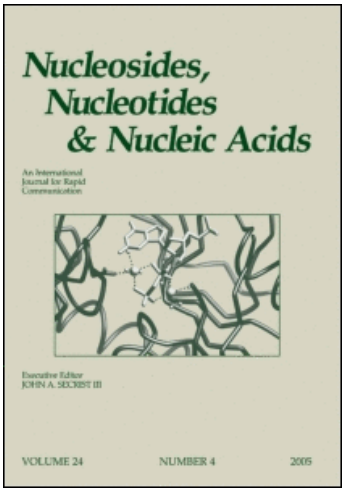

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The Analogue of Thymidine Triphosphate Containing a Methylene Group in Place of the 5' Oxygen Can Serve as a Substrate for Reverse Transcriptase

M. A. Bell[a]; G. R. Gough[a]; P. T. Gilham[ab]
[a] Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA [b] Department of Biological Sciences, Purdue University, West Lafayette, IN, USA

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The Analogue of Thymidine Triphosphate Containing a Methylene Group in Place of the 5′ Oxygen Can Serve as a Substrate for Reverse Transcriptase

M. A. Bell, G. R. Gough, and P. T. Gilham*

Department of Biological Sciences, Purdue University, West Lafayette, Indiana, USA

ABSTRACT

The thymidine 5′-triphosphate analogue containing a methylene group in place of the 5′ oxygen atom can be prepared using modifications of published procedures and can substitute for the natural thymidine triphosphate in chain extension reactions catalyzed by Moloney-MLV reverse transcriptase. Using rabbit β-globin mRNA as the template together with an appropriate primer, the enzyme readily makes full-length DNA transcripts in which all thymidine 5′ oxygen atoms have been replaced with methylene groups. In sequence analyses using the partial depurination procedure, the analogue DNA transcript produces electrophoretic gel patterns identical with those of the corresponding natural DNA transcript. Experiments on second strand synthesis using the four regular triphosphates show that the analogue DNA transcript, like the natural transcript, can serve as a template. The two DNA duplexes (natural/natural and analogue/natural) formed by these reactions produce similar electrophoretic cleavage patterns when treated with either of the endonucleases *Hae*III and *Eco*RI. However, further studies on template properties indicate that, while the enzyme makes a full-length product when using the analogue substrate with a natural DNA strand as template, it appears unable to use the analogue transcript as template with the analogue

*Correspondence: P. T. Gilham, Department of Biological Sciences, Purdue University, West Lafayette, IN 47907, USA; E-mail: pgilham@purdue.edu.

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triphosphate as substrate during second strand synthesis. Preliminary experiments have also been carried out with a DNA polymerase. No products are detected in reactions using Taq polymerase with PCR protocols containing the analogue triphosphate as the only source of thymidine.

Key Words: Reverse transcriptase; Phosphonate; Thymidine triphosphate; Nucleotide analogue.

INTRODUCTION

Studies on nucleic acid molecules containing specific chemical modifications can yield new insights into their structures and functions and suggest new directions for drug design in the therapeutic control of human diseases. The modification of particular interest in this laboratory has been the substitution of a methylene group for the 5′ oxygen within the nucleic acid backbone structure. The initial observations were made in work on RNA synthesis, where it was shown that the adenosine 5′-diphosphate analogue containing the methylene substitution for the 5′ oxygen could be readily polymerized by polynucleotide phosphorylase.[1,2] The oligonucleotide products of this reaction are apparently capable of assuming conformations that are essentially isosteric with their natural counterparts since they were shown to form triple-stranded complexes with uridine oligomers that are analogous to those formed from adenosine oligomers containing the natural phosphodiester linkage. Extension of this work indicated that the corresponding analogue of adenosine 5′-triphosphate can substitute for ATP as a substrate for T3 RNA polymerase.[3] We now show that the similar analogue of the deoxyribonucleotide thymidine 5′-triphosphate is an acceptable substrate for M-MLV reverse transcriptase.

