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Polymerization of 2′-Fluoro- and 2′-*O*-Methyl-dNTPs by Human DNA Polymerase α, Polymerase γ, and Primase

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ABSTRACT. Studies were undertaken to assess the ability of human polymerase α (pol α) and polymerase γ (pol γ) to incorporate 2′-fluoro- and 2′-*O*-methyldeoxynucleotides into DNA. *In vitro* DNA synthesis systems were used to detect incorporation and determine K_m and V_{max} for 2′-FdATP, 2′-FdUTP, 2′-FdCTP, 2′-FdGTP, 2′-*O*-MedATP, 2′-*O*-MedCTP, 2′-*O*-MedGTP, 2′-*O*-MedUTP, dUTP, UTP, and FIAUTP, in addition to normal deoxynucleotides. Pol α incorporated all 2′-FdNTPs except 2′-FdATP, but not 2′-*O*-MedNTPs. Pol γ incorporated all 2′-FdNTPs, but not 2′-*O*-MedNTPs. In general, 2′-fluorine substitution decreased V_{max}/K_m; however, the magnitude of the changes was nucleotide dependent, with dATP and dUTP being the most affected. Misinsertion frequencies for pol α and pol γ of 2′-FdNTPs compared with their normal nucleotides were: FIAUTP > 2′-FdCTP > 2′-FdGTP > 2′-FdATP (pol γ only) > 2′-FdUTP. Because kinetics of insertion of pol α can be affected by the nature of the primer, we examined the ability of pol α to polymerize 2′-fluoro- and 2′-*O*-MedATP and dGTP when elongating a primer synthesized by DNA primase. Under these conditions, both 2′-FdATP and 2′-FdGTP were polymerized, but 2′-*O*-MedATP and 2′-*O*-MedGTP were not. Primase alone could not readily polymerize these analogs into RNA primers. Previous studies showed that 2′-deoxy-2′-fluorocytosine (2′-FdC) is incorporated by several non-human DNA polymerases. The current studies showed that human polymerases can polymerize numerous 2′-FdNTPs but cannot polymerize 2′-*O*-MedNTPs. BIOCHEM PHARMACOL **59**;9:1045–1052, 2000. © 2000 Elsevier Science Inc.

KEY WORDS. 2′-fluoronucleoside triphosphate; polymerase α; polymerase γ; human; 2′-*O*-methoxynucleoside triphosphate; DNA primase

RNA-based aptamers generated using systematic evolution of ligands by exponential enrichment (SELEX) technology [1] are being developed as therapeutic protein inhibitors [2–4]. Because nucleases can degrade ribose-based aptamers rapidly, modified nucleosides including 2′-FdC,‖ 2′-FdU, 2′-*O*-MedA, and 2′-*O*-MedG are used in place of normal ribonucleosides to increase resistance to nuclease digestion and in some cases increase binding affinity [5]. Despite this increase in resistance, it is assumed that degradation of these aptamers to nucleosides can occur *in vivo*. Since these aptamers are developed for therapeutic use, there is an interest in the potential for these nucleosides to be incorporated into nuclear and mitochondrial DNA. This interest stems, in part, from numerous studies that have correlated or implicated toxicity of nucleoside analogs including fludarabine [6], araC [7], gemcitabine [8], 2′,2′-difluorodeoxyguanosine [9], ddC, AZT [10], and fialuridine [11, 12] with incorporation into cellular and/or mitochondrial DNA. In addition, insertion of modified nucleosides into cellular DNA may lead to DNA modifications that could be precursors to cancer and other biological diseases.

The ability of nucleotide analogs to inhibit and to serve as substrates for DNA synthesis has been reviewed by Wright and Brown [13]. Several specific studies have examined the ability of various polymerases to incorporate 2′-FdUTP and 2′-FdCTP into DNA, using whole DNA and/or oligonucleotide substrates. Aoyama *et al.* [14] demonstrated that 2′-FdCTP can be incorporated into DNA templates using mouse cell pol γ and *Xenopus laevis* pol α.

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‖ *Abbreviations*: 2′-FdC, 2′-deoxy-2′-fluorocytosine; 2′-FdU, 2′-deoxy-2′-fluorouridine; 2′-*O*-MedA, 2′-deoxy-2′-*O*-methyladenosine; 2′-*O*-MedG, 2′-deoxy-2′-*O*-methylguanosine; 2′-FdUTP, 2′-fluoro-2′-deoxyuridine triphosphate; 2′-FdCTP, 2′-fluoro-2′-deoxycytidine triphosphate; 2′-FdGTP, 2′-fluoro-2′-deoxyguanosine triphosphate; 2′-FdATP, 2′-fluoro-2′-deoxyadenosine triphosphate; 2′-*O*-MedGTP, 2′-*O*-methyl-2′-deoxyguanosine triphosphate; 2′-*O*-MedATP, 2′-*O*-methyl-2′-deoxyadenosine triphosphate; 2′-*O*-MedCTP, 2′-*O*-methyl-2′-deoxycytidine triphosphate; 2′-*O*-MedUTP, 2′-*O*-methyl-2′-deoxyuridine triphosphate; FIAUTP, 2′-1(2′-deoxy-2′-fluoro-β-D-arabinofuranosyl)-5-iodouracil; 2′-FdCMP, 2′-fluoro-2′-deoxycytidine monophosphate; 2′-FdUMP, 2′-fluoro-2′-deoxyuridine monophosphate; 2′-FdAMP, 2′-fluoro-2′-deoxyadenosine monophosphate; TBE, Tris–borate–EDTA; pol α, polymerase α; and pol γ, polymerase γ.

