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Enzymic Synthesis of Oligonucleotides Containing Methylphosphonate Internucleotide Linkages

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ABSTRACT: Thymidine 5′-*O*-(pyrophosphoryl methylphosphonate) (dTTPαCH3) has been chemically synthesized by condensation of thymidine 5′-*O*-(methylphosphonate) with pyrophosphate. This novel nucleotide, which contained an α-phosphorus atom as methylphosphonate, was used as a substrate of terminal deoxynucleotidyltransferase (TDTase) in the presence of oligonucleotide (5′-GCTGTATCGTCA-AGGCACTC-3′) as an initiator. The reaction products were separated into two components by reverse-phase high-performance liquid chromatography (RP-HPLC). These products were, after purification, digested with nuclease P1 and alkaline phosphatase followed by separation of digested products by RP-HPLC. The result showed the presence of one of the isomers of 2′-deoxycytidyl-3′-methylphosphonyl-5′-thymidine (dCpCH3T) and 2′-deoxycytidyl-3′-methylphosphonyl-5′-thymidyl-3′-methylphosphonyl-5′-thymidine (dCpCH3TpCH3T), respectively. Fast atom bombardment mass spectrometry of these products further supported identification of the dinucleotide and the trinucleotide. These results indicated that dTTPαCH3 was used as a substrate of TDTase, resulting in methylphosphonate linkages. Produced oligomers were resistant to hydrolysis by snake venom phosphodiesterase I.

Oligonucleoside methylphosphonates, backbone-modified oligomers containing uncharged methylphosphonate internucleotide linkages, have been chemically synthesized and used by several laboratories. For example, owing to their stability to nucleases and permeability into mammalian cells (Agarwal & Riftina, 1979; Miller et al., 1981), these analogues have been used as antisense oligonucleotides to inhibit replication of a number of DNA or RNA viruses including human immunodeficiency virus (HIV)[1] (Miller et al., 1985; Smith et

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al., 1986; Agris et al., 1986; Zaia et al., 1988; Sarin et al., 1988; Kulka et al., 1989). These backbone-modified oligomers have also been useful for studying the interaction of nucleic acids and proteins (Agarwal & Riftina, 1979; Miller et al., 1980, 1982; Nobel et al., 1984).

These analogues contain a chiral center at each methylphosphonate and, when obtained by chemical synthesis, consist of 2^n diastereomers (n is the number of methylphosphonate internucleotide linkages). Callahan et al. (1986) reported that a hexamer containing a methylphosphonate linkage of R_p configuration formed Z-DNA in high salt concentrations to the same extent as a normal hexamer containing the usual phosphodiester internucleotide linkage, while a hexamer containing a methylphosphonate linkage of S_p configuration did not. In addition, Bower et al. (1987) reported that self-complementary oligonucleotides having a single methylphosphonate linkage with R_p configuration hybridized intermolecularly more stably than the S_p isomers. These data indicate that the configuration of methylphosphonate at the internucleotide linkage greatly affects the biochemical and biological properties of these analogues, such as their ability to form a duplex. Stereoselective chemical synthesis of these analogues has not been well documented, except for the pioneering study by Lešnikowski et al. (1987a, 1988) who succeeded in synthesizing oligonucleotides of thymidine methylphosphonate stereoselectively.

It is well-known that enzymic catalysis is generally stereospecific, and it appeared possible that stereospecific methylphosphonate internucleotide linkages can be synthesized by an enzymic reaction. In this work we synthesized a novel nucleoside triphosphate containing methylphosphonate at the α position and found that this analogue was incorporated by terminal deoxynucleotidyltransferase (TDTase) at the 3′ terminus of the initiator oligonucleotide. The result suggested that the TDTase reaction with dTTPαCH3 gave an S_p configuration at the methylphosphonate linkage. The reaction products were resistant against snake venom phosphodiesterase I.

Materials and Methods

Thymidine and 2′-deoxycytidine were obtained from Yamasa Shoyu. Thymidine 5′-*O*-(methylphosphonate) (dTMPCH3) was prepared essentially according to the methods described by Myers et al. (1965). Anhydrous pyridine was purchased from Dojindo Laboratories. DMF was distilled under reduced pressure and stored with 4-Å molecular sieves. DEAE-Toyopearl 650M was from Toyo Soda Mfg. Dowex 50W-X8 (100–200 mesh) was from Muromachi Kagaku Kogyo. Nuclease P1 (800 units/mg) and *Crotalus atrox* venom phosphodiesterase I (25 units/mg) were purchased from Pharmacia. Calf thymus terminal deoxynucleotidyltransferase (10000 units/mg) was purchased from Takara Shuzo. Calf intestinal alkaline phosphatase (2600 units/mg) was from Boeringer Mannheim.

Oligonucleotides used as initiators for TDTase were synthesized with an Applied Biosystems 380A DNA synthesizer by the phosphoramidite method and purified by reverse-phase and anion-exchange high-performance liquid chromatography followed by gel filtration.