MATERIALS AND METHODS

Materials

Nucleosides, 1,3-dicyclohexylcarbodiimide, 1,1′-carbonyldiimidazole, and pyridinium trifluoroacetate were obtained from either Sigma Chemical Co. or Aldrich Chemical Co. Chloromethylphosphonic dichloride was obtained from Alpha Products, and converted to diphenyl chloromethylphosphonate using a published procedure.[4] Pyridine was purified by distillation and then maintained in an anhydrous state by storage over calcium hydride. Dimethylformamide was dried over 4A molecular sieves. Silica gel 60 and aluminum-backed silica gel 60 F254 TLC plates were obtained from EM science. 3′-*O*-Acetylthymidine was prepared as described in the literature.[5] The Wittig reagent diphenyl (triphenylphosphoranylidene)methylphosphonate was prepared by condensing diphenyl chloromethylphosphonate with triphenylphosphine using a published procedure.[6] *Crotalus Adamanteus* venom phosphodiesterase I, NAP columns containing DNA grade G-25 Sephadex, and ultrapure deoxyribonucleoside 5′-triphosphates were purchased from Pharmacia Biotech Inc. Rabbit β-globin mRNA and Moloney-MLV reverse transcriptase

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(RNase H^-, Superscript II) were obtained from Gibco BRL Products. Ribonuclease inhibitor and Microcon microconcentrators were purchased from Promega and Amicon, respectively. Oligodeoxyribonucleotide primers were synthesized by Integrated DNA Technologies, Inc., and labeled at their 5′ ends with radioactive phosphate using phage T4 polynucleotide kinase and [γ-^{32}P]ATP.

Chromatography

HPLC was performed on a Varian 5000 Liquid Chromatograph utilizing either a Supelcosil LC-SI silica gel column (0.5 cm × 15 cm) from Supelco that had been derivatized with polyethyleneimine (PEI) prior to use[7] or a Varian Micropak SAX-10 column (0.4 cm × 30 cm). The solvent system used for elution of products from the Supelcosil PEI column was aqueous 0.05 M KH_2PO_4 containing 30% (v/v) methanol and a linear gradient of 0–0.2 M $(NH_4)_2SO_4$ at pH 6 with a flow rate of 1.0 mL/min. The solvent system used with the Varian SAX-10 column was 0.025 M KH_2PO_4 containing 30% (v/v) methanol with a flow rate of 1.0 mL/min. Silica gel chromatography was carried out on columns (2.5 cm × 30 cm) of silica gel 60 packed in a chloroform slurry, and elution of products was achieved with various concentrations of methanol in chloroform. Thin layer chromatography used aluminum-backed silica gel 60 F254 plates (4 cm × 7 cm), and development was carried out in glass chambers using various concentrations of methanol in chloroform. Preparative paper chromatography was conducted in the descending mode on Whatman 3 MM paper, using solvent systems: A, *iso*propanol/conc. NH_4OH/water (7:1:2, v/v/v); B, *iso*butyric acid/1 M aqueous NH_4OH (10:6, v/v); or C, *n*-propanol/conc. NH_4OH/water (55:10:35, v/v/v).

Electrophoresis

Denaturing polyacrylamide gel electrophoresis was performed using 11% or 15% acrylamide cross-linked with N,N′-ethylenebisacrylamide and cast in Tris borate-EDTA buffer containing 7 M urea. Gel polymerization was initiated by the addition of 10% (w/v) ammonium persulfate and N,N,N′,N′-tetramethylethylenediamine.

Diphenyl ester of 3′-*O*-acetyl-5′-phosphonomethyl-5′-deoxythymidine. 3′-*O*-Acetylthymidine (427 mg, 1.5 mmol) was dissolved in 4.1 mL of anhydrous dimethyl sulfoxide. To this solution were added with stirring dicyclohexylcarbodiimide (929 mg, 4.5 mmol), pyridinium trifluoroacetate (145 mg, 0.75 mmol), and pyridine (61.0 μL, 0.75 mmol). The solution was sealed under argon and stirring was continued overnight at room temperature. The resulting 5′-aldehyde was treated directly with diphenyl (triphenylphosphoranylidene)methylphosphonate (763 mg, 1.5 mmol), and the mixture was stirred for a further two days at 37°C under argon. It was then cooled to room temperature and dissolved in 150 mL ethyl acetate. The solution was extracted with 10% NaCl, then dried over sodium sulfate and evaporated to an oil under reduced pressure. Silica gel chromatography with $CHCl_3$ containing a stepwise gradient (0–2.5%) of MeOH yielded the diphenyl ester of 5′,6′-dehydro-3′-*O*-acetyl-5′-phosphonomethyl-5′-deoxythymidine in 89% yield. The ester (266 mg,

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0.5 mmol) was dissolved in 13.2 mL ethanol and added to 10% palladium on activated carbon (115 mg). This mixture was then stirred under H_2 for 3 days at room temperature. The reaction gave the reduced diphenyl ester of 3′-*O*-acetyl-5′-phosphonomethyl-5′-deoxythymidine in quantitative yield as demonstrated by ^{31}P-NMR and TLC.