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TABLE 1. Primer and templates

Primer	5′-CGATATTCACAAAG
G template	3′-GCTATAAGTGTTTCTTGCCAATCTCGTAC-5′
A template	3′-GCTATAAGTGTTTCTTACCGGTTTAGATC-5′
T template	3′-GCTATAAGTGTTTCAATCCGGTAATGTAC-5
C template	3′-GCTATAAGTGTTTCAACTTGGGACTCTAC-5′
$d(TC)_{30}$ template	5′-TCTCTCTCTCTCTCTCTCTCTCTCTCTC-3′
$d(C)_{40}$ template	5′-CCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCCC-3′

Once incorporated, 2′-FdC appeared to serve as a poor template for these two enzymes [15]. 2′-FdCTP and to a lesser extent 2′-FdUTP both are incorporated into gapped calf thymus DNA templates with *Escherichia coli* DNA pol II [16]. Likewise, thermostabile DNA polymerases can incorporate both 2′-FdCTP and 2′-FdUTP *in vitro* [17]. These studies indicated that fluorine modification of the 2′-position of the NTPs does allow for DNA incorporation by selected DNA polymerases despite the known fact that they highly discriminate against modifications of NTPs. Presently, no published data exist on the effects of *O*-methyl modification of the 2′ position of dNTPs on DNA incorporation. Because these nucleosides have potential use in therapeutic aptamers, we were interested in the ability of mammalian polymerases, specifically human pol γ [18] and pol α [19], to incorporate the 2′-modified nucleosides.

The purpose of these studies was to determine if and to what extent 2′-FdNTPs and 2′*O*-MedNTPs can be utilized by human pol γ and pol α as substitutes for normal deoxynucleotides. To accomplish this, these studies utilized methods developed by Goodman and coworkers [20] to measure the K_m and V_{max} for each of these nucleotides using pol α and pol γ.

MATERIALS AND METHODS

2′-FdCTP, 2′-FdUTP, 2′-FdGTP, 2′-FdATP, 2′-*O*-MedGTP, 2′-*O*-MedATP, 2′-*O*-MedCTP, and 2′-*O*-MedUTP were obtained from JBL Scientific, Inc. FIAUTP was provided by Eli Lilly & Co. dATP, dGTP, dTTP, dCTP, and UTP were obtained from United States Biochemicals. dUTP was obtained from Pharmacia Biotech. The templates and primer were synthesized and HPLC-purified by Operon Technologies, Inc. Acetylated BSA was obtained from Promega. Pol α was expressed and purified using a baculovirus expression system [18]. Pol γ [19] was obtained from Dr. William Copeland, National Institute of Environmental Health Sciences.

Purification of Modified Nucleotides

All 2′-FdNTPs and 2′-*O*-MeNTPs were purified by thin-layer chromatography. Multiple 2.0-μL aliquots were spotted onto Whatman 250-μm thick K5F silica gel plates (Whatman Inc.). The spots were allowed to air-dry, and the plates were developed using isopropanol:ammonium hydroxide:H_2O (4.5:3:2.5). The separated mono-, di-, and triphosphates were visualized using long-wave UV light. The triphosphate spots were scraped with individual, sterile scalpels into 1.5-mL microfuge tubes. One milliliter of sterile, deionized H_2O was added to each tube, and the tubes were incubated at 37° overnight on a rotating wheel. The samples were centrifuged in a Beckman GS-15R centrifuge using a F2402H rotor (Beckman Instruments, Inc.) at 16,000 *g* for 10 min. The supernatant was removed and filtered through a 2-μm syringe filter. An additional 1.0 mL of H_2O was added to the pelleted silica; the sample was vortexed, microfuged, and filtered as above. The samples were dried in a SAVANT Speed Vac (SAVANT) and resuspended in 50–100 μL of H_2O. The concentrations of the triphosphates were determined using a Hewlett-Packard 845X UV-visible spectrophotometer (Hewlett-Packard GmbH).

Primers and Templates

The primer and templates used for the pol α and γ and primase assays are shown in Table 1. All reactions were running start reactions, with the modified nucleotide inserted in the third position from the 5′ end of the primer.

For the G and A templates, two dAMPs were inserted before the insertion of the modified nucleotide. For the T and C templates, two dTMPs were inserted before the insertion of the modified nucleotide. Primase and primase-coupled pol α assays used $d(TC)_{30}$ and $d(C)_{40}$ templates.

Labeling and Annealing

The primer (1.0 nmol) was end-labeled using 120 μCi of [γ-^{32}P]dATP (3000 Ci/mmol, NEN Life Sciences) and 50 U of T_4 polynucleotide kinase (10 U/μL, Boehringer Mannheim) in a final volume of 100 μL of 1x kinase buffer (Boehringer Mannheim). After 30 min at 37°, the unincorporated nucleotides were removed using Centri Sep spin columns (Princeton Separations). Specific activities of the labeled primer ranged from 5×10^4 to 5×10^5 cpm/pmol.

Labeled primer and template (1:1.1) were annealed in H_2O at a primer concentration of 5 μM. The samples were incubated at 65° for 15 min in a heat block. Then the heat block was turned off, and the samples were allowed to cool slowly to room temperature. The annealed samples were stored at −20° until used, but not past 2 weeks post end-labeling.

