed-phase HPLC was performed on a Partisil 10 ODS-3 column (4.6 mm × 25 cm) (Whatman, Inc.). The column was eluted at a flow rate of 2.5 mL/min with a linear gradient of 0–25% acetonitrile in 0.10 M ammonium acetate buffer, pH 5.8 (total volume 50 mL).

Preparation of Oligothymidylate Methylphosphonate Analogues. The same general synthetic procedures were used as previously described for the preparation of alternating methylphosphonate/phosphodiester oligothymidylate analogues and oligonucleotide *p*-chlorophenyl phosphotriesters (Miller et al., 1980a,b). Protected oligothymidylate methylphosphonate analogues d-[(MeO)$_2$Tr]T*p*(TṗT*p*)$_3$Tṗ-(Tṗ)$_n$TOAc isomers I and II (n = 0–4) were each prepared by reaction of the triethylammonium salt of d[(MeO)$_2$Tr]-T*p*(TṗT*p*)$_3$Tṗ isomer I or isomer II with dTOAc or d-(Tṗ)$_n$TOAc (n = 1–4) in anhydrous pyridine in the presence of MST or TSNI. Reactions employing MST were run for 3 h while those employing TSNI were run overnight at room temperature. After workup, the protected oligomers were purified by silica gel PTLC or by preparative silica gel HPLC. The reaction conditions and yields are given in Table I.

The pure protected oligomers were treated with 1.0 mL of solution containing 0.017 M tetra-*n*-butylammonium fluoride in tetrahydrofuran/pyridine/water (8:1:1 v/v) at room temperature for 24 h, followed by treatment with 2 mL of 50% concentrated ammonium hydroxide in pyridine at 4 °C for 2.5 days. After evaporation of the solvents the residue was treated with 0.5 mL of 80% acetic acid at 37 °C for 30 min. The resulting d-T*p*(TpT*p*)$_3$T(pT)$_{1-5}$ analogues were each purified by preparative HPLC on a Pellionex AL WAX column (Leutzinger et al., 1978). Fractions containing the pure oligomers were combined, and the ammonium acetate was removed by passing the oligomer solution through a DEAE-cellulose column (2.5 × 4 cm) as previously described (Miller et al., 1980b). The oligomer was eluted from the DEAE column with 1 M ammonium bicarbonate. After removal of the buffer by evaporation, the oligomer was dissolved in 50% ethanol/water, and the solution was stored at 0 °C. For use in the physical and biological experiments, aliquots containing the required amount of oligomer were evaporated to dryness, and the oligomer was dissolved in the buffer used in the particular experiment.

The deprotected oligomers were characterized by digestion with snake venom phosphodiesterase and bacterial alkaline phosphatase. Twelve nanomoles of oligomer was treated with 2 μg of snake venom phosphodiesterase and 0.9 unit of bacterial alkaline phosphatase in 25 μL of buffer containing 10 mM Tris-HCl, pH 8.0, and 2 mM magnesium chloride for 24 h at 37 °C. The digest was briefly heated at 100 °C and then analyzed by reversed-phase HPLC. The areas under the product peaks were measured, and the ratio of d-T*p*T/d-T was calculated (Table II).

Interactions of d-Tp(TpTp)$_3$T(pT)$_{1-5}$ with Poly(dA). Ultraviolet spectra were recorded on a Varian 219 spectrophotometer equipped with a thermostated cell compartment and temperature readout accessory. The continuous variation experiments and melting experiments were carried out as previously described (Miller et al., 1971). Melting curves were corrected for the change in absorbance of poly(dA) with temperature. No change in absorbance was observed for d-T*p*(TpT*p*)$_3$T(pT)$_{1-5}$ over the temperature range investigated. The following molar extinction coefficients were used: poly(dA), $\sum_{257nm}$ = 9100; d-(Tp)$_{11}$T, $\sum_{266nm}$ = 8700; d-T*p*(TpT*p*)$_3$T(pT)$_{1-5}$ isomers I and II, $\sum_{265nm}$ = 8300 and $\sum_{265nm}$ = 8600, respectively.