5′-Phosphonomethyl-5′-deoxythymidine. The monophenyl ester of 5′-phosphonomethyl-5′-deoxythymidine was produced by dissolving the diphenyl ester (90 mg, 0.175 mmol) in a mixture of 1.0 mL 1,4-dioxane and 1.0 mL 1 M NaOH. After 2 h at room temperature, the solution was neutralized with Dowex 50W-X2 (H^+ form) ion-exchange resin until a pH of ca. 7 was indicated. The solution was then filtered and evaporated under reduced pressure to a minimal volume. The products were fractionated by paper chromatography using solvent system A, affording the pure monophenyl ester in 95% yield. The monophenyl ester (1,315 AU_{260}, 0.137 mmol) was then incubated in a mixture of 2.5 mL of 100 mM ammonium acetate (pH 8.9) containing 14 mM $MgCl_2$ and 10 μL of snake venom phosphodiesterase I (56 units/mL). The hydrolysis reaction was allowed to proceed for 3 days at room temperature with daily additions of 5 μL of phosphodiesterase I. The reaction mixture was co-evaporated (3 × 5 mL) with water, and the resulting oil dissolved in a minimal volume of water and purified by paper chromatography using solvent system C. Elution from the paper gave the ammonium salt of 5′-phosphonomethyl-5′-deoxythymidine in 83% yield; its purity was established by chromatography on the Micropak SAX-10 column.

5′-Phosphonomethyl-5′-deoxythymidine diphosphate. Sodium pyrophosphate decahydrate (2.23 g, 5.0 mmol) was converted to its pyridinium form by passage through a column of Dowex 50W-X2 (pyridinium form) ion-exchange resin using 5% aqueous pyridine as eluant. The product was concentrated in vacuo and added to tri-*n*-butylamine (2.4 mL, 10 mmol). The mixture was dried by repeated co-evaporation with anhydrous pyridine (4 × 10 mL), and then with anhydrous DMF (2 × 10 mL). The product was finally brought up to a final volume of 12.5 mL with anhydrous DMF to give a stock solution of pyrophosphate with concentration of 0.4 mmol/mL. 5′-Phosphonomethyl-5′-deoxythymidine (213 AU_{260}, 0.02 mmol) was passed through a column of Dowex 50W-X2 (pyridinium form) ion-exchange resin, then concentrated and treated with 1 equiv. of tri-*n*-butylamine (5.3 μL, 0.02 mmol). The nucleotide was dried by repeated co-evaporation in vacuo with anhydrous pyridine (2 × 5 mL), and then with anhydrous DMF (3 × 10 mL). A solution of the nucleotide (0.02 mmol) in 0.5 mL of anhydrous DMF was treated with 1,1′-carbonyldiimidazole (0.11 mmol). After two hours at room temperature the mixture was treated with the pyrophosphate stock solution (275 μL, 0.11 mmol), and stirred at room temperature for 2 days under argon. It was then filtered, and the precipitate was washed with 1 mL of MeOH and then 1 mL of DMF. The filtrate and the combined washings were concentrated and the products separated by paper chromatography using solvent system B. After drying, the paper was washed with ethanol to remove *iso*butyric acid, and the triphosphate analogue band was eluted with water (yield, 41%). The product was converted to its sodium salt by passage

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through a column of Dowex 50W-X8 (Na^+ form), and was shown to be pure by chromatography on the PEI silica column.