HPLC was carried out with a Shimadzu LC-6A controlled by system controller SCL-6A. Reverse-phase chromatography was carried out with a YMC-312 ODS column (6 × 150 mm, purchased from Yamamura Chemical Labolatories) at a flow rate of 1 mL/min. The nucleotides were eluted with one of the following gradient systems and monitored by a Shimadzu UV detector SPD-6A at 260 nm. Linear gradients of solution A (0.05 M TEAA, pH 6.8) and solution B (CH_3CN) were used. Gradient I was 3% B at 0 min and 30% B at 20 min. Gradient II was 10% B at 0 min and 25% B at 15 min; then from 15 to 20 min, 25% of B was used. Gradient III was 10% B at 0 min and 14% B at 10 min; then, from 10 to 30 min, 14% of B was used. Gradient IV was 0% B at 0 min, 5% B at 5 min, and 14% B at 20 min; then, from 20 to 35 min, 14% of B was used. Gradient V was 10% B at 0 min and 19% B at 15 min. Gradient VI was a linear gradient of water and B (0% B at 0 min, 5% B at 2 min, and 14% B at 20 min). Elution conditions are given in individual figure legends.

FAB-MS spectra were recorded in the positive-ion mode on a JEOL DX300 mass spectrometer equipped with a JMA-2000S mass data analysis system. Samples were mixed with glycerol on the probe tip. Xenon was used for the fast atom gun at 3 keV with a total discharge current of 20 mA.

Synthesis of dTTPαCH3. dTTPαCH3 was prepared according to the general procedure for the synthesis of nucleoside triphosphate (Hoard & Ott, 1965) with slight modifications. Anhydrous tributylammonium salt of thymidine 5′-*O*-(methylphosphonate) (0.1 mmol) was dissolved in 1 mL of DMF. The solution was mixed with 0.5 mmol of 1,1′-carbonyldiimidazole in 1 mL of DMF and stirred for 1 h at room temperature. The mixture was further mixed with 5 mL of DMF containing 1 mmol of tri-*n*-butylammonium pyrophosphate and maintained at room temperature for 1 h. After removal of the precipitate by centrifugation, the supernatant was evaporated under vacuum to dryness, and crude dTTPαCH3 was purified on a DEAE-Toyopearl column (14.5 × 3.5 cm) with a linear gradient of TEAB (0.05–0.25 M, pH 7.5) followed by further elution with TEAB (0.25 M, pH 7.5). The absorbance of each eluted fraction (10 mL) was measured at 260 nm. Fractions 38–42 were pooled, and ethanol was added to 50% followed by repeated evaporation in vacuo to remove TEAB. Yield was approximately 20–30%. The molecular weight of the synthesized compound was measured by FAB-MS.

Synthesis of dCpCH3T, dTpCH3T, and dCpCH3TpCH3T. Di- and trinucleoside methylphosphonates have been prepared essentially according to the procedure described by Miller et al. (1979), except for the use of dimethoxytrityl group for protection of 5′OH, MSNT as a condensing reagent, and *tert*-butylamine for removal of the cyanoethyl group. After removal of protecting groups, the diastereomers were separated and purified by RP-HPLC.

Alkaline Hydrolysis of Trimer to Dimers (dCpCH3T, dTpCH3T). An aqueous solution (50 μL) containing 0.1 A_{260} unit of an isolated isomer of dCpCH3TpCH3T was mixed with an equal volume of 28% NH_4OH and incubated at 37 °C for 4 h followed by analysis with RP-HPLC.

[1] Abbreviations: TDTase, terminal deoxynucleotidyltransferase; HIV, human immunodeficiency virus; FAB-MS, fast atom bombardment mass spectrometry; RP-HPLC, reverse-phase high-performance liquid chromatography; HPTLC, high-performance thin-layer chromatography; DEAE, diethylaminoethyl; ODS, octadecylsilyl; TEAB, triethylammonium bicarbonate; TEAA, triethylammonium acetate; DMF, *N*,*N*-dimethylformamide; MSNT, 1-(mesitylsulfonyl)-3-nitro-1,2,4-triazole; Tris·HCl, tris(hydroxymethyl)aminomethane hydrochloride; dNTP, deoxynucleoside 5′-triphosphate; dTMPCH3, thymidine 5′-*O*-(methylphosphonate); dTTPαCH3, thymidine 5′-*O*-(pyrophosphoryl methylphosphonate); dA, 2′-deoxyadenosine; dG, 2′-deoxyguanosine; dC, 2′-deoxycytidine; dT, thymidine; dCpCH3T, 2′-deoxycytidyl-3′-methylphosphonyl-5′-thymidine; dTpCH3T, thymidyl-3′-methylphosphonyl-5′-thymidine; dCpCH3TpCH3T, 2′-deoxycytidyl-3′-methylphosphonyl-5′-thymidyl-3′-methylphosphonyl-5′-thymidine.