DNA Polymerase Catalyzed Reactions. Enzymatic reactions were run in duplicate under the conditions described below. The reaction mixtures were preincubated for 10 min at the appropriate temperature in the absence of enzyme. The reactions were then initiated by addition of enzyme. Aliquots (4 μL for DNA polymerase I reactions, 6 μL for DNA polymerase α reactions) were withdrawn at appropriate times and added to 1 mL of ice-cold 5% trichloroacetic acid solution. The solution was filtered on a glass fiber filter (Whatman GF/C). The filter was washed with four 1-mL aliquots of 2 N hydrochloric acid and four 1-mL aliquots of 95% ethanol. After being dried under a heat lamp, the filters were counted in Betafluor (National Diagnostics, Inc.) scintillation fluid.

(*A*) *E. coli DNA Polymerase I Reactions.* Reactions were carried out in 30 μL of solution containing 20 mM Tris-HCl, pH 7.6, 5 mM magnesium chloride, 0.1 M potassium chloride, 0.6 μg of bovine serum albumin, 50 μM poly(dA) (base concentration), 5 μM oligothymidylate (base concentration), and 100–200 μM ^{3}H-labeled thymidine triphosphate, sp act. 250–500 mCi/mmol. Reactions were initiated by addition of 0.125–0.25 unit of DNA polymerase I.

(*B*) *Calf Thymus DNA Polymerase α Reactions.* Reactions were carried out in 40 μL of solution containing 20 mM potassium phosphate, pH 7.2, 6 μg of bovine serum albumin, 1

Table I: Synthesis of Protected Oligothymidylate Methylphosphonates

oligomer	isomer	d-[(MeO)$_2$Tr]-Tp(TṗTp)$_3$Tṗ (μmol)	5'-OH component (μmol)	condensing agent (μmol)	yield (%)
d-[(MeO)$_2$Tr]Tp(TṗTp)$_3$TṗTOAc	I	5.0	d-TOAc (7.5)	MST (30)	37
d-[(MeO)$_2$Tr]Tp(TṗTp)$_3$TṗTṗTOAc	I	5.0	d-TṗTOAc (7.5)	MST (30)	48
	II	2.5	d-TṗTOAc (3.8)	MST (15)	7
d-[(MeO)$_2$Tr]Tp(TṗTp)$_3$T(ṗT)$_2$TOAc	I	5.0	d-(Tṗ)$_2$TOAc (7.5)	MST (30)	51
	II	2.5	d-(Tṗ)$_2$TOAc (3.8)	MST (15)	7
d-[(MeO)$_2$Tr]Tp(TṗTp)$_3$T(ṗT)$_3$TOAc	I	2.5	d-(Tṗ)$_3$TOAc (3.8)	TSNI (15)	13
	II	2.5	d-(Tṗ)$_3$TOAc (3.8)	TSNI (15)	9
d-[(MeO)$_2$Tr]Tp(TṗTp)$_3$T(ṗT)$_4$TOAc	I	5.0	d-(Tṗ)$_4$TOAc (7.5)	TSNI (20)	50
	II	5.0	d-(Tṗ)$_4$TOAc (7.5)	TSNI (20)	40

Table II: Characterization of Oligothymidylate Methylphosphonates

oligomer		elution position on Pellionex AL WAX[a]	enzymatic hydrolysis products[b] (dTpT/dT) obsd	theory
d-Tp(TpTp)$_3$TpT	isomer I	0.21 M	3.7/1.0	4.0/1.0
d-Tp(TpTp)$_3$T(pT)$_2$	isomer I	0.27 M	1.9/1.0	2.0/1.0
	isomer II	0.26 M	1.9/1.0	
d-Tp(TpTp)$_3$T(pT)$_3$	isomer I	0.36 M	1.4/1.0	1.3/1.0
	isomer II	0.34 M	1.2/1.0	
d-Tp(TpTp)$_3$T(pT)$_4$	isomer I	0.43 M	0.8/1.0	1.0/1.0
	isomer II	0.43 M	0.9/1.0	
d-Tp(TpTp)$_3$T(pT)$_5$	isomer I	0.56 M	0.6/1.0	0.8/1.0
	isomer II	0.56 M	0.7/1.0	