Primer Extension Reactions on RNA Templates

All extension reactions were 20 μL in volume and contained 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3.0 mM $MgCl_2$, 1.0 mM DTT, 200 U of reverse transcriptase, 0.125 pmol of oligonucleotide primer (either unlabeled or labeled at the 5′ terminal phosphate with ^{32}P), 0.25 pmol rabbit β-globin mRNA, 20 U of RNase inhibitor, 0.5 mM each of dATP, dGTP, and dCTP, and either 0.5 mM dTTP or 1.0 mM of the thymidine triphosphate analogue. Prior to the addition of the enzyme, DTT, and RNase inhibitor, each reaction mixture was heated to 70°C for 5–10 min. The complete reaction mixture was incubated at 37°C for 2 h, and then heated for 10 min at 70°C. A portion of each reaction mixture was mixed with an equal volume of 90% formamide containing 0.1% xylene cyanol FF and 0.1% bromophenol blue. Samples were heated to 70°C for 10 min and subjected to electrophoresis through 15% denaturing gels for 3 h at 100 volts. Gels were dried and the labeled bands of products were visualized by autoradiography in the usual way. In order to obtain an approximate estimate of product chain lengths, portions of the reaction mixtures were treated as above and subjected to electrophoresis through 11% denaturing gels at 1700 volts alongside a set of ^{32}P-labeled standard markers of chain length 100x where x = 1–6.

Sequence Analysis of DNA Products

The 5′-labeled DNA products from the reverse transcriptase reactions described above were purified on NAP-10 columns using procedures supplied by the manufacturer. The resulting samples were concentrated to dryness and then further purified on Microcon spin columns again using the manufacturer′s protocols, and the final solutions were evaporated to dryness in vacuo. Samples were dissolved in 10 μL of water and subjected to depurination/β-elimination reactions as described.[8] The cleavage products were then separated by electrophoresis on an 11% denaturing gel at 1700 volts for 5 h.

Second Strand Synthesis Using Reverse Transcriptase

DNA copies of globin mRNA using either dTTP or its analogue were prepared as described above except that unlabeled primer was used. Each reaction mixture (20 μL) was then mixed with 10 μL of 0.3 M NaOH and kept at 37°C for 30 min in order to degrade the mRNA template. The alkaline mixture was then neutralized by addition of 10 μL of 0.3 M HCl. The DNA product in each reaction mixture was purified as described above for the sequence analysis experiments. Each chain extension on the DNA template was performed in a volume of 20 μL containing 50 mM Tris-HCl (pH 8.3), 75 mM KCl, 3.0 mM $MgCl_2$, 0.125 pmol of an octadecanucleotide primer labeled at its 5′ terminal phosphate with ^{32}P, along with 0.5 mM each of dATP, dGTP, and dCTP, and either 0.5 mM dTTP or 1.0 mM of

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the thymidine triphosphate analogue. Each reaction mixture was first heated to 70°C for 5–10 min, and then incubated at 37°C for 2 h with 1.0 mM DTT and 200 U of reverse transcriptase. Each mixture was then heated at 70°C for 10 min and the products were either directly analyzed by gel electrophoresis as indicated above or subjected to endonuclease digestion prior to analysis. Restriction endonuclease digests were carried out by mixing 10 μL from each second strand synthesis reaction with 10 μL of a buffered solution of either *Eco*RI or *Hae*III as described in the literature.[8] Each digest was allowed to proceed at 37°C for 30 min. and then subjected to electrophoretic analysis on a 15% denaturing gel. Chain extensions on a chemically-synthesized template were performed with a 90-nucleotide DNA strand and appropriate primer (both kindly provided by Dr. H. L. Weith). The conditions and analyses were identical with those used above for templates that were produced enzymatically.

Polymerase Chain Reactions

PCR reactions (50 μL) contained 10 mM Tris-HCL (pH 9.0), 50 mM KCl, 5 U Taq DNA polymerase, $MgCl_2$ (optimum concentration: 4.0 mM), 0.5 pmol each of "top" and "bottom" primers (one of which was 5′ end-labeled with ^{32}P), 0.025 μg of template (either pUC18 or pUC18 with an insert of A-T tracts), and 20 μM each of dATP, dGTP, dCTP, and either 20 μM dTTP or 240 μM of its analogue. The PCR cycle, consisting of denaturation at 92°C for 2 min, annealing at 45°C for 5 min, and polymerization at 70°C for 10 min, was repeated 30 times. Products were analyzed by electrophoresis on 15% gels using the conditions described above.