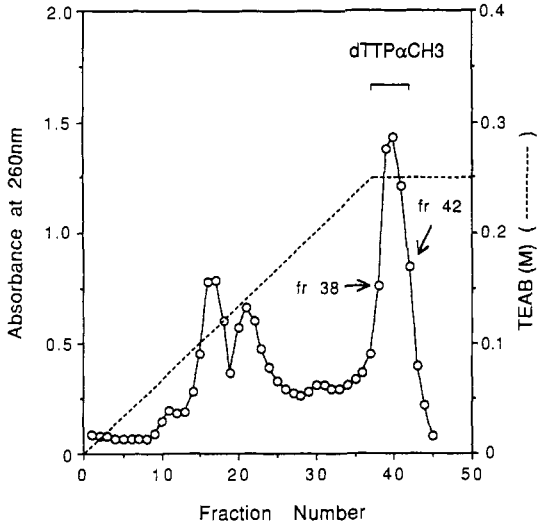

FIGURE 1: Purification of dTTPαCH3 by anion-exchange chromatography. The reaction mixture for preparation of dTTPαCH3 was chromatographed on a DEAE-Toyopearl column (14.5 × 3.5 cm) with a linear gradient of TEAB (0.05–0.25 M, pH 7.5) followed by isocratical elution with TEAB (0.25 M, pH 7.5). Absorbance of each fraction was monitored at 260 nm. Fractions 38–42, shown by the bracket and arrows, were used for enzymic reaction as purified dTTPαCH3.

Elongation at 3′ Termini of the Initiator Oligonucleotide with dTTPαCH3 by TDTase. The reaction mixture (100 μL) for enzymic elongation of the initiator oligonucleotide contained 100 mM sodium cacodylate (pH 7.2), 25 mM $MgCl_2$, 0.1 mM dithiothreitol, 0.5 A_{260} unit of the initiator oligonucleotide (5′-GCTGTATCGTCAAGGCACTC-3′), and 2.0 A_{260} units of dTTPαCH3. The reactions were initiated by the addition of 50 units of TDTase and incubated at 37 °C for 24 h. Aliquots of the reaction mixture were taken and subjected to RP-HPLC to separate into two components of UV-absorbing materials (peaks 3 and 4 of Figure 5). Each peak was purified and further analyzed by 20% polyacrylamide gel electrophoresis.

Identification of Elongated Nucleotides. The reaction products isolated as indicated above (0.2 A_{260} unit) were digested with nuclease P1 and alkaline phosphatase. The reaction mixture (40 μL) containing 20 mM sodium acetate (pH 5.3), 0.1 mM $ZnCl_2$, and 4 units of nuclease P1 was incubated at 37 °C for 30 min. At 30 min, a 40-μL solution containing 50 mM Tris·HCl (pH 8.0), 0.1 mM EDTA, and 23 units of alkaline phosphatase was further added to the reaction mixture and incubated for additional 30 min at 37 °C. At the end of the reaction, aliquots of the reaction mixture were subjected to RP-HPLC, and each isolated reaction product (dCpCH3T and dCpCH3TpCH3T) was characterized by FAB-MS.

Snake Venom Phosphodiesterase I Digestion of Oligomers Elongated with dTTPαCH3. The reaction mixture (100 μL) contained 100 mM Tris·HCl (pH 8.9), 100 mM NaCl, 14 mM $MgCl_2$, 0.2 A_{260} unit of purified elongated oligonucleotide, and 1 unit of snake venom phosphodiesterase I. The reaction mixture was incubated at 37 °C for up to 2 h. At the various times indicated, aliquots of the reaction mixture were analyzed with RP-HPLC.

RESULTS

Synthesis of dTTPαCH3. Thymidine 5′-*O*-(methylphosphonate) (dTMPCH3) was converted to its triphosphate analogue by the reaction with pyrophosphate in DMF. In this reaction, dTMPCH3 was activated by the reaction with 1,1′-carbonyldiimidazole to give the presumed intermediate,

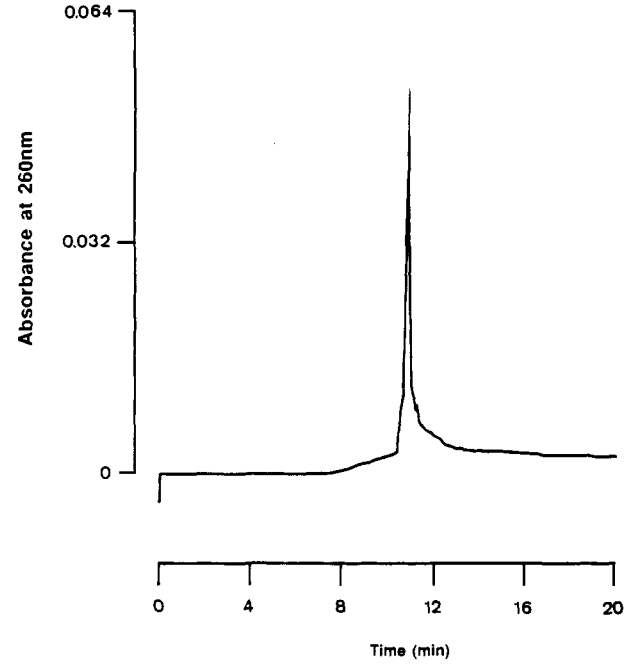

FIGURE 2: RP-HPLC analysis of purified dTTPαCH3. This figure shows the chromatogram of fraction 39 of Figure 1. HPLC was carried out with gradient I.