[a] Ammonium acetate concentration at which oligomer is eluted from a Pellionex AL WAX column. [b] Conditions for hydrolysis are given under Experimental Procedures.

mM dithiothreitol, 6 mM magnesium acetate, 50 μM poly(dA) (base concentration), 5 μM oligothymidylate (base concentration), and 100 μM ^{3}H-labeled thymidine triphosphate, sp act. 250–500 mCi/mmol. Reactions were initiated by addition of 0.6–1.1 units of DNA polymerase α.

Results

Synthesis of Oligothymidylate Methylphosphonates. The syntheses of d-Tp(TpTp)$_4$T isomers I and II have been previously described (Miller et al., 1980a), and the preparations of the oligothymidylate derivatives, d-Tp(TpTp)$_3$T(pT)$_n$ (n = 1–5), closely follow these procedures. The reaction conditions and isolated yields are given in Table I. The deprotected oligomers were purified by high-performance liquid chromatography and were characterized by hydrolysis with a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase as indicated in Table II. The designations isomer I and isomer II refer to the configuration of the methylphosphonate linkage, which is the same throughout each isomer. The absolute configuration of the type I and type II methylphosphonate group has not as yet been determined.

Interaction of Oligothymidylate Methylphosphonates with Poly(deoxyadenylic acid). Complex formation between the oligothymidylate methylphosphonates and poly(dA) was determined under the salt conditions used in the DNA polymerase I and DNA polymerase α experiments (see the following section). The stoichiometries, melting temperatures, and temperatures at which the complexes are melted are summarized in Table III for the DNA polymerase I ionic strength conditions. Similar results were obtained for complexes in the DNA polymerase α buffer, although the melting temperatures were approximately 2 °C lower (data not shown). The melting profiles of all the complexes are sigmoidal in shape and show the expected increase in T_m as the oligomer chain length increases.

Ability of Oligothymidylate Methylphosphonates To Serve as Primers for DNA Polymerase. Table IV compares the initial rates of polymerization reactions initiated by d-Tp(TpTp)$_4$T and d-Tp(TpTp)$_3$T(pT)$_n$ (n = 1–5) with those initiated by d-(Tp)$_{11}$T on a poly(dA) template. The reactions were carried out at 37 °C by using either *E. coli* DNA polymerase I or calf thymus DNA polymerase α and were monitored by following the incorporation of ^{3}H-labeled thy-

Table III: Complex Formation between Oligothymidylate Methylphosphonate Analogues and Poly(dA)[a]

oligomer		stoichiometry of complex	T_m (°C)	temp at which complex is completely melted (°C)
d-(Tp)$_{11}$T		1T:1A	36.5	47
d-Tp(TpTp)$_4$T	isomer I	1T:1A	32.4	40
	isomer II	2T:1A	2[b]	15[b]
d-Tp(TpTp)$_3$TpT	isomer I	1T:1A	31.4	39
d-Tp(TpTp)$_3$T(pT)$_2$	isomer I	1T:1A	33.5	40
	isomer II	2T:1A	8.0	20
d-Tp(TpTp)$_3$T(pT)$_3$	isomer I	1T:1A	36.5	49
	isomer II	mixture of 1T:1A and 2T:1A	13.5	25
d-Tp(TpTp)$_3$T(pT)$_4$	isomer I	1T:1A	39.0	50
	isomer II	1T:1A	19.0	31
d-Tp(TpTp)$_3$T(pT)$_5$	isomer I	1T:1A	41.8	52
	isomer II	1T:1A	24.2	34

[a] Buffer: 20 mM Tris-HCl (pH 7.2), 5 mM magnesium chloride, and 0.1 M potassium chloride; total nucleotide concentration 2.5×10^{-5} M. [b] Buffer: 0.1 M sodium cocodylate (pH 6.8); total nucleotide concentration 2.5×10^{-5} M.