RESULTS

The introduction of the phosphonomethyl group into the 5′ position of the deoxyribonucleoside thymidine was accomplished using modifications of the procedures described by Jones and Moffatt[9] for preparation of the corresponding ribonucleotide analogues of UMP and AMP from the starting materials 2′,3′-*O*-isopropylideneuridine and 2′,3′-*O*-isopropylideneadenosine, respectively. For the work with thymidine we employed acetyl to protect the 3′-hydroxyl: 3′-acetylthymidine was treated with dimethyl sulfoxide and dicyclohexylcarbodiimide to yield the 5′-aldehyde. This material was then treated with the Wittig reagent diphenyl triphenylphosphoranylidenemethylphosphonate, and the resulting product was subjected to catalytic hydrogenation to reduce the double bond. The acetyl and one of the phenyl groups were removed by mild alkaline treatment to yield the monophenyl ester of 5′-phosphonomethyl-5′-deoxythymidine, and removal of the second phenyl group was achieved by exposure to snake venom phosphodiesterase. Finally, the pyrophosphate group was added using a modification of the procedure used by Hoard and Ott[10] for conversion of deoxyribonucleoside 5′-phosphates to their corresponding 5′-triphosphates.

Reverse transcriptase activity was tested using rabbit β-globin mRNA as template along with the primer pAATTTCCTTTATT labeled at its 5′ end with ^{32}P. In the expected primer/template complex, the 5′ terminal nucleotide of the primer

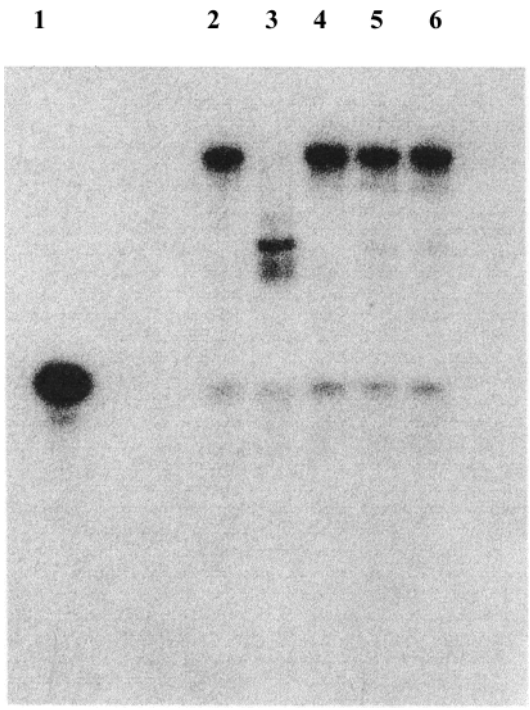

Figure 1. Gel patterns produced by reverse transcriptase primer extensions on rabbit β-globin mRNA. Lane 1 contained the labeled primer used. Lanes 2–6 were the products from reactions containing 0.05 mM each of dATP, dCTP, and dGTP. The reactions in Lanes 2 and 5 contained 0.05 mM dTTP, while those in 4 and 6 contained the analogue thymidine triphosphate at 1.0 mM concentration. The reactions in Lanes 2–4 were run overnight while those in Lanes 5 and 6 were run for 2 h.

should be located opposite nucleotide #577 of the mRNA template (GenBank Accession # V00879). Chain extension experiments with either thymidine triphosphate or its analogue yielded full-length transcripts of similar chain length (Fig. 1). One of the control reactions for these chain extensions contained the three triphosphates dATP, dCTP, and dGTP only, and while this control indicated some activity, it is clear that no complete transcript was formed. We attribute this minor activity either to trace amounts of dTTP contaminating one or more of the other three triphosphates or to a low level of non-Watson Crick incorporation. Assignments of the chain lengths of the two transcripts were made by electrophoresis on a longer polyacrylamide gel (Fig. 2). The positions of the two transcripts are consistent with their expected chain lengths of 577 each. Next, a partial sequence analysis was carried out to check that the analogue thymidine residues were undergoing insertion only in positions opposite the adenosine moieties in the RNA template. The analysis of partial depurination reactions on the two transcripts produced identical cleavage patterns upon electrophoresis, and these patterns are readily accounted for on the basis of the expected purine positions in the transcripts (Fig. 3). For example, the series of 13 contiguous purine cleavages that give rise to the chain length set of