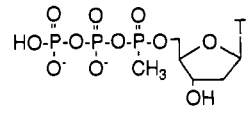

FIGURE 3: Structure of dTTPαCH3.

thymidine 5′-*O*-(methylphosphonic imidazolide), which subsequently condensed with pyrophosphate. Aliquots were taken from time to time and analyzed with HPTLC. Decrease of starting material and appearance of new spot on HPTLC indicated the progress of the reaction. After 1 h, the reaction mixture was subjected to DEAE-Toyopearl column chromatography as shown in Figure 1. It can be seen that there are three major peaks in this chromatogram. A separate experiment indicated that the first two major peaks were dTMPCH3 and other byproducts. The second major peak was identified as dTTPαCH3 by FAB-MS. The purity of the synthesized product was assessed by RP-HPLC as shown in Figure 2. dTTPαCH3 has a chiral center at the α-phosphorus (Figure 3). The configuration (S_p or R_p) of the synthesized dTTPαCH3 is not clear at the present moment. The reaction product was in the form of a triethylammonium salt, but this was converted to a sodium salt for the FAB-MS analysis by the method previously reported (Hoard & Ott, 1965). The FAB-MS of this product gave the expected molecular ion signals: m/z 547 $[M + H]^+$ and 569 $[M + Na]^+$ as the trisodium salt. dTTPαCH3 was converted to dTMPCH3 upon treatment with 0.1 N NaOH or alkaline phosphatase, supporting the identification of this product (data not shown).

Elongation of the Initiator Oligonucleotide with dTTPαCH3 Catalyzed by TDTase. dTTPαCH3 synthesized in the preceding section was now tested as a possible substrate for TDTase. Since TDTase requires oligonucleotide as an initiator, 5′-GCTGTATCGTCAAGGCACTC-3′ was used as the initiator for this reaction. The absence of nuclease activity of TDTase used in this experiment was confirmed by the analysis of the reaction mixture without the substrate nucleotide. In addition, efficient polymerizing reaction was observed after a 24-h incubation of the initiator oligonucleotide

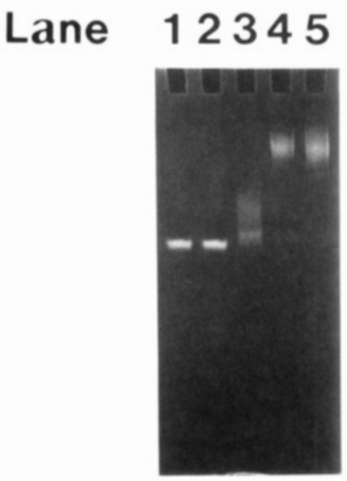

FIGURE 4: Absence of the nuclease activity of TDTase. Samples applied to lanes are 10 μL of the reaction mixture (100 μL) for elongation of the initiator oligonucleotide as described under Materials and Methods, after a 24-h incubation at 37 °C, containing (1) the initiator oligonucleotide, (2) the initiator oligonucleotide and TDTase, (3) the initiator oligonucleotide, TDTase, and 0.02 μmol of dTTP, (4) the initiator oligonucleotide, TDTase, and 0.2 μmol of dTTP, and (5) the initiator oligonucleotide, TDTase, and 1 μmol of dTTP. After the reaction mixture was subjected to the polyacrylamide gel electrophoresis, the gel was stained with ethidium bromide for 45 min. The electric current direction was from the top to the bottom of the gel. Lane 2 shows no sign of reduction of the initiator oligonucleotide by TDTase.

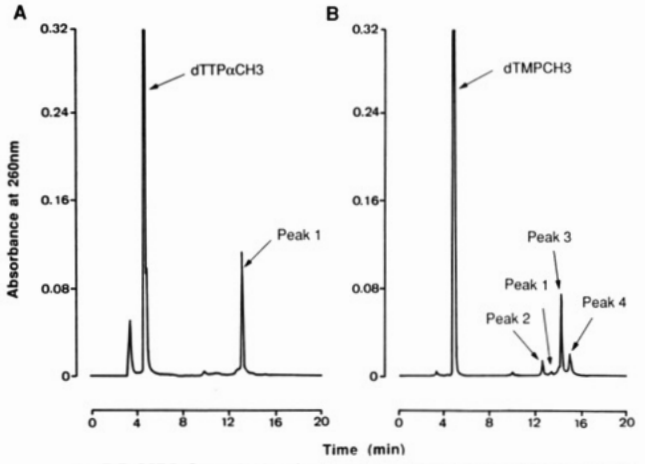

FIGURE 5: RP-HPLC analysis of the elongated product of the initiator oligonucleotide with dTTPαCH3 catalyzed by TDTase. (A) Reaction mixture at 0 time: The largest peak seen in this figure represents dTTPαCH3 (retention time 4.62 min), and peak 1 represents the initiator oligonucleotide. The small peak eluting faster than dTTPαCH3 was not identified. (B) Reaction mixture after a 24-h incubation at 37 °C: The largest peak represents $dTMPCH_3$ (retention time 4.86 min) which was identified by a separate experiment. Although the retention time of dTTPαCH3 was close to that of dTMPCH3 under this HPLC condition used for analyzing the elongated oligomer, it was possible to distinguish clearly both compounds by using anion-exchange chromatography (data not shown). Peaks 2–4 represent the reaction products. HPLC was carried out with gradient II. The reaction conditions were described under Materials and Methods.