Incorporation Reaction Conditions

The annealed primer/template was incubated with human pol α under the following conditions: 5 pmol of a specific primer/template in a total reaction volume of 5.0 μL was incubated at 37° for 8 min in 50 mM Tris–HCl (pH 7.5), 10 mM $MgCl_2$, 1 mM dithiothreitol, and 1 μg/mL of acetylated BSA. Each reaction also contained 0.1 U pol α, 80 μM dATP or dTTP as the running start nucleotide, and various concentrations of the normal or modified nucleotide. The annealed primer/template was incubated with human pol γ under the following conditions: 5 pmol of a specific primer/template in a total reaction volume of 5.0 μL was incubated at 37° for 10 min in 25 mM HEPES (pH 7.6), 5 mM $MgCl_2$, 0.1 mM EDTA, 2 mM β-mercaptoethanol, and 50 μg/mL of acetylated BSA. Each reaction also contained 2.0 ng pol γ, 10 μM dATP or dTTP as the running start nucleotide, and various concentrations of the normal or modified nucleotide.

All reactions were quenched by the addition of 5 μL of a 2x dye stock solution consisting of 98% formamide, 20 mM EDTA, 0.025% SDS, 0.05% (w/v) bromophenol blue, 0.05% (w/v) xylene cyanol, and 0.05% (w/v) orange G. Products were separated by denaturing polyacrylamide gel electrophoresis (16% acrylamide, 8 M urea), electrophoresed at 50 W/gel using 0.5x TBE in the upper reservoir and 1.5 M sodium acetate (pH 7) in 1x TBE in the lower reservoir [21]. The amount of radiolabeled products was quantified using a Fuji PhosphorImager (Fuji Photo Film Co., Ltd.). K_m and V_{max} values were obtained using a linear regression of a Hanes–Woolf plot. The IC_{50} values of selected nucleotides were derived using a Dixon plot.

Elongation Reactions

Elongation reactions were performed as for the incorporation reactions, but included appropriate dNTPs to allow elongation past the site of 2′-FdNTP incorporation.

Primase Assays

Primase assays were performed as previously described [22]. Assays contained primase (p49/p58 complex), 50 μM DNA (total nucleotide), 0.1 to 1 mM [α-^{32}P]NTPs, 1 mM dithiothreitol, and 0.1 mg/mL of BSA. After 60 min at 37°, assays were quenched by adding 2.5 vol. of gel loading buffer. Products were separated by denaturing polyacrylamide gel electrophoresis (20% acrylamide, 8 M urea) and quantified using a Molecular Dynamics PhosphorImager.

Primase-Coupled Pol α Assays

Assays were performed as previously described [23], and typically contained pol α–primase, 50 μM DNA (total nucleotide), 200 μM NTPs, 1 mM dithiothreitol, 0.1 mg/mL of BSA, and 5 μM [α-^{32}P]dNTPs. After 60 min at 37°, assays were quenched by adding 2.5 vol. of gel loading buffer. Products were separated by denaturing polyacrylamide gel electrophoresis (20% acrylamide, 8 M urea) and quantified using a Molecular Dynamics PhosphorImager.

RESULTS

DNA Pol α and Pol γ

Initial studies focused on determining the effects of 2′-fluoro and 2′-*O*-Me substitution on polymerization by pol α and pol γ. In each case, polymerization of both the normal and modified nucleoside triphosphates was measured using the "running start" assay developed by Goodman and coworkers [20]. Table 2 provides a summary of steady-state kinetic parameters and misinsertion frequencies for all nucleotides tested, and Figs. 1-3 provide representative phosphorimages for selected nucleotides.

The effects of 2′-fluorine substitution on incorporation and kinetic constants were examined first (Figs. 1 and 2). All 2′-fluoro-substituted nucleotides were polymerized by pol γ; however, V_{max}/K_m decreased in a nucleotide-dependent manner (as compared with normal deoxynucleotides). Misinsertion frequencies indicated that pol γ will polymerize 2′-FdNTPs 6-fold (2′-FdATP) to 200-fold (2′-FdUTP) less frequently when the normal deoxynucleotide and 2′-FdNTP are present at equimolar concentrations. In pol γ reactions, primer shortening has been observed and attributed to the exonuclease function of the polymerase [24]. This primer shortening was reduced in a concentration-dependent manner when 2′-FdGTP or 2′-FdCTP (Fig. 1) was added to the reaction, suggesting that the exonuclease function was being inhibited. 2′-Fluorine substitution produced similar effects on kinetic parameters in assays using pol α, with the exception that no incorporation was observed with 2′-FdATP. To ensure that 2′-FdATP could not be incorporated by pol α, a separate lot of 2′-FdATP was purified and tested. Pol α was not able to incorporate this second lot of material. 2′-FdGTP, 2′-FdCTP, and 2′-FdUTP all had lower V_{max}/K_m values than their respective normal deoxynucleotides and FIAUTP. Elongation reactions indicated that both pol α and pol γ could elongate primers after insertion of 2′-FdNTP (Fig. 3). Again, pol α could not elongate the primer in the presence of 2′-FdATP.

Among uridine and thymidine triphosphates, V_{max}/K_m increased from UTP < 2′-FdUTP < dUTP < dTTP < FIAUTP with a large difference between 2′-FdUTP and dUTP (Table 2). The K_m values and misinsertion frequencies were similar for dUTP and dTTP using human pol γ, supporting previous work with porcine pol γ [25]. Both human pol α and pol γ incorporated FIAUTP with essentially equal and possibly greater efficiency than normal dTTP, supporting previous work with other mammalian polymerases [26].