Table IV: Ability of Oligothymidylate Methylphosphonate Analogues To Initiate DNA Polymerase Catalyzed Polymerization on a Poly(dA) Template at 37 °C

	initial rate relative to d-$(Tp)_{11}T$-initiated reaction[a]			
	DNA polymerase I		DNA polymerase α	
primer	isomer I	isomer II	isomer I	isomer II
d-$(Tp)_{11}T$	1.000		1.000	
d-T*p*(TpT*p*)$_4$T	0.000	0.000	0.000	0.000
d-T*p*(TpT*p*)$_3$TpT	0.000		0.000	
d-T*p*(TpT*p*)$_3$T(pT)$_2$	0.011	0.000	0.006	0.004
d-T*p*(TpT*p*)$_3$T(pT)$_3$	0.120	0.000	0.149	0.015
d-T*p*(TpT*p*)$_3$T(pT)$_4$	0.535	0.047	0.437	0.072
d-T*p*(TpT*p*)$_3$T(pT)$_5$	0.984	0.331	0.721	0.229

[a] The reaction conditions are given under Experimental Procedures.

midylic acid into trichloroacetic acid precipitable material. The initial incorporation of label was linear with time for reactions catalyzed by either enzyme. No incorporation of label was observed in the absence of either primer or template molecules.

Neither d-T*p*(TpT*p*)$_4$T nor d-T*p*(TpT*p*)$_3$TpT initiated polymerization on the poly(dA) template in the presence of DNA polymerase I. Polymerization was observed for reactions initiated by d-T*p*(TpT*p*)$_3$T(pT)$_{2-5}$ isomer I. The initial rates of polymerization increased as the chain length of the oligothymidylate tail of the analogue increased. The rate of polymerization initiated by d-T*p*(TpT*p*)$_3$T(pT)$_5$ isomer I was almost identical with that of the reaction initiated by d-$(Tp)_{11}T$ at 37 °C. In contrast, d-T*p*(TpT*p*)$_3$T(pT)$_n$ isomer II served as a primer only when n was 4 or 5. Although the initial rates of the reactions primed by the shorter oligomers were considerably less than that for the reaction initiated by d-$(Tp)_{11}T$, the extent of these polymerizations approached that of d-$(Tp)_{11}T$ after prolonged incubation.

Similar results were obtained for reactions catalyzed by calf thymus DNA polymerase α. No priming activity was observed for d-T*p*(TpT*p*)$_4$T or d-T*p*(TpT*p*)$_3$TpT, while d-T*p*-(TpT*p*)$_3$T(pT)$_{2-5}$ showed increasing priming ability as the chain length of the oligothymidylate tail increased. In contrast to the results obtained with DNA polymerase I, the d-T*p*-(TpT*p*)$_3$T(pT)$_{2\text{ and }3}$ isomer II analogues each served as primers in the DNA polymerase α catalyzed reactions. Although the initial rates of these reactions were quite small, prolonged incubation resulted in polymerization levels comparable to those obtained in the d-$(Tp)_{11}T$/poly(dA) control reactions. On the other hand, no polymerization was observed, even after prolonged incubation, when DNA polymerase I was the catalyst.