and thymidine triphosphate (dTTP) with TDTase (Figure 4). In the experiment shown in Figure 5, the reaction mixture was subjected to RP-HPLC and UV-absorbing material was monitored. It can be seen from this figure that the amount of the initiator oligonucleotide (peak 1) was reduced after 24-h reaction and an additional three peaks (peaks 2–4) appeared, suggesting that the enzymic reaction took place. In a separate experiment, the reaction products were not observed at all

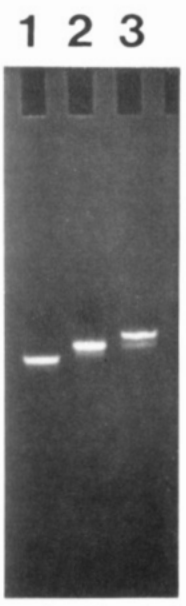

FIGURE 6: Polyacrylamide gel electrophoresis analysis of purified peaks 3 and 4 of Figure 4 in the presence of 7 M urea. Samples applied to lanes are (1) the initiator oligonucleotide, (2) peak 3, and (3) peak 4. The gel was stained with ethidium bromide for 30 min.

Table I: Characterization of Diastereomers of dCpCH3T, dTpCH3T, and dCpCH3TpCH3T

compound	FAB-MS, m/z $[M + H]^+$
isomer 1 of dCpCH3T	530
isomer 2 of dCpCH3T	530
isomer 1 of dTpCH3T	545
isomer 2 of dTpCH3T	545
isomer 1 of dCpCH3TpCH3T	832
isomer 2 of dCpCH3TpCH3T	832
isomer 3 of dCpCH3TpCH3T	832
isomer 4 of dCpCH3TpCH3T	832

when TDTase was omitted (data not shown). A similar result was obtained with a different oligomer as the initiator (data not shown). Peaks 3 and 4 were isolated and purified by RP-HPLC followed by gel filtration. As can be seen in Figure 6, polyacrylamide gel electrophoresis of these purified products indicated that the molecular weights of peaks 3 and 4 were larger than that of the initiator. This suggested that the addition of nucleotide to the initiator took place. Peak 2 was not characterized in this study.

Preparation of Standard Di- or Trinucleoside Methylphosphonate for Analysis of the Enzymic Reaction Products. For analysis of the elongated oligomers described in the preceding section, it was necessary to prepare standard dCpCH3T and dCpCH3TpCH3T. dTpCH3T was also prepared as a standard for determining the dimer units as constituents of four isomers of the trimer (dCpCH3TpCH3T). In view of the chemical structure of synthesized dimers, there should exist two diastereomers, S_p and R_p isomers, for dCpCH3T and dTpCH3T. For dCpCH3TpCH3T, there should exist four diastereomers, S_pS_p, S_pR_p, R_pS_p, and R_pR_p. As shown in Figure 7A, these diastereomers were isolated by RP-HPLC. The isolated diastereomers were named isomers 1 and 2 of dCpCH3T, isomers 1 and 2 of dTpCH3T, and isomers 1–4 of dCpCH3TpCH3T as indicated in this figure. These diastereomers were characterized by FAB-MS as indicated in Table I. FAB-MS data fitted exactly with the presumed nucleotide composition as shown in this table.

Alkaline Hydrolysis of Chemically Synthesized Trimer. To determine the possible configuration of the chemically