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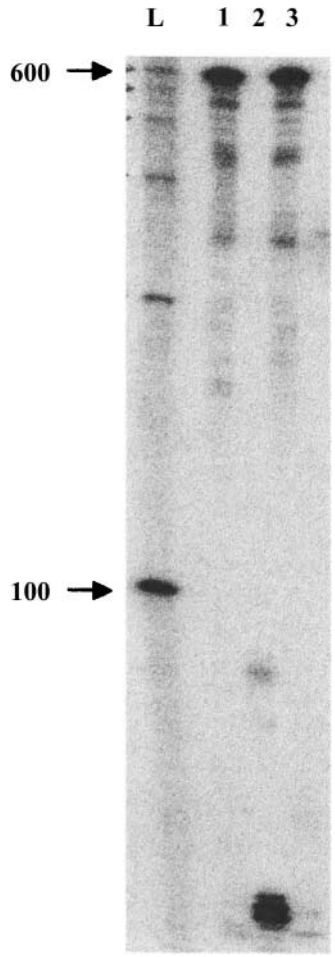

Figure 2. Gel electrophoretic analysis of DNA's made using reverse transcriptase with rabbit β-globin mRNA as template. The reactions in Lanes 1–3 were run for 2 h and each contained 0.5 mM dATP, dCTP, and dGTP. The reaction in Lane 1 also contained 0.05 mM dTTP, while that in Lane 3 contained 1.0 mM of the thymidine triphosphate analogue. Chain length markers were located in Lane L.

65–77 correspond to the location of the 13 contiguous pyrimidines (at positions 500–512) in the template.

For second strand synthesis the first series of experiments employed the set of 4 regular nucleoside triphosphates with either normal or analogue DNA as template. The templates were prepared from globin RNA as described above, except that the primer used was unlabeled at its 5′ end (Fig. 4). The 5′ labeled primer selected for the copying reactions was assigned the sequence complementary to the area occupied by nucleotides #168 to #185. The double-stranded products from these reactions were expected to have recognition sites for the restriction endonucleases, *Hae*III and *Eco*RI, at positions #108 and #156, respectively. The results of the chain extension

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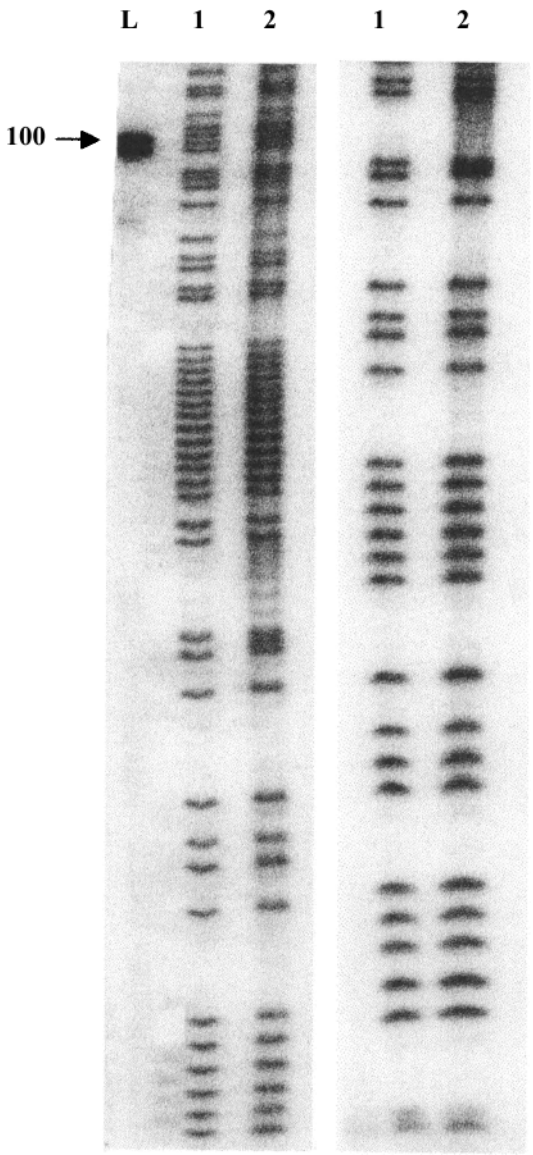