The effects of temperature on polymerization reactions initiated by d-$(Tp)_{11}T$ and d-T*p*(TpT*p*)$_3$T(pT)$_{4\text{ and }5}$ were determined. The results of a typical experiment are shown in Figure 1 for polymerizations catalyzed by DNA polymerase I with d-$(Tp)_{11}T$ and d-T*p*(TpT*p*)$_3$T(pT)$_4$ as primers. The data are presented in the form of an Arrhenius plot. The apparent activation energies calculated from these plots are given in Table V.

The rates of the DNA polymerase I catalyzed polymerization primed by d-T*p*(TpT*p*)$_3$T(pT)$_4$ are very temperature dependent. The rate of the isomer I primed reaction diminishes rapidly with decreasing temperature and is barely detectable below 19 °C. In contrast, the isomer II primed reaction has a rate maximum at 27 °C. At low temperatures, isomer II is a better primer than is isomer I. The rates of polymerizations initiated by d-T*p*(TpT*p*)$_3$T(pT)$_5$ isomer I increased over the temperature range 5–35 °C, while isomer II showed a rate maximum at approximately 25 °C. Similar temperature dependence was observed for reactions catalyzed by DNA polymerase α.

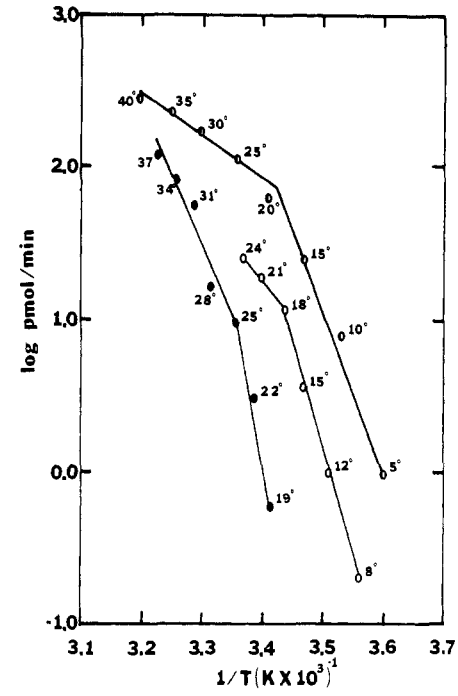

FIGURE 1: Arrhenius plot of the effect of temperature on the rate of *E. coli* DNA polymerase I catalyzed polymerizations on a poly(dA) template using d-$(Tp)_{11}T$ (◐) and d-T*p*(TpT*p*)$_3$T(pT)$_4$ isomer I (●) and isomer II (○) as primers. The reaction conditions are given under Experimental Procedures.

Table V: Apparent Activation Energies for Reactions Catalyzed by DNA Polymerase I on a Poly(dA) Template[a]

primer		$E_{act,app}$ (kcal/mol)[b]
d-$(Tp)_{11}T$		44.5 (5–20)
		11.9 (20–35)
d-T*p*(TpT*p*)$_3$T(pT)$_4$	isomer I	91.5 (19–25)
		32.7 (25–35)
	isomer II	66.9 (8–18)
		21.5 (18–24)
d-T*p*(TpT*p*)$_3$T(pT)$_5$	isomer I	56.7 (5–20)
		23.8 (20–35)
	isomer II	38.9 (5–25)

[a] The reaction conditions are given under Experimental Procedures. [b] The apparent activation energies were determined from Arrhenius plots similar to Figure 1 and are the average values obtained from at least two experiments. The temperature range (degree Celcius) is in parentheses.

The apparent activation energies of DNA polymerase I catalyzed polymerizations initiated by d-T*p*(TpT*p*)$_3$T(pT)$_4$ isomers I and II and d-Tp(TpT*p*)$_3$T(pT)$_5$ isomer I are between 1.3 and 2.7 times greater than those observed for d-$(Tp)_{11}T$-initiated polymerization (Table V). The apparent activation energy for the d-T*p*(TpT*p*)$_3$T(pT)$_5$, isomer II initiated reaction is similar to that of the d-$(Tp)_{11}T$-initiated reaction over the temperature range 5–25 °C. Similar results were obtained for the DNA polymerase α catalyzed reactions.