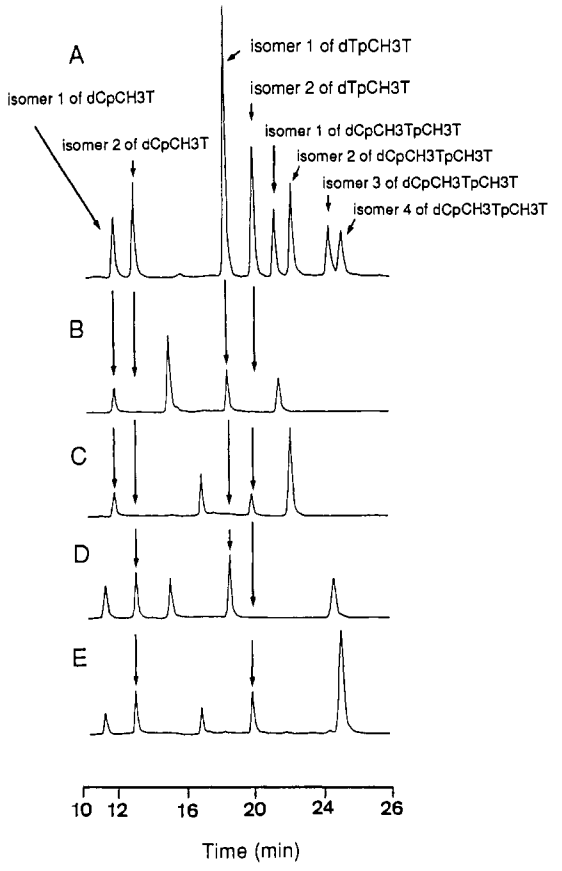

FIGURE 7: (A) RP-HPLC analysis of chemically synthesized dinucleotides and trinucleotides. Isomer 1 of dCpCH3T (11.6 min), isomer 2 of dCpCH3T (12.8 min), isomer 1 of dTpCH3T (18.2 min), isomer 2 of dTpCH3T (19.8 min), isomer 1 of dCpCH3TpCH3T (21.1 min), isomer 2 of dCpCH3TpCH3T (22.0 min), isomer 3 of dCpCH3TpCH3T (24.3 min), and isomer 4 of dCpCH3TpCH3T (25.0 min) were identified as shown in Table. I. (B–E) RP-HPLC analysis of hydrolyzed products of each isomer of dCpCH3TpCH3T: (B) products from isomer 1 of dCpCH3TpCH3T; (C) products from isomer 2 of dCpCH3TpCH3T; (D) products from isomer 3 of dCpCH3TpCH3T; (E) products from isomer 4 of dCpCH3TpCH3T. All figures show the elution profiles after 10-min elutions. HPLC were carried out with gradient III. Reaction conditions were described under Materials and Methods.

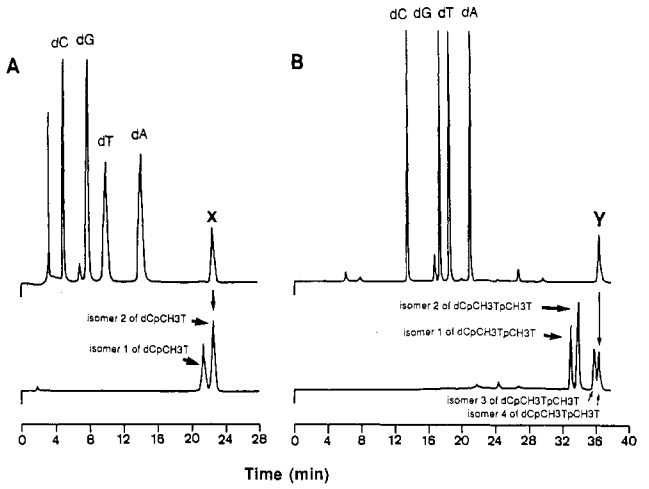

FIGURE 8: Characterization of the products made by elongation of the initiator oligonucleotide with dTTPαCH3 by TDTase. (A) RP-HPLC pattern of digests of peak 3 (see Figure 4) by nuclease P1 and alkaline phosphatase. Gradient VI was used. (B) RP-HPLC pattern of digests of peak 4 (see Figure 4) by nuclease P1 and alkaline phosphatase. Gradient IV was used. Lower panels of A and B indicate RP-HPLC patterns of chemically synthesized standard dimer or trimer.

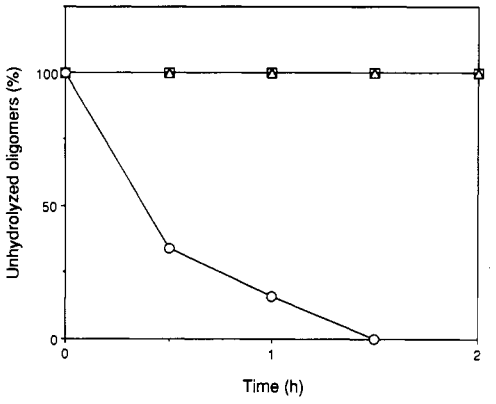

FIGURE 9: Stability of modified oligomers against snake venom phosphodiesterase I (3′-exonuclease). Hydrolysis of oligomers was determined by RP-HPLC with gradient V. (○) Initiator oligonucleotide; (△) peak 3; (□) peak 4. Reaction conditions were described under Materials and Methods.

synthesized trimers (dCpCH3TpCH3T), each isomer of the trimer was subjected to partial hydrolysis, which yielded dinucleotides. Figure 7 shows the RP-HPLC of this reaction mixture. As shown in this figure, isomer 1 of dCpCH3TpCH3T contained isomer 1 of dCpCH3T and isomer 1 of dTpCH3T (Figure 7B); isomer 2 of dCpCH3TpCH3T contained isomer 1 of dCpCH3T and isomer 2 of dTpCH3T (Figure 7C); isomer 3 of dCpCH3TpCH3T contained isomer 2 of dCpCH3T and isomer 1 of dTpCH3T (Figure 7D); isomer 4 of dCpCH3TpCH3T contained isomer 2 of dCpCH3T and isomer 2 of dTpCH3T (Figure 7E).

