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Influence of template primary structure on 3′-azido-3′-deoxythymidine triphosphate incorporation into DNA[1]

Edward G. Bridges[a], Robert B. LeBoeuf[b], Douglas A. Weidner[a] and Jean-Pierre Sommadossi[a]

[a]*Department of Pharmacology and Division of Clinical Pharmacology and* [b]*Department of Physiology and Biophysics, Center for AIDS Research and the Comprehensive Cancer Center, University of Alabama at Birmingham, Birmingham, AL 35294, USA*

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Summary

In the present study, templates containing a specific segment of the Gγ-globin gene were constructed and incorporation of 3′-azido-3′-deoxythymidine 5′-triphosphate (AZT-TP) or 2′,3′-dideoxythymidine 5′-triphosphate (ddTTP) into these templates was compared to that observed in M13 bacteriophage DNA. Investigations on the intrinsic fidelity of T7 DNA polymerase in reactions with AZT-TP and without deoxythymidine 5′-triphosphate, resulted in DNA synthesis beyond the first T site, suggesting that other normal deoxynucleotides misincorporated at these T sites. Modified T7 DNA polymerase incorporated AZT-TP into T sites of elongating DNA strands. Chain termination at noncomplementary sites was also observed with AZT-TP when a genomic DNA template was used and interestingly, this phenomenon was not detected in the presence of a M13 DNA template. These DNA template-dependent effects were not detected with either ddTTP, 2′,3′-

Correspondence to: J.-P. Sommadossi, Professor of Pharmacology, University of Alabama at Birmingham, VH 113, Box 600, University Station, Birmingham, Alabama 35294, USA. Fax: (205) 934-8240.

Abbreviations: AZT, 3′-azido-3′-deoxythymidine; AZT-TP, 3′-azido-3′-deoxythymidine 5′-triphosphate; D4T-TP, 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-triphosphate; ddTTP, 2′,3′-dideoxythymidine 5′-triphosphate; ddCTP, 2′,3′-dideoxycytidine 5′-triphosphate; DTT, dithiothreitol; dNTPs, deoxynucleoside triphosphates; PCR, polymerase chain reaction.

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dideoxycytidine 5′-triphosphate, or 2′,3′-didehydro-2′,3′-dideoxythymidine 5′-triphosphate (D4T-TP). A variation in the extent of chain termination at T sites was observed with D4T-TP suggesting that each 2′,3′-dideoxynucleoside may exhibit unique chain termination patterns along with the template sequence.

AZT-TP, 2′,3′-dideoxynucleotide

Introduction

Nucleoside analogs have been extensively used in antiviral chemotherapy. In order to better understand the mechanism of action of these compounds, many studies have focused on the interaction between their triphosphate derivatives and the probable molecular targets, either viral or host DNA polymerases. Most studies performed to date on the incorporation of nucleotide analogs, such as AZT-TP, by host or viral DNA polymerases into DNA have used either synthetic polymeric or bacteriophage DNA as templates (Furman et al., 1986; Cheng et al., 1987; St. Clair et al., 1987; Kedar et al., 1990; Huang et al., 1990; White et al., 1989; Elwell et al., 1987). However, these experimental systems do not consider potential sequence-dependent variations in the rate and accuracy of analog incorporation along the template as demonstrated with other nucleoside triphosphates (Townsend and Cheng, 1987; Ohno et al., 1989; Ohno et al., 1988).

In the present study, we investigated the effects of template sequence on the incorporation of AZT-TP and other 2′,3′-dideoxynucleoside triphosphates, including ddTTP, D4T-TP, and ddCTP using a modified T7 bacteriophage DNA polymerase lacking 3′ to 5′ exonuclease activity and exhibiting a high replication fidelity (Patel et al., 1991).