Discussion

We have examined the ability of d-T*p*(TpT*p*)$_4$T and a series of 3′-oligothymidylate derivatives of this analogue, d-T*p*-(TpT*p*)$_3$T(pT)$_{1-5}$, to initiate polymerizations catalyzed by DNA polymerase on a poly(dA) template. The oligothymidylate derivatives were prepared by methods analogous

to the phosphotriester approach used to synthesize oligodeoxyribonucleotides (Miller et al., 1980b). Thus, 3′-*O*-acetylthymidine or 3′-*O*-acetyloligothymidylate *p*-chlorophenyl phosphotriesters, d-$(T\dot{p})_nTOAc$ (n = 1–4), were added to either the all *R* or all *S* methylphosphonate isomers of d-$[(MeO)_2Tr]Tp(T\dot{p}Tp)_3T\dot{p}$ (Miller et al., 1980a). As shown in Table I, reactions carried out on a 5-μmol scale generally proceeded in satisfactory yield, while the yields for the 2.5 μmol scale reactions were quite low. These low yields can be attributed to the difficulty of excluding small traces of moisture from the condensation reaction mixtures.

The *p*-chlorophenyl phosphate protecting groups were selectively removed with tetra-*n*-butylammonium fluoride in aqueous tetrahydrofuran (Ogilvie & Beaucage, 1979), a reagent which does not attack methylphosphonate linkages (Miller et al., 1980a). The percentage of unwanted internucleotide phosphotriester bond cleavage increased as the chain length of the oligomers increased. However, the use of pyridine aldoximate, a reagent which does not cause internucleotide phosphotriester bond cleavage (Reese et al., 1978), was precluded since this reagent hydrolyzes methylphosphonate linkages.

The deprotected oligonucleotides were completely hydrolyzed to d-T and d-T*p*T by a combination of snake venom phosphodiesterase and bacterial alkaline phosphatase. The expected ratios of products were obtained for each analogue (Table II). Although snake venom phosphodiesterase alone hydrolyzed the oligothymidylate tail of d-$Tp(TpTp)_3T(pT)_n$, further hydrolysis of the remaining d-$Tp(TpTp)_3T$ was incomplete even after prolonged incubation. Previous studies have shown that this enzyme completely hydrolyzes the phosphodiester but not the methylphosphonate linkages of d-$Tp(TpTp)_4T$ in an exonucleolytic manner starting at the 3′ end of the analogue (Miller et al., 1980a). The inability of snake venom phosphodiesterase to completely hydrolyze the methylphosphonate-flanked phosphodiester bonds of d-$Tp(TpTp)_3T(pT)_n$ suggests that the d-pT initially formed in the reaction serves as an inhibitor of hydrolysis of these phosphodiester bonds.

The analogues d-$Tp(TpTp)_3T(pT)_{1-5}$ with the type I methylphosphonate configuration each formed 1T:1A duplexes with poly(dA) (Table III). Two ionic strength conditions were chosen for these experiments: 0.1 M potassium chloride, 20 mM Tris-HCl, and 20 mM magnesium chloride, the buffer used for the DNA polymerase I reactions, and 20 mM potassium phosphate and 6 mM magnesium acetate, the buffer used for the DNA polymerase α reactions. The stabilities of the duplexes in the DNA polymerase I buffer system were slightly greater than those measured in the DNA polymerase α buffer system and reflect the greater ionic strength of this buffer. As expected, the T_m values of the duplexes increased as the chain length of the analogues increased.