2′-*O*-MedNTPs were not incorporated by either pol α or pol γ at concentrations up to 5 mM. 2′-*O*-MedNTPs were weak inhibitors of pol α (IC_{50} for 2′-*O*-MedUTP = 222 μM; 2′-*O*-MedATP = 752 μM; 2′-*O*-MedGTP = 714

TABLE 2. K_m, V_{max}, V_{max}/K_m and misinsertion frequencies for 2′-modified nucleotides

Nucleotide	Pol	K_m^* (μM)	V_{max}^* (I_3/I_2)	V_{max}/K_m	$MF^{\dagger}_{(comparator)}$
dGTP	α	0.185 ± 0.054	1.465 ± 0.339	7.919	
	γ	0.870 ± 0.039	1.730 ± 0.602	1.988	
2′FdGTP	α	1.233 ± 0.539	0.298 ± 0.017	0.242	$0.031_{(dGTP)}$
	γ	2.894 ± 1.236	0.910 ± 0.355	0.314	$0.16_{(dGTP)}$
dCTP	α	0.641 ± 0.167	1.498 ± 0.478	2.337	
	γ	0.097 ± 0.012	2.691 ± 0.767	27.742	
2′FdCTP	α	1.006 ± 0.095	0.178 ± 0.003	0.177	$0.076_{(dCTP)}$
	γ	0.799 ± 0.161	1.288 ± 0.179	1.612	$0.058_{(dCTP)}$
2′FdATP	α	No incorporation	No incorporation		
	γ	4.070 ± 1.474	0.714 ± 0.143	0.175	$0.04_{(dATP)}$
dATP	α	0.249 ± 0.038	0.498 ± 0.232	2	
	γ	0.102 ± 0.017	0.458 ± 0.021	4.49	
2′FdUTP	α	31.02 ± 8.72	0.301 ± 0.024	0.01	$0.007_{(dTTP)}$
	γ	29.67 ± 1.56	0.734 ± 0.123	0.025	$0.005_{(dTTP)}$
dTTP	α	1.646 ± 0.529	2.341 ± 0.657	1.422	
	γ	0.149 ± 0.034	0.781 ± 0.108	5.242	
UTP	α	44.63 ± 13.06	0.134 ± 0.039	0.003	$0.002_{(dTTP)}$
	γ	47.21 ± 10.36	0.254 ± 0.101	0.005	$0.001_{(dTTP)}$
dUTP	α	3.150 ± 0.750	0.598 ± 0.104	0.19	$0.134_{(dTTP)}$
	γ	0.140 ± 0.062	0.478 ± 0.116	3.414	$0.677_{(dTTP)}$
FIAUTP	α	1.231 ± 0.269	0.655 ± 0.107	0.532	$0.374_{(dTTP)}$
	γ	0.070 ± 0.027	0.556 ± 0.246	7.943	$1.515_{(dTTP)}$
2′-O-Med-G, C, A, U-TP	α	No incorporation	No incorporation		
	γ	No incorporation	No incorporation		

*Mean ± SD for 3 experiments.

†Misinsertion frequency = $\frac{V_{max}\text{ (modified nucleotide)}/K_m\text{ (modified nucleotide)}}{V_{max}\text{ (normal nucleotide)}/K_m\text{ (normal nucleotide)}}$.

μM; 2-*O*-MedCTP = 792 μM); however, no inhibition of pol γ or of the exonuclease function of pol γ was observed.

Primase Activity

We measured the effects of 2′-FdATP, 2′-FdGTP, 2′-*O*-MedATP, and 2′-*O*-MedGTP on the synthesis of RNA primers by DNA primase, a reaction essential for the initiation of all new strands of DNA [27]. The effects of 2′-FdGTP and 2′-*O*-MedGTP were examined initially in assays containing $(dC)_{40}$ and [α-^{32}P]GTP (Fig. 4). Both compounds inhibited primase competitively with respect to GTP and gave K_i values of 62 ± 10 μM (2′-FdGTP) and 80 ± 10 μM (2′-*O*-MedGTP). For comparison, the K_m of GTP was 330 ± 50 μM; however, there was no apparent incorporation of the compounds, based on the absence of products of altered electrophoretic mobility [28]. Inhibition of primase by 2′-FdATP and 2′-*O*-MedATP was measured in assays containing poly(dT) and 200 μM [α-^{32}P]ATP. Both compounds were weak inhibitors of primase activity [IC_{50} = 180 μM (2′-FdATP) and 400 μM (2′-*O*-Me-dATP)]. There was no apparent incorporation of the compounds, as evidenced by the absence of products of altered electrophoretic mobility (data not shown).

To confirm that primase does not polymerize these analogs readily, we examined primer synthesis on $d(TC)_{30}$, a template that requires both ATP and GTP for primer synthesis. Figure 4 shows the primers synthesized in the presence of ATP and GTP, and demonstrates that omission of either NTP eliminated primer synthesis. Importantly, no detectable primers were synthesized in assays containing either 2′-FdGTP and [α-^{32}P]ATP or 2′-FdATP and [α-^{32}P]GTP, indicating that 2′-FdNTPs are not readily polymerized by primase. Identical studies using 2′-*O*-Me-dATP and 2′-*O*-MedGTP in place of the 2′-FdNTPs showed that primase also does not polymerize these analogs (data not shown).