Materials and Methods

Nucleotides, templates and oligonucleotides

Molecular biology grade dNTPs, ddCTP and ddTTP were purchased from Boehringer–Mannheim Biochemicals (Indianapolis, IN). AZT-TP, synthesized and purified according to previously published methods (Schinazi et al., 1989) was a generous gift of Dr. Raymond F. Schinazi (Emory University, Atlanta, GA). Purity of AZT-TP was >99% as assessed by HPLC analysis and TTP was not detected as a contaminant. D4T-TP was kindly provided by Dr. Michael J.M. Hitchcock (Pharmaceutical Group, Bristol-Myers Squibb, Wallingford, CT). Single-stranded circular M13mp18 bacteriophage DNA was purchased from US Biochemical (Cleveland, OH). The 17-base M13 universal sequencing primer, 'Primer U', and the 20-base oligomer, 'Primer A' (5′-TTCTCCCACCATAGAAGATA-3′) were purchased from Promega

(Madison, WI) and Oligos Etc. Inc. (Wilsonville, OR), respectively. The 20-base oligomer, 'Primer B' (5'-CTACTTATCCGTACCTATTT-3') was synthesized using a Dupont Coder 300 DNA synthesizer and subsequently purified using Nensorb prep columns (Dupont, Boston, MA).

Polymerase chain reaction, cloning and preparation of Gγ-globin single-stranded DNA

PCR was performed on the human γ-globin cDNA plasmid, pγIVS(-)SP3, containing the upstream promoter region, the first and part of the second exon of the Gγ-globin gene. Amplification primers flanked a 1151 bp fragment of the upstream promoter region and encompassed nucleotide positions 77 to 97 and −1074 to −1094, respectively (Numbering is 5' to 3', −1 indicating the first base 5' of the cap site). The sense and antisense primers contained 6 nucleotide *Sal*I and *Kpn*I restriction endonuclease sites, respectively, on their 5' ends. PCR was performed for 30 cycles according to previously described procedures (LeBoeuf et al., 1989). The PCR-amplified Gγ-globin DNA fragment was cloned into M13mp18 and used to transform *E. coli* according to previously described procedures (LeBoeuf et al., 1989). To confirm that the recombinant M13 contained the Gγ-fragment, the clone was used as template in a PCR reaction using M13 forward and reverse universal sequencing primers as amplimers. PCR reaction products were analyzed by Southern blot analysis using a ^{32}P-labeled Gγ-globin cDNA probe prepared by Nick translation (Sambrook et al., 1989). Recombinant M13 containing the Gγ-fragment was used to prepare single-stranded DNA for sequencing as described (Sambrook et al., 1989). The entire globin cDNA in M13 was sequenced and was identical to the published sequence.

DNA primer extension and strand elongation assays

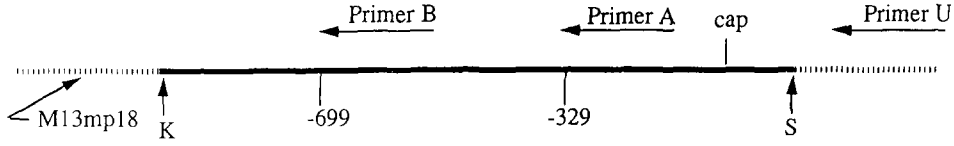
DNA primers were 5' end-labeled with ^{32}P as previously described (Townsend and Cheng, 1987). The labeled primer (1 pmol) was annealed to single-stranded Gγ-globin or M13mp18 template DNA (1 μg) by heating at 65°C for 3 min in the same composition buffer used for T7 sequencing reactions and then cooling slowly to 32°C. The DNA elongation reaction mixture (6 μl) contained 15 mM Tris-HCl (pH 7.5); 8 mM $MgCl_2$; 19 mM NaCl; 4 mM DTT; 33 μg/ml bovine serum albumin; 0.2 μg of labeled template/primer; 1.5 unit of T7 DNA polymerase (Sequenase version 2.0, US Biochemical); 33.3 μM each of dATP, dCTP, and dGTP; and specified concentrations of TTP, ddTTP, ddCTP, D4T-TP or AZT-TP. When sequencing reactions within 50 bases from the primer were performed (primer extension assay), $MnCl_2$ at a final concentration of 3.8 mM was added to the reaction mixture (Tabor and Richardson, 1989). Reactions were carried out at 37°C for 5 min, and terminated by addition of 4 μl of stop solution containing 95% formamide, 20 mM EDTA, 0.05% Bromophenol Blue, and 0.05% Xylene Cyanol FF. After heating at 90°C for 3 min, the reaction mixture was quickly cooled on ice and loaded onto either a 6 or 12% polyacrylamide gel (31 × 38.5 cm, 0.4 to 1.2 mm