In contrast to the behavior of the analogues with type I methylphosphonate configuration, d-$Tp(TpTp)_3T(pT)_n$ isomer II forms either 2T:1A triplexes or 1T:1A duplexes with poly(dA). The type of complex formed depends upon the length of the 3′-terminal oligothymidylate tail. The T_m values of the isomer II/poly(dA) complexes are 17–30 °C lower than those of the isomer I/poly(dA) duplexes. This behavior is similar to that previously observed for d-$Tp(TpTp)_4T$ isomer I vs. d-$Tp(TpTp)_4T$ isomer II. The influence of the methylphosphonate configuration on the stoichiometry and the stability of the analogue/poly(dA) complexes may reflect differences in the hydration of the methylphosphonate/phosphodiester backbone of the oligomer in the duplex vs. the single-strand forms (Miller et al., 1980a).

The ability of the oligonucleoside methylphosphonates to serve as initiators of polymerization catalyzed by either *E. coli* DNA polymerase I or calf thymus DNA polymerase α on a poly(dA) template was examined. The initial rates of polymerization observed are compared with those observed for d-$(Tp)_{11}T$-initiated polymerizations in Table IV. Neither d-$Tp(TpTp)_4T$ nor d-$Tp(TpTp)_3TpT$ served as primers for the polymerization reactions, even after prolonged (24-h) incubation. The presence of two thymidylate residues at the 3′ end of the analogue resulted in priming activity by d-$Tp(TpTp)_3T(pT)_2$ isomer I. Further additions of 3′-thymidylate residues gave increasingly more efficient primers. Thus the initial rate of polymerization initiated by d-$Tp(TpTp)_3T(pT)_5$ isomer I was nearly the same as that of d-$(Tp)_{11}T$-initiated polymerization. In contrast to the results obtained with isomer I, isomer II showed considerably less ability to initiate polymerization at 37 °C.

The results suggest that the presence of methylphosphonate linkages in the backbone of the primer molecule can significantly perturb the priming ability of the oligonucleotides. Since all the oligomers tested with the type I methylphosphonate configuration form stable duplexes with poly(dA), the lack of priming ability does not result from inability of the oligomer to bind to the template. This observation suggests that the presence of the methylphosphonate groups may perturb the binding of the polymerase to the initiator/template complex. Thus binding sites on the polymerase may interact directly with the charged phosphodiester backbone of the oligonucleotide primer. This conclusion is in agreement with recent studies by Fisher & Korn (1981) on the effects of magnesium ion concentration on polymerization reactions. Their results suggest that phosphodiester groups of the primer interact via a magnesium complex with binding sites of KB cell DNA polymerase α. Introduction of nonionic methylphosphonate groups could thus directly eliminate binding interactions between the primer backbone and the enzyme or could perturb the interactions of adjacent phosphodiester groups with the enzyme.

Previous studies have shown that a free 3′-hydroxyl group is required for initiation of polymerization (Huberman & Kornberg, 1970). NMR investigations have suggested that the zinc atom associated with *E. coli* DNA polymerase I binds to the 3′-hydroxyl group of the primer molecule (Springgate et al., 1973). The observation that d-$Tp(TpTp)_3T(pT)_2$ isomer I but not d-$Tp(TpTp)_3TpT$ isomer I can initiate polymerization suggests that at least two contiguous phosphodiester groups are required in molecules of this type for polymerization to occur. These phosphodiesters could serve in addition to the 3′-hydroxyl group as binding points between the polymerase and the 3′ end of the primer molecule.

In the case of the analogues with the type II methylphosphonate backbone configuration, only d-$Tp(TpTp)_3T(pT)_{4\text{ and }5}$ isomers, which form 1T:1A duplexes with poly(dA), are able to prime polymerization reactions catalyzed by DNA polymerase I at 37 °C. However, the results shown in Table III indicate that at this temperature the duplexes should be completely melted out. These observations therefore suggest that complex formation between the initiator and the template is not required in order for initiation of polymerization to occur. Thus, the polymerase may stabilize binding of the initiator with the template. Similar suggestions have been made for replication of single-strand DNA by T4 phage DNA polymerase (Goulian et al., 1968), for replication of homopolymers by calf thymus DNA polymerase α (Cassani & Bollum, 1969; Chang

et al., 1972), and for replication of single-stranded "hook" type primer/templates by KB cell DNA polymerase α (Fisher & Korn, 1979).