Primase-Coupled Pol α Activity

In vivo, pol α normally elongates primase-synthesized primers to initiate the synthesis of new DNA strands. Importantly, we have found previously that the effects of nucleotide analogs and template lesions on coupled activity often vary dramatically from the effects observed when pol α elongates an exogenously added primer [29–31]. To determine if pol α interacted differently with 2′-fluoro- and 2′-*O*-Me-dATP and -dGTP when elongating primase-synthesized primers than when elongating exogenously added primer-templates, assays were performed containing

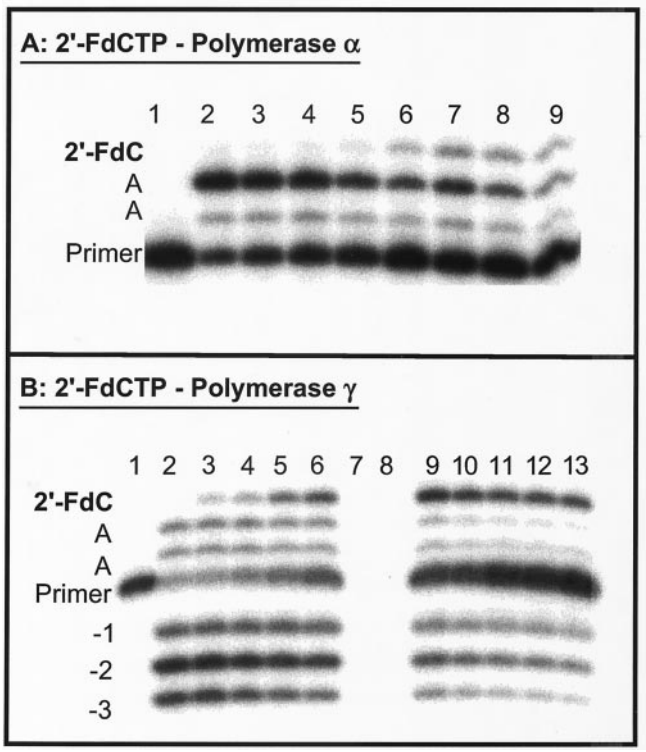

FIG. 1. Incorporation of 2′-FdCTP by pol α (panel A) or pol γ (panel B). The reaction conditions were as described in Materials and Methods. The bands representing the primer, nucleotides incorporated (dAMP or 2′-FdCMP), and degradation by the exonuclease (-1, -2, -3) are labeled in the figure. For pol α (panel A), all reactions contained 80 μM dATP, pol α (except for Lane 1, which contained no enzyme), and the following concentrations of 2′-FdCTP: (Lane 1) 0 μM; (Lane 2) 0 μM; (Lane 3) 0.05 μM; (Lane 4) 0.1 μM; (Lane 5) 1.0 μM; (Lane 6) 10 μM; (Lane 7) 20 μM; (Lane 8) 50 μM; and (Lane 9) 100 μM. For pol γ (panel B), all reactions contained 10 μM dATP, pol γ (except for Lane 1, which contained no enzyme), and the following concentrations of 2′-FdCTP: (Lane 1) 0 μM; (Lane 2) 0 μM; (Lane 3) 0.05 μM; (Lane 4) 0.1 μM; (Lane 5) 0.5 μM; (Lane 6) 1.0 μM; (Lanes 7 and 8) no samples; (Lane 9) 5 μM; (Lane 10) 10 μM; (Lane 11) 20 μM; (Lane 12) 50 μM; and (Lane 13) 100 μM.