Characterization of the Products Made by the Elongation of the Initiator Oligonucleotide with dTTPαCH3 by TDTase. Now that we have synthesized standard di- or trinucleotides involving methylphosphonate, it was possible to characterize the reaction products as described in Figure 5. The reaction products of TDTase as shown in Figure 5 (peaks 3 and 4) were subjected to nuclease P1 and alkaline phosphatase digestion followed by RP-HPLC analysis. As can be seen in Figure 8A, digestion of peak 3 gave normal nucleoside (dA, dG, dC, and dT) and the slowly eluted product (designated as peak x). From the elution time of the chemically synthesized standard dimer, peak x was identified to be isomer 2 of dimer (dCpCH3T). In a similar fashion, digestion of peak 4 gave dA, dG, dC, and dT. In addition, material designated as peak y was eluted at much later time (Figure 8B). Comparison of this pattern with that of chemically synthesized standard isomers identified peak y as isomer 4 of trimer (dCpCH3TpCH3T). Further confirmation of the structure of peaks x and y came from the measurement of molecular weight of each peak by FAB-MS (peak x, m/z 530 $[M + H]^+$; peak y m/z 832 $[M + H]^+$). From these results, it was concluded that peak 3 represents the product that has one residue of dTMPCH3 added to the 3′ terminus of the initiator oligonucleotide. Similarly, peak 4 is the product resulting from addition of two residues of dTMPCH3.

3′-Exonuclease Cannot Digest Phosphodiester Bonds Involving Methylphosphonate. Oligonucleotides having one or two terminal methylphosphonate internucleotide linkages as described in the preceding section (peaks 3 and 4 of Figure 5) were tested for possible digestion by snake venom phosphodiesterase I (3′-exonuclease). It is clear from Figure 9 that these oligonucleotides, with terminal methylphosphonate linkages were completely resistant to this enzyme for up to 2 h, while the initiator oligonucleotide without a terminal

methylphosphonate linkage was completely hydrolyzed in 1.5 h.

Discussion

Nucleotide linkages in DNA or RNA are synthesized through enzymic reaction, but the substrates for these enzymes do not appear to be limited to naturally occurring nucleotides. For example, deoxynucleotide 5′-*O*-(1-thiotriphosphate) (dNTPαS) containing phosphorothioate at the α-phosphorus can be incorporated into DNA or RNA by appropriate polymerases (Armstrong et al., 1979; Yee et al., 1979; Burgers & Eckstein, 1979; Brody & Frey, 1981; Romaniuk & Eckstein, 1982; Brody et al., 1982; Bartlett & Eckstein, 1982). It therefore appeared to us that methylphosphonate derivatives of nucleic acid can be synthesized through an enzymic elongation of dNTPαCH3 to initiator oligomers. If such a synthesis is possible, this would be a convenient way to synthesis possible anti-messenger RNA which contains methylphosphonate. TDTase appeared to be suitable for synthesis of such methylphosphonate nucleotides because of two distinct characteristics of this enzyme. First, this enzyme does not require a template. Second, this enzyme does not have exo- and endonuclease activities as prokaryotic DNA polymerases do. One disadvantage of using TDTase for synthesis of methylphosphonate derivative nucleotides was that the substrate for this reaction (dNTPαCH3) has never been synthesized. Fortunately, however, the synthesis of this novel substrate turned out to be relatively easy. The condensing reaction of thymidine methylphosphonic imidazolide with pyrophosphate was the key reaction in this synthesis. This reaction went much easier than expected from other similar condensing reactions (Hoard & Ott, 1965). This is probably because the electron density at phosphorus in the methylphosphonic imidazolide was decreased compared with the phosphonic imidazolide. This would make the nucleophilic attack of pyrophosphate to this compound occur easily.

The TDTase reaction with dTTPαCH3 resulted in only one or two additions, which was confirmed by the use of nuclease P1 and alkaline phosphatase followed by RP-HPLC analysis. A similar method was used for identification of chemically synthesized oligonucleotides containing a single methylphosphonate linkage (Stec et al., 1985a,b). Upon analysis of the reaction product which has one added residue of dTMPCH3 to the initiator oligomer, it became clear that only one configuration of methylphosphonate linkage has been synthesized (Figure 8A). The same result was obtained with two additions of dTMPCH3 as indicated in Figure 8B. Similarly, reaction products from dNTPαS and DNA polymerases or reverse transcriptase have been shown to be of one configuration (Burgers & Eckstein, 1979; Brody & Frey, 1981; Romaniuk & Eckstein, 1982; Brody et al., 1982; Bartlett & Eckstein, 1982).