wedge gel) containing 7 M urea, 100 mM Tris-HCl, 89 mM boric acid, and 2.5 mM EDTA at pH 8.3. Gels were electrophoresed at 35 watts for 3 h, transferred to Whatman 3MM paper and dried. Autoradiograms were obtained by exposure of Kodak XAR film at room temperature without intensifying screens. DNA sequences were confirmed by the Sanger dideoxy method with a Sequenase version 2.0 sequencing kit (US Biochemical) according to manufacturer's specifications.

Results and Discussion

In the present report, a template containing an upstream region of the Gγ-globin gene was constructed to study incorporation of AZT-TP into DNA using a modified T7 DNA polymerase lacking 3′ to 5′ exonuclease activity. M13mp18 bacteriophage DNA was also used as a template to compare AZT-TP incorporation with that observed with globin templates. To assess the effects of flanking sequences, different primers were used varying both in their template sequence and in their first site of nucleotide analog incorporation as described in Materials and Methods. The incorporation of AZT-TP was compared to other 2′,3′-dideoxynucleotides, including ddTTP, D4T-TP and ddCTP, which lack the 3′-hydroxyl group and have a ribose moiety similar to the naturally occurring 2′-deoxynucleotides.

A primer extension assay was used to evaluate site-specific effects of local DNA template sequence on DNA primer extension when nucleotide analogs were substituted for natural substrates. In complete primer extension reaction mixtures containing TTP in the thymidine (T) pool, both AZT-TP and ddTTP were utilized by modified T7 DNA polymerase with chain termination occurring at the expected T sites from either Primer A or B (Fig. 1, lane T and 1–3). In Primer B reactions, extension products were also observed at a noncomplementary G position (G-716) in the presence of AZT-TP (Fig. 1, Primer B, lane 3). We further investigated nucleotide analog incorporation under conditions in which the T reaction pool was incomplete. Extension of the labeled primer-template was assessed in the presence of either ddTTP or AZT-TP without competing TTP. The addition of ddTTP caused termination of DNA synthesis at the first T site from both Primer A and B (Fig. 1, lane 5). Similar data were obtained when D4T-TP was substituted for TTP (data not shown). Furthermore, in the absence of competing dCTP, ddCTP terminated primer extension at the first C site from both primers (Fig. 1, lane 6). In the presence of Primer A, AZT-TP also correctly incorporated at the first T site with some chains extending further (Fig. 1, Primer A, lane 4). In contrast, a large fraction of chains extended beyond the first site encoding for the insertion of TTP from Primer B in the presence of AZT-TP (Fig. 1, Primer B, lane 4). Termination of chain synthesis by AZT-TP at three sites of TTP insertion from Primer B suggests that AZT-TP is a poor substrate along this sequence. Conversely, in the presence of ddTTP or D4T-TP chain termination occurred