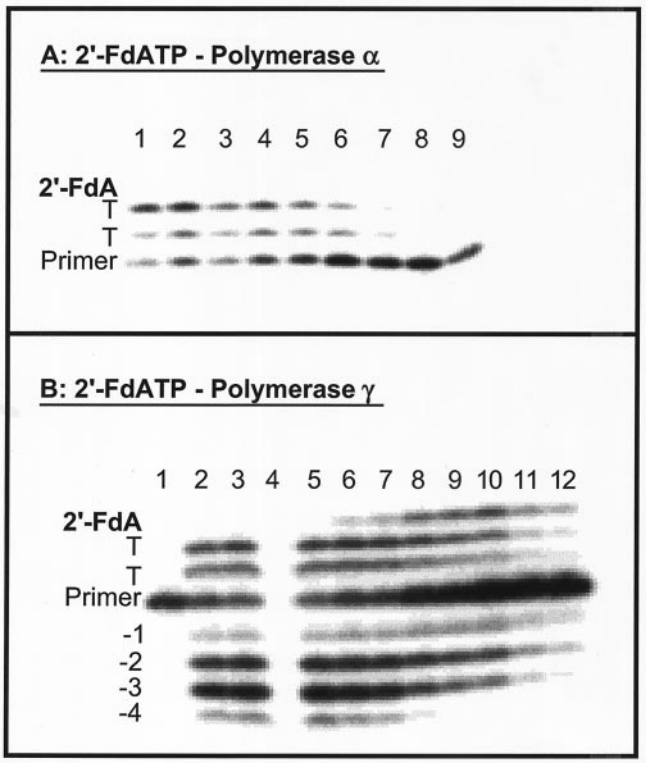

FIG. 2. Incorporation of 2′-FdATP by pol α (panel A) or pol γ (panel B). The reaction conditions were as described in Materials and Methods. The bands representing the primer, nucleotides incorporated (dTMP or 2′-FdAMP), and degradation by the exonuclease (-1, -2, -3, -4) are labeled in the figure. For pol α (panel A), all reactions contained 80 μM dTTP, pol α (except for Lane 9, which contained no enzyme), and the following concentrations of 2′-FdATP: (Lane 1) 0 μM; (Lane 2) 0.01 μM; (Lane 3) 0.05 μM; (Lane 4) 0.1 μM; (Lane 5) 1.0 μM; (Lane 6) 10 μM; (Lane 7) 20 μM; (Lane 8) 50 μM; and (Lane 9) 0 μM. For pol γ (panel B), all reactions contained 10 μM dTTP, pol γ (except for Lane 1, which contained no enzyme), and the following concentrations of 2′-FdATP: (Lane 1) 0 μM; (Lane 2) 0 μM; (Lane 3) 0.05 μM; (Lane 4) no samples; (Lane 5) 0.1 μM; (Lane 6) 0.5 μM; (Lane 7) 1 μM; (Lane 8) 5 μM; (Lane 9) 10 μM; (Lane 10) 20 μM; (Lane 11) 50 μM; and (Lane 12) 100 μM.

$d(TC)_{30}$, ATP, GTP, 5 μM dATP, 5 μM dGTP pol α–primase, and pol α. Most of the products were 50–60 nucleotides long, indicating that primase initiated primer synthesis near the 3′-end of the template and pol α elongated the primers to near the 5′ end of the template (Fig. 5) [23]. The addition of either 2′-FdATP (10 μM) or 2′-FdGTP (10 μM) had no effect on either the amount or the length of the products. Omission of dATP resulted in products consisting of Ö(dG)pAp(dG)pAp(dG)Ö that were up to 10 nucleotides shorter than when both dATP and dGTP were present (Fig. 5) [29]. Similar results were obtained when dGTP was omitted from the assays, except that the elongated primer contained Ö(dA)pGp(dA)pGp(dA). The addition of 2′-FdATP to primase-coupled pol α assays lacking dATP or of 2′-FdGTP to assays lacking dGTP increased the length of the resulting products, indicating that pol α–primase polymerized the 2′-FdATP and 2′-FdGTP, respectively. Interestingly, whereas adding 2′-FdGTP to assays lacking dGTP resulted in products as long as those synthesized in assays containing dATP and dGTP, adding 2′-FdATP to assays lacking dATP resulted in only a modest increase in the length of the products. When similar studies were performed with the 2′-O-MedNTPs, no detectable incorporation was observed (data not shown).

DISCUSSION

This work examined the ability of human pol α and pol γ to incorporate 2′-modified nucleotides into DNA templates. The results demonstrated that, with the exception of 2′-FdATP, both polymerases were able to incorporate and extend 2′-FdNTPs over a limited template but were unable to incorporate or extend 2′-O-methylnucleotides. The

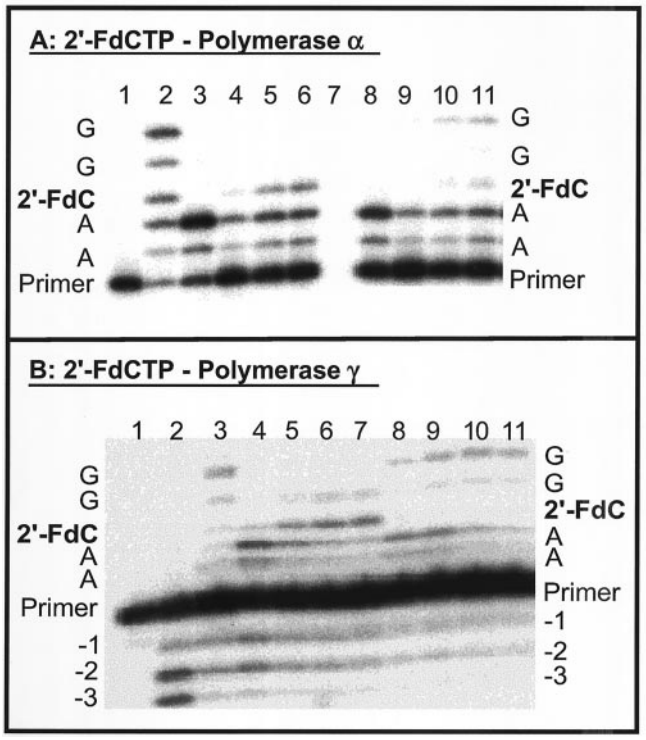

FIG. 3. Elongation past 2′-FdCTP by pol α (panel A) or pol γ (panel B). The reaction conditions were as described in Materials and Methods. The bands representing the primer, nucleotides incorporated (dAMP, dGMP, or 2′-FdCMP), and degradation by the exonuclease (-1, -2, -3) are labeled in the figure. For pol α (panel A), all reactions contained pol α (except for Lane 1, which contains no enzyme). Lane 2 contained 80 μM each of dATP, dCTP, and dGTP. Lanes 3–6 contained 80 μM dATP and the following concentrations of 2′-FdCTP: (Lane 3) 0 μM; (Lane 4) 10 μM; (Lane 5) 20 μM; and (Lane 6) 50 μM. (Lane 7) no sample. Lanes 8–11 contained 80 μM dATP, 80 μM dGTP, and the following concentrations of 2′-FdCTP: (Lane 8) 0 μM; (Lane 9) 10 μM; (Lane 10) 20 μM; and (Lane 11) 50 μM. For pol γ (panel B), all reactions contained pol γ (except for Lane 1, which contained no enzyme). Lane 3 contained 10 μM each of dATP, dCTP, and dGTP. Lanes 2 and 4–7 contained 10 μM dATP and the following concentrations of 2′-FdCTP: (Lane 2) 0 μM; (Lane 4) 1.0 μM; (Lane 5) 5 μM; (Lane 6) 10 μM; and (Lane 7) 20 μM. Lanes 8–11 contained 10 μM dATP, 10 μM dGTP, and the following concentrations of 2′-FdCTP: (Lane 8) 1.0 μM; (Lane 9) 5 μM; (Lane 10) 10 μM; and (Lane 11) 20 μM.