As for the absolute configuration of the methylphosphonate linkage synthesized by TDTase, the following observations are important: Peak 3 of Figure 5 (single addition of dTMPCH3 to the initiator oligonucleotide) was hydrolyzed with nuclease P1 and alkaline phosphatase to give isomer 2 of dCpCH3T (Figure 8A). Chemically synthesized dinucleotide (dCpCH3T) consisted of two isomers; one eluted from the RP-HPLC column faster than the other. The hydrolysates of peak 3 of Figure 5 gave the slow eluting isomer. Peak 4 of Figure 5 represents the reaction product which has two nucleotide additions by TDTase to the initiator oligonucleotide. When this reaction product was hydrolyzed by enzymes, it gave trinucleotide (dCpCH3TpCH3T). Upon alkaline hydrolysis of the corresponding chemically synthesized trinucleotide, two dinucleotides, dCpCH3T and dTpCH3T, were produced (Figure 7E with arrows). The latter was identified to be of S_p configuration by comparison of the behavior of this compound on the RP-HPLC column with that which has been published (Leśnikowski et al., 1987b). In general, enzymic nucleotide linkage formation produces one configuration and not a mixture. The fact that dTpCH3T in S_p configuration was isolated from the reaction product involving TDTase and dTTPαCH3 strongly indicates that all the linkage would be of the S_p configuration. In addition, dinucleotide (isomer 2 of dCpCH3T, S_p configuration) which was produced by nuclease treatments of the enzymic reaction product (peak 3) was eluted later than chemically synthesized isomer 1 of dCpCH3T. This fact further supports the notion that TDTase synthesizes the S_p configuration because the S_p isomer of dinucleoside methylphosphonate has been reported to elute from RP-HPLC columns later than the R_p isomer (Stec et al., 1985a,b; Koziolkiewicz et al., 1986). It should be pointed out, however, that the relationship between configuration and their behavior on RP-HPLC column is not always consistent with the notion that the R_p isomer is eluted faster than the S_p isomer (Kan et al., 1980; Callahan et al., 1986). Despite these reports, with all the available information put together, we can tentatively conclude that TDTase adds one or two nucleotides to the initiator oligomer in the S_p configuration.

This paper represents the first attempt for enzymic synthesis of an oligonucleotide containing a methylphosphonate linkage. Although only two residues were added to the initiator oligonucleotide, the partial success of such an enzymic reaction strongly suggests that, under certain conditions, enzymes such as DNA polymerases, reverse transcriptase, and others may be used for sequence-specific synthesis of oligonucleoside methylphosphonate. Further experiments are in progress to explore such possibilities.

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Registry No. TDTase, 9027-67-2; dTTpαCH3, 128600-38-4; dTMPCH3, 73264-62-7; 5′-GCTGTATCGTCAAGGCACTC-3′, 128659-06-3; dTMPCH3·Bu_3N, 128600-39-5; dTTPαCH3·Et_3N, 128600-40-8; S_p-dCpCH3T, 128631-43-6; R_p-dCpCH3T, 128706-15-0; S_p-TpCH3T, 71830-19-8; R_p-TpCH3T, 71830-20-1; S_pS_p-dCpCH3TpCH3T, 128600-41-9; S_pR_p-dCpCH3TpCH3T, 128704-92-7; R_pS_p-dCpCH3TpCH3T, 128704-93-8; R_pR_p-dCpCH3TpCH3T, 128704-94-9; 5′-GCTGTATCGTCAAGGCACTC-PCH3T-3′, 128659-07-4; 5′-GCTGTATCGTCAAGGACTC-PCH3T-PCH3T-3′, 128659-08-5.

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Single-Stranded DNA-Dependent ATPase from HeLa Cells That Stimulates DNA Polymerase α–Primase Activity: Purification and Characterization of the ATPase†

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ABSTRACT: A single-stranded DNA-dependent ATPase that cofractionates during the early stages of purification of a multiprotein DNA polymerase α complex from HeLa cells has been purified to homogeneity. The ATPase is part of a 16S multienzyme DNA polymerase α complex that is fully active in SV40 DNA replication in vitro. The ATPase hydrolyzes ATP to ADP in a reaction that is completely dependent on the presence of DNA. DNA in single-stranded form is strongly preferred as a cofactor, and polydeoxynucleotides with adenine or thymidine residues are highly effective. Glycerol gradient sedimentation showed that the purified ATPase sedimented at an $s_{20,w}$ of 7 S, and polyacrylamide gel electrophoresis under denaturing conditions reveals two polypeptides with relative molecular weights of 83 000 and 68 000. Both of these polypeptides have purine nucleotide binding sites as revealed by photoaffinity cross-linking experiments. ATP binds to the two subunits more efficiently than GTP, and CTP or UTP does not cross-link with the two polypeptides. DNA synthesis catalyzed by purified HeLa cell DNA polymerase α–primase is stimulated in the presence of ATPase and ATP at an optimum concentration of 2 mM. Analysis of the DNA product by gel electrophoresis indicates that with poly(dT) but not phage M13 DNA as template the ATPase overcomes a lag and decreases the length of nascent DNA chains synthesized by the DNA polymerase α–primase complex.

Many reactions in DNA metabolism such as replication, repair, and recombination are driven by chemical energy derived from the hydrolysis of ATP. Enzymes catalyzing the hydrolysis of ATP in a DNA-dependent reaction have been found in prokaryotes and eukaryotes (Kornberg, 1980; Hachmann & Lezius, 1976; Assairi & Johnston, 1979; Cobianchi et al., 1979; Boxer & Korn, 1980; DeJong et al., 1981; Hyodo & Suzuki, 1981; Thomas & Meyer, 1982; Biamonti et al., 1983; Tawaragi et al., 1984; Hubscher & Stalder, 1985; Hockensmith et al., 1986; Sugino et al., 1986; Tsurimoto & Stillman, 1990). Most of these enzymes are DNA-dependent

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