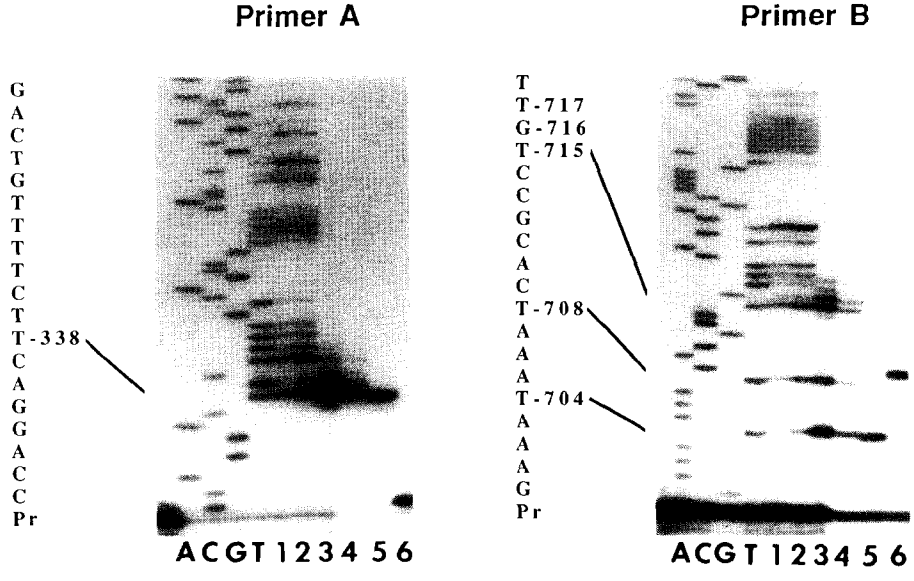


Fig. 1. Effects of AZT-TP on primer extension using T7 DNA polymerase and Gγ-globin template-primer complex. The upper panel is a schematic diagram of the Gγ-globin template. The template was constructed with a 1151 base pair fragment of the Gγ-globin gene inserted into the *SalI*/ *KpnI* sites of M13mp18 as described in Materials and Methods. To assess the effects of flanking sequences on nucleotide analog incorporation, different primer sites were selected to vary both template sequence and the first site of analog insertion. Incubations were performed in the presence of T7 DNA polymerase, ^{32}P end-labeled Primer A or B annealed to γ-globin single-stranded DNA, 33.3 μM each of dATP, dCTP and dGTP, the indicated amount of analog, and the reactions electrophoresed on a 12% polyacrylamide/7 M urea gel. The labeled primer/template was extended as described under Materials and Methods. Lanes A, C, G and T, dideoxynucleotide sequencing ladders generated with 30 μM dNTPs and 3.3 μM ddATP, ddCTP, ddGTP, and ddTTP, respectively. Lane 1, 31.6 μM AZT-TP and 1.67 μM TTP; lane 2, 32.0 μM AZT-TP and 1.30 μM TTP; lane 3, 33.0 μM AZT-TP and 0.30 μM TTP; lane 4, 33.3 μM AZT-TP; lane 5, 33.3 μM ddTTP; lane 6, 33.3 μM ddCTP.

at the first T position suggesting that these 2′,3′-dideoxynucleotides are efficiently used as substrates. Thus, the variation in substrate specificity observed between different primer-templates and nucleotide analogs appears to be unique to AZT-TP and not a general property of 2′,3′-dideoxynucleotides.

The present studies would suggest that template selection may be important to accurately address the basis for 2′,3′-dideoxynucleotides positioning in

DNA, since the rate and accuracy of their incorporation vary with template sequence (Fig. 1). To further investigate the effects of template sequence on AZT-TP incorporation into elongating DNA, extension reactions were performed under conditions that would allow synthesis of larger molecular weight DNA fragments by modified T7 DNA polymerase. In reactions containing only the natural dNTPs, DNA chains were extended to high molecular weight DNA with no evidence of major pause sites (Fig. 2, lane 4;