Results obtained with calf thymus DNA polymerase α were similar to those of the DNA polymerase I experiments. In this case, however, d-T*p*(TpT*p*)$_3$T(pT)$_{2\text{ and }3}$ isomers II, which form 2T:1A triplexes with poly(dA), can also initiate polymerization. Possibly in this system the polymerase selectively stabilizes duplex formation between the analogues and the template.

The effects of temperature on polymerization reactions initiated by d-(Tp)$_{11}$T and d-T*p*(TpT*p*)$_3$T(pT)$_{4\text{ and }5}$ were examined (Figure 1 and Table V). Arrhenius plots of the rate vs. temperature data were biphasic with a break point occurring between 20 and 25 °C. This behavior is in contrast to the linear plot observed for the polymeric primer/template system poly(d-A-T) whose apparent activation energy is 17 kcal/mol over the temperature range 4–40 °C (McClure & Jovin, 1975). The biphasic nature of the plots for the oligonucleotide-primed polymerizations may result from a change in the rate-determining step of the polymerization reaction as the temperature is increased.

For reactions catalyzed by DNA polymerase I, the order of apparent activation energies for the oligonucleotide-initiated reactions is d-T*p*(TpT*p*)$_3$T(pT)$_4$ > d-T*p*(TpT*p*)$_3$T(pT)$_5$ > d-(Tp)$_{11}$T. Both d-T*p*(TpT*p*)$_3$T(pT)$_4$ isomers I and II and d-T*p*(TpT*p*)$_3$T(pT)$_5$ isomers I and II form 1T:1A duplexes with poly(dA). The CD spectra of these duplexes are virtually identical with that of the d-(Tp)$_{11}$T/poly(dA) duplex (data not shown). This suggests that the conformation of the analogue/poly(dA) duplexes are very similar if not identical with that of d-(Tp)$_{11}$T/poly(dA). Thus it is unlikely that the increased apparent activation energies of the analogue-initiated polymerizations vs. that of the d-(Tp)$_{11}$T-initiated polymerization are due to differences between the conformation of the analogue/poly(dA) duplexes and that of the d-(Tp)$_{11}$T/poly(dA) duplex.

The increased apparent activation energies could be a consequence of unfavorable interactions between the polymerase and the methylphosphonate groups of the analogue primer backbone. Such altered interactions could influence the rate of polymerization by directly affecting the formation of the enzyme/primer/template complex. Alternatively, the rates of subsequent steps in the polymerization process, such as translocation of the polymerase, could be altered by unfavorable interactions. The methylphosphonate groups in d-T*p*(TpT*p*)$_3$T(pT)$_{4\text{ and }5}$ are located some distance removed from the site of the polymerization reaction. Thus interactions between the polymerase and the initiator backbone may extend up to at least five nucleotide units from the 3′-OH terminus. This would be consistent with the model of Fisher & Korn, which suggests that up to seven phosphodiester linkages may be involved in primer/polymerase binding. The observation that the apparent activation energies of the type I analogues are greater than those of the type II analogues suggests that such interactions may be influenced by the configuration of adjacent methylphosphonate groups.

The results presented in this paper suggest that DNA polymerase interacts with charged phosphodiester groups of the backbone of the primer molecule. Such interactions appear to occur with phosphodiester groups located at the 3′ terminus of the primer as well as with groups located at sites remote from the 3′ end. Although d-T*p*(TpT*p*)T isomer I does not serve as an initiator of DNA polymerization, preliminary experiments have shown that it does inhibit d-(Tp)$_{11}$T-initiated polymerization on a poly(dA) template catalyzed by DNA polymerase α. Further characterization of this inhibitory reaction may provide additional insights into the interactions of DNA polymerase with primer/template complexes.

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