Figure 3. Depurination patterns produced from normal and analogue transcripts made with rabbit β-globin mRNA as template. The left-hand pattern shows bands with chain lengths of 32 to 111 while the bands in the right-hand pattern have chain lengths of 13 to 65. Lane 1 has products deriving from the use of dTTP in the transcription reaction and Lane 2 are those deriving from the use of the analogue dTTP.

reactions are shown in Fig. 5. Chain extensions using either the normal DNA template or the template prepared from the nucleotide analogue gave full-length second strands of 185 nucleotides. Treatment of the double-stranded products with either *Hae*III or *Eco*RI before electrophoresis showed the expected shortened chains of

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AATTTCCTTT ATTAGCCAGA AGTCAGATGC TCAAGGGGCT TCATGATGTC
CCCATAATTT TTGGCAGAGG GAAAAAGATC TCAGTGGTAT TTGTGAGCCA
GGGCATTGGC CACACCAGCC ACCACCTTCT GATAGGCAGC CTGCACCTGA
GGAGTGAATT CTTTGCCAAA ATGATGAGAC AGCACAATAA CCAGCACGTT
GCCCAGGAGC CTGAAGTTCT CAGGATCCAC GTGCAGCTTG TCACAGTGCA
GTTCACTCAG CTTAGCAAAG GTGCCTTTGA GGTTGTCCAG GTGACTCAGA
CCCTCACTGA AGGCAGCCAG CACCTTCTTG CCATGAGCCT TCACCTTAGG
ATTGTTCATA ACAGCATTTG CAGAGGACAG GTCCCCAAAG GACTCGAAGA
ACCTCTGGGT CCATGGGTAG ACAACCAGCA GCCTGCCCAG GGCCTCACCA
CCAACTTCTT CCACATTCAC CTTGCCCCAC AGGGCAGTGA CCGCAGACTT
CTCCTCACTG GACAGATGCA CCATTCTGTC TGTTTTGGGG GATTGCAAGT
AAACACAGTT GTGTCAAAAG CAAGTGT

Figure 4. Sequence of DNA used for study of second strand synthesis. The primer used was complementary to 18-nucleotide run (underlined) located at positions 168–185. The locations of the endonuclease HaeIII site (GGCC) and EcoRI site (GAATTC) are also underlined.

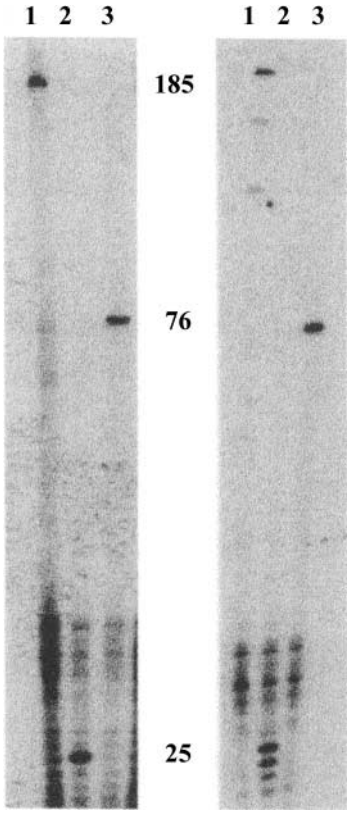

Figure 5. Gel patterns derived from second strand syntheses using the four regular nucleoside triphosphates and the labeled primer and DNA template shown in Fig. 4. The left-hand panel contains products from the normal DNA template, while the right-hand panel shows products using the analogue DNA as template. For each panel, Lane 1 shows the full extension reaction out to a chain length of 185; Lane 2 is the pattern obtained by treatment of the reaction product with endonuclease EcoRI before electrophoresis; and Lane 3 is the pattern obtained by treatment of the reaction mixture with endonuclease HaeIII.

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TACCAGGTAA TATACCACAA CGTGTGTTTC TCTGGTTGAC TTCTCTGTTT
TACAGAGAAG TCACTTCTCT GTCTATAGTG AGTCGTATTA
                      GATATCAC TCAGCATAAT
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Figure 6. Chemically synthesized oligonucleotide and primer for chain extension reactions containing the analogue thymidine triphosphate.

chain lengths 76 and 25, respectively. The observed cleavages of the duplex containing the analogue strand are worthy of note; the location of multiple phosphonate internucleotide linkages near or within the recognition sites seemed to have little effect on the restriction enzymes' activities.