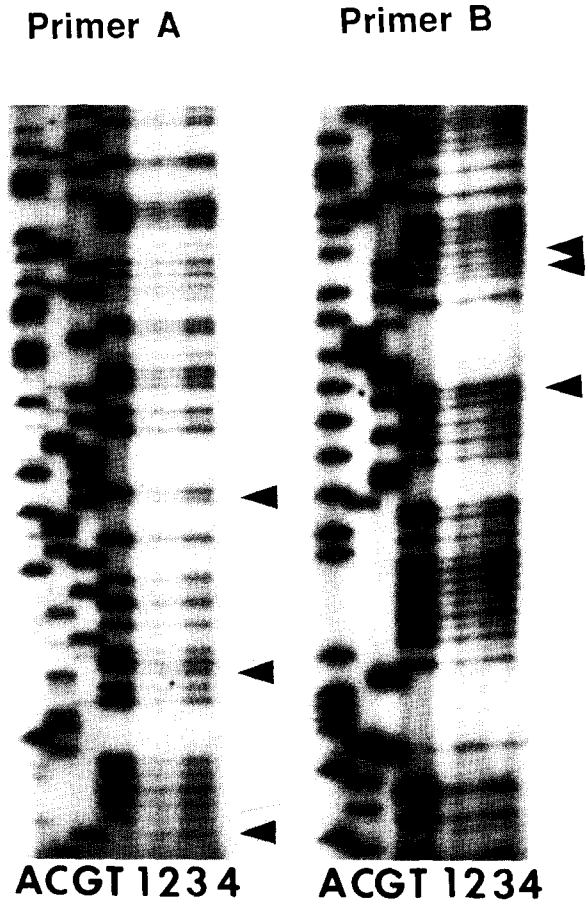


Fig. 2. Effects of AZT-TP on DNA strand elongation using T7 DNA polymerase and Gγ-globin template. Incubations were performed in the presence of T7 DNA polymerase, ^{32}P end-labeled Primer A or B annealed to M13mp18 containing the γ-globin insert, 33.3 μM each of dATP, dCTP and dGTP, the indicated amount of TTP/analog and the reactions electrophoresed on a 6% polyacrylamide/ 7 M urea gel. The labeled primer/template was extended as described in Materials and Methods. Lanes A, C, G and T, dideoxynucleotide sequencing ladders generated with 30 μM dNTPs and 3.3 μM ddATP, ddCTP, ddGTP and ddTTP, respectively; lane 1, 31 μM AZT-TP and 2.3 μM TTP; lane 2, 31.5 μM AZT-TP and 1.7 μM TTP; lane 3, 32.5 μM AZT-TP and 0.8 μM TTP; lane 4, 33.3 μM TTP. Solid symbols indicate position of noncomplementary chain termination in the presence of AZT-TP. Note Primer B, lane 4 has been cut off.

data not shown for primer B). In reactions using either Primer A or B and AZT-TP in the T pool, termination occurred at the expected T sites (Fig. 2, lanes 1–3). In addition, chain termination also occurred at certain noncomplementary positions in the presence of AZT-TP. When incorporation of another thymidine analog, D4T-TP, was assessed using Primers A and B, termination of DNA synthesis was different as compared to that of AZT-TP and ddTTP. D4T-TP did not exhibit noncomplementary chain termination as

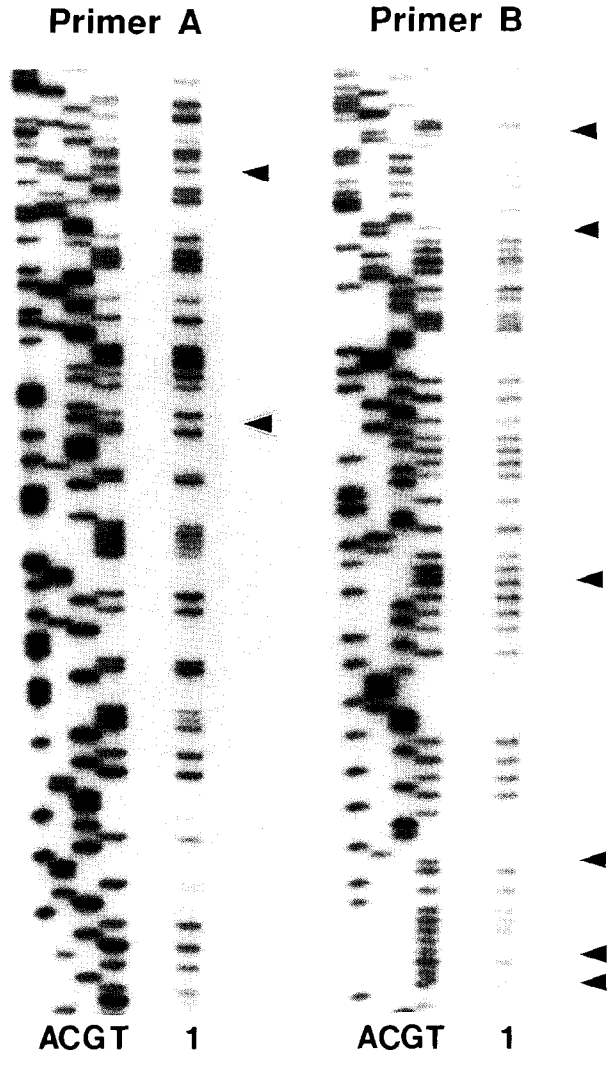


Fig. 3. Effects of D4T-TP on DNA strand elongation using T7 DNA polymerase and Gγ-globin template. Conditions are identical to those described in Fig. 2 except lane 1 contains 33.3 μM dNTPs, including 20 μM TTP and 13.3 μM D4T-TP. Solid symbols indicate T positions where chain termination does not occur in the presence of D4T-TP.