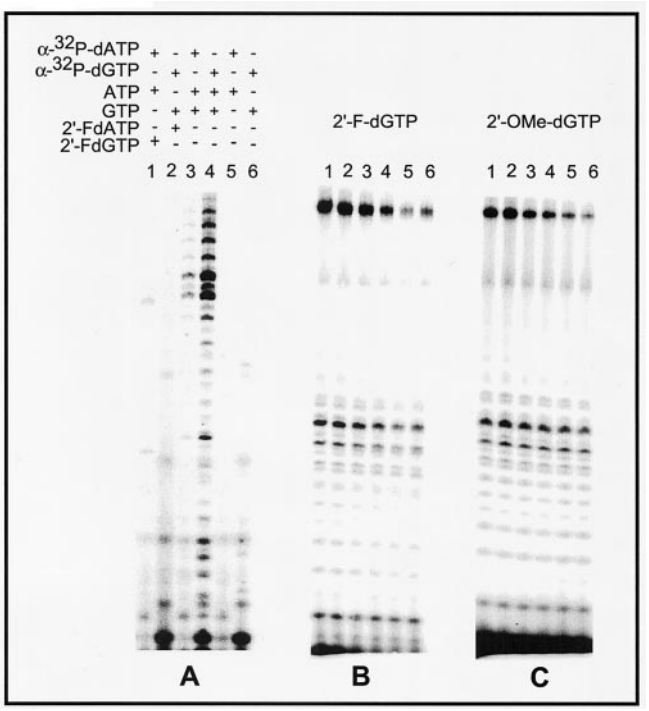

FIG. 4. Incorporation by α primase of 2′-FdGTP or 2′-FdATP (panel A) and inhibition of α primase by 2′-FdGTP (panel B) or 2′-*O*-MedGTP (panel C). The reaction conditions were as described in Materials and Methods. Panel A: (Lane 1) [α-^{32}P]dATP, ATP, and 2′-FdGTP; (Lane 2) [α-^{32}P]dGTP, GTP, and 2′-FdATP; (Lane 3) [α-^{32}P]dATP, ATP, and GTP; (Lane 4) [α-^{32}P]dGTP, ATP, and GTP; (Lane 5) [α-^{32}P]dATP and ATP; and (Lane 6) [α-^{32}P]dGTP and GTP. Panel B: (Lane 1) 0 μM 2′-FdGTP; (Lane 2) 25 μM 2′-FdGTP; (Lane 3) 50 μM 2′-FdGTP; (Lane 4) 75 μM 2′-FdGTP; (Lane 5) 100 μM 2′-FdGTP; and (Lane 6) 125 μM 2′-FdGTP. Panel C: (Lane 1) 0 μM 2′-*O*-MedGTP; (Lane 2) 50 μM 2′-*O*-MedGTP; (Lane 3) 100 μM 2′-*O*-MedGTP; (Lane 4) 150 μM 2′-*O*-MedGTP; (Lane 5) 200 μM 2′-*O*-MedGTP; and (Lane 6) 250 μM 2′-*O*-MedGTP.

effect of the 2′-fluoro substitution was nucleotide dependent. 2′-Fluoro substitution of dGTP decreased V_{max}/K_m 30-fold (pol α) and 6-fold (pol γ), whereas substitution of dUTP decreased V_{max}/K_m 19-fold (pol α) and 130-fold (pol γ). The most striking effect on substitution, however, was observed with 2′-FdATP, where incorporation by pol α was abolished completely and K_m with pol γ was increased 40-fold as compared with dATP (Table 2). The reason that 2′-FdATP was not incorporated remains unclear, but may relate to the strong inhibitory properties of the nucleotide relative to the other 2′-FdNTPs. Two lines of evidence indicate that the lack of incorporation of 2′-FdATP during elongation of primer-templates by pol α was not due to low levels of a potent inhibitor: (i) the same result was obtained with two different lots of 2′-FdATP, and (ii) 2′-FdATP was incorporated readily during primase-coupled pol α activity. This result may indicate that pol α interacts differently with dATP and its analogs than it does with the other dNTPs. Consistent with this idea is the finding that pol α greatly prefers to polymerize dATP opposite abasic template lesions, even though the template "base" provides no base-pairing information [32].