A second series of experiments tested the capacity of the analogue triphosphate to serve as a substrate for second strand synthesis. However, all attempts to use this triphosphate with the analogue DNA strand as template failed. In order to test second strand synthesis using a natural DNA strand as template, it was necessary to employ a template uncontaminated with normal nucleoside triphosphates. A chemically-synthesized 90-nucleotide polymer (Fig. 6) and an appropriate ^{32}P-labeled primer were used. Fig. 7 shows the gel patterns of the products of these extension

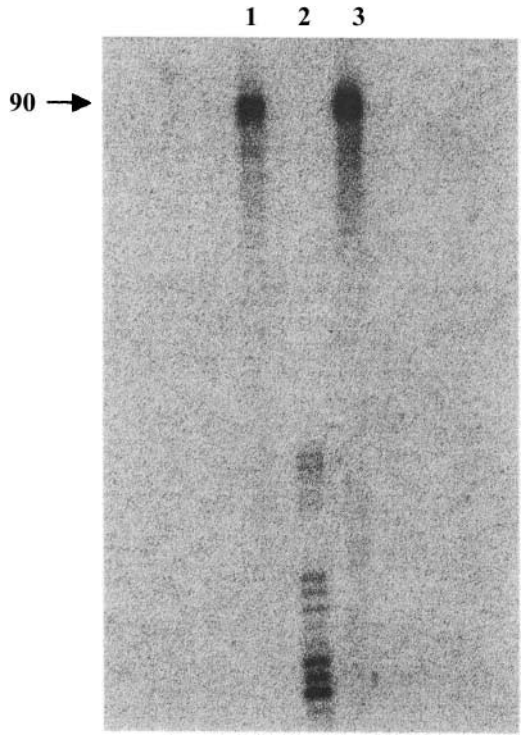

Figure 7. Chain extension reactions using the synthetic DNA/primer shown in Fig. 6. Lane 1 shows the gel pattern obtained for the extension reaction using all four regular nucleoside triphosphates. Lane 2 is the pattern obtained for the same reaction mixture except for the omission of the dTTP. Lane 3 is the pattern for the same reaction mixture as in Lane 1 except for the substitution of the dTTP with the analogue triphosphate.

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reactions: the product containing the analogue thymidine residues matches that obtained with the 4 regular triphosphates. Again, in this study, the control reaction containing only the triphosphates of A, G, and C indicated traces of chain extension, and these also can be attributed to some contamination of the commercially-obtained monomers with dTTP.

Finally, a series of experiments were conducted to test the ability of Taq DNA polymerase to accept the analogue in place of dTTP. PCR reactions were carried out under a wide variety of conditions, using pUC18 plasmids as templates along with the appropriate labeled primers. While control reactions with the 4 regular triphosphates yielded the expected PCR product, none was detected in reactions containing the analogue in place of the dTTP.

DISCUSSION

In view of these results, it seems possible that exposure of a retrovirus in vivo to the dTTP analogue could interfere with its replication cycle. Inhibition of the cycle might occur at any of the various steps of reverse transcription, integration, and transcription. One biochemical step that merits further detailed study is that of second strand synthesis by reverse transcriptase. The experiments described above indicate that the analogue-containing DNA strand can serve as template in second strand synthesis when dTTP is present in the reaction, but not when the analogue is the only source of thymidine. Specifically, it would be of interest to know the effects on first and second strand syntheses when both natural and analogue triphosphates are present. However, a definitive study of the relative incorporation of the two substrates will require the use of differential labels such as, for example, α-^{32}P in dTTP and ^{14}C in its analogue. Another aspect that should be investigated further is the interaction of the phosphonate analogue triphosphates with DNA-dependent DNA polymerases. The lack of product formation in the PCR reactions with Taq DNA polymerase may be due to complete rejection of the analogue as substrate. Alternatively, the polymerase may be behaving in a manner similar to that of the reverse transcriptase by incorporating the analogue during first strand formation to generate a template that is unacceptable for second strand synthesis.

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MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016

MARCEL DEKKER, INC.
270 Madison Avenue, New York, New York 10016