observed with AZT-TP, with DNA strands being terminated only at certain T sites (Fig. 3, lane 1), which suggests that D4T-TP incorporation is also dependent on the template sequence. The chain termination patterns observed with AZT-TP and D4T-TP are not due to a 3′ to 5′ exonuclease activity since the modified T7 DNA polymerase lacks such activity. Therefore, each 2′,3′-dideoxynucleotide may exhibit unique chain termination characteristics that vary with template sequence.

Lastly, we compared whether a M13 DNA template also directed noncomplementary chain terminations by modified T7 DNA polymerase in the presence of AZT-TP. Although noncomplementary chain termination was detected in the presence of AZT-TP (Fig. 4, M13mp18, lanes 1–3), these same

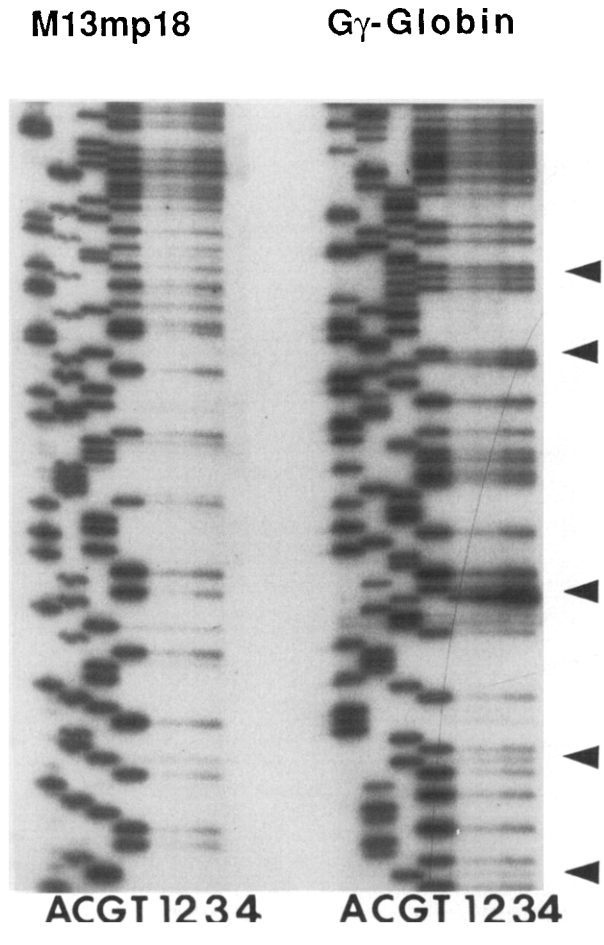


Fig. 4. Effects of AZT-TP on DNA strand elongation using either a M13mp18 or a Gγ-globin template. Conditions are identical to those described in Fig. 2 except ^{32}P end-labeled Primer U annealed to M13mp18 DNA and M13mp18 containing the γ-globin insert was used. A portion of Gγ-globin, lane 4 has been cut off. Solid symbols indicate positions of noncomplementary chain termination in the presence of AZT-TP.

events were also seen in the presence of ddTTP as detected by longer autoradiography exposures (M13mp18, lane T). Reactions primed from the universal primer (Primer U) complementary to M13 containing the globin insert produced a chain termination pattern in the presence of AZT-TP at all T sites and at five noncomplementary positions (Fig. 4, Gγ-globin, lanes 1–3). These studies demonstrate variations in substrate specificity of 2′,3′-dideoxynucleotide analogs with template sequence that was not evident in a template (M13) commonly used in nucleotide analog incorporation studies. The genomic template may potentially form secondary structures or influence the kinetics of analog insertion by the DNA polymerase. These findings suggest the importance of template selection for insertion kinetic studies, as recently reported (Parker, et al., 1991). Ongoing studies with mammalian DNA polymerases will address whether incorporation of 2′,3′-dideoxynucleosides into replicating DNA may vary based on genomic DNA template sequence.

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