The fact that primer-coupled pol α incorporated 2′-FdATP, whereas pol α alone did not, provides another example of the fundamental differences between primase-coupled pol α activity and the use of exogenous primer-templates. Previous comparisons of these two activities showed that during coupled activity (i) pol α discriminates less efficiently against acyclic nucleotide analogs; (ii) araNTPs are not chain terminators; (iii) the cognate NTP is polymerized readily when a required dNTP is not present; and (iv) abasic lesions in the template strand are not strong chain terminators. Since the primary role of pol α during

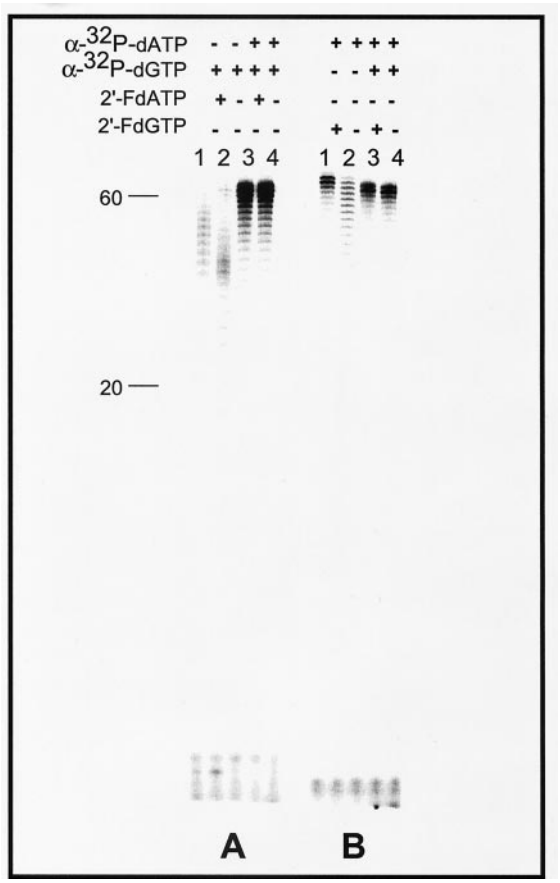

FIG. 5. Incorporation of 2′-FdATP (panel A) or 2′-FdGTP (panel B) during coupled reactions with pol α and α primase. The reaction conditions were as described in Materials and Methods. Panel A: (Lane 1) [α-^{32}P]dGTP and 2′-FdATP; (Lane 2) [α-^{32}P]dGTP; (Lane 3) [α-^{32}P]dGTP, [α-^{32}P]dATP, and 2′-FdATP; and (Lane 4) [α-^{32}P]dGTP and [α-^{32}P]dATP. Panel B: (Lane 1) [α-^{32}P]dATP and 2′-FdGTP; (Lane 2) [α-^{32}P]dATP; (Lane 3) [α-^{32}P]dATP, [α-^{32}P]dGTP, and 2′-FdGTP; and (Lane 4) [α-^{32}P]dATP and [α-^{32}P]dGTP.

DNA replication is thought to be elongation of primase-synthesized primers, it is clearly important to examine both primase-coupled pol α activity and elongation of exogenously added primer-templates to understand how nucleotide analogs may affect pol α activity. The fact that primase-coupled pol α incorporated 2′-FdGTP to a greater extent than 2′-FdATP and that no incorporation of 2′-O-MedNTPs was observed indicates that, in these studies, overall results using primase-coupled pol α versus pol α alone were not widely divergent.

The ability of both DNA and RNA polymerases [33] to polymerize 2′-FdNTPs but not 2′-O-MedNTPs is likely related to the size of the substituent. For other polymerases, DNA pol I and reverse transcriptase, the ability to discriminate between NTPs and dNTPs arises from an unfavorable steric contact between the 2′-OH and the enzyme [34, 35]. Since an O-methyl group will be larger than an OH, and both pol α and pol γ discriminate against 2′-O-MedNTP polymerization more potently than they discriminate against NTP polymerization, it seems likely that these enzymes also discriminate against 2′-O-MedNTP polymerization via steric exclusion. Consistent with this hypothesis, replacement of the 2′-OH with a fluorine, a substituent much smaller than an OH, resulted in compounds that both pol α and pol γ readily polymerized.

These studies used human pol α, pol γ, and primase in an effort to assess the potential of 2′-modified nucleotides, used in aptamers, to be incorporated into nuclear and mitochondrial DNA. These studies represent a first approximation of incorporation and clearly do not account for absorption, biodistribution, uptake, and phosphorylation that must occur prior to enzymatic incorporation. Still, they did demonstrate that once these steps have been accomplished, 2′-FdNTPs but not 2′-O-MedNTPs could be incorporated into the DNA by these DNA polymerases. This incorporation did not result in immediate chain termination characteristic of chain terminators. There was also no evidence of 'masked' chain termination as described with difluoronucleotides [8, 9]. Whether the 2′-FdNTPs would lead to chain termination following successive incorporation, as suggested with FIAUTP [12], was not examined; however, recent studies in this laboratory would suggest that DNA incorporation levels would not be great enough to lead to significant numbers of successive 2′-FdNTPs. Those studies found approximately 200 pmol 2′-FdU/μmol dT in cellular DNA (and lower levels in cellular RNA) of tissues of rats and woodchucks administered 2′-FdU. These concentrations are approximately 20-fold lower than those observed with similar doses of FIAU [36]. 2′-FdU was also found in tissues from rats administered a 2′-FdUMP-containing aptamer. These findings support the notion that aptamers can be degraded, and the resulting 2′-fluoronucleosides can be transported, phosphorylated, and incorporated into cellular DNA.

Previous studies have suggested that DNA polymerase inhibition assays may not be quantitatively useful for evaluating toxicity [37]. These studies suggest that DNA polymerase assays still play a vital role in assessing the potential of a compound to be incorporated into DNA.

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