Purification and characterization of porcine liver DNA polymerase γ : utilization of dUTP and dTTP during *in vitro* DNA synthesis

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ABSTRACT

Porcine liver DNA polymerase γ has been demonstrated to preferentially incorporate dTMP over dUMP during *in vitro* DNA synthesis. When polymerase activity was measured in standard reactions containing saturating levels of either dTTP or dUTP, the polymerization rate was slightly faster in the reaction containing dTTP. However, under conditions where both dTTP and dUTP competed, at an equal molar concentration, approximately 3-times more thymine residues were incorporated than uracil residues into DNA. Similarly, preferential incorporation of dTMP was observed on several substrates including poly (dA)•oligo p(dT), poly (rA)•oligo p(dT) and poly (dA-dT). The discrimination against dUMP incorporation was even more apparent with reduced levels of dUTP. These observations were consistent with the finding that the K_m for DNA polymerase γ was about 3-fold lower for dTTP (0.4 μ M) than for dUTP (1.1 μ M). On the other hand, the V_{max} for these two reactions was very similar. Discrimination against dUMP incorporation was also observed during inhibition of polymerase γ by dideoxynucleoside triphosphates. Dideoxythymidine triphosphate preferentially inhibited dUMP incorporation compared to that of dTMP, whereas ddATP, ddCTP and ddGTP inhibited both reactions equally.

INTRODUCTION

Uracil residues can be introduced into DNA either by deamination of cytosine residues (1) or by incorporation of dUMP in place of dTMP during polymerization (2-4). Studies performed *in vivo* (5-8) or with cell-free systems (9-11) indicate that the frequency of stable dUMP incorporation depends largely upon the relative intracellular pool size of dUTP and dTTP. When the level of dUTP increases in relationship to that of dTTP, uracil residues accumulate in the genome of most organisms (5-11). Shlomai and Kornberg (3) have estimated that under normal physiological conditions *Escherichia coli* stably incorporate 1 uracil residue into DNA for every 300 thymine nucleotides polymerized. *In vitro* experiments have illustrated that *E. coli* DNA polymerases I, II, and III efficiently utilize dUTP in lieu of dTTP for DNA synthesis (3,11). In bacteria, uracil residues that enter DNA are rapidly eliminated by base-excision repair (3,5,12). This process is initiated by uracil-DNA glycosylase which cleaves the N-glycosylic bond between uracil and deoxyribose to produce an apyrimidinic site in DNA (13). Repair then proceeds *via* the sequential action of apurinic/apyrimidinic (AP) endonucleases, DNA polymerase and DNA ligase (14-16).

Estimates of the stable dUMP incorporation frequency in mammalian cells appear to be at least 10^3 -fold lower than those reported for prokaryotic systems (6,7). This may, in part, reflect the

absence of a mammalian dCTP deaminase which directly converts dCTP to dUTP (9) and/or the presence of an abundant dUTP nucleotidohydrolase which hydrolyses dUTP to dUMP and PPi (17). In human lymphoid cells the dUTP to dTTP ratio has been estimated to be less than $1/10^5$ (7). This low dUTP/dTTP ratio would be expected to promote infrequent incorporation of dUMP, even if mammalian DNA polymerases utilize these two deoxyribonucleoside triphosphates with equal efficiency. In contrast, the dUTP pool in HeLa cells has been estimated at 1% that of dTTP (18). Thus, alternative mechanisms may exist for excluding or eliminating dUMP incorporation into DNA of some systems. Mammalian cells have the ability to excise uracil residues from chromosomal (7,10,19) or viral DNA (9) by the uracil-excision repair pathway. Another possible mechanism for reducing dUMP incorporation might be discrimination against dUTP utilization by mammalian DNA polymerases.

Four major classes of DNA polymerases have been isolated in mammalian cells and designated DNA polymerase α , β , γ and δ . DNA polymerase α and β are nuclear enzymes which function predominantly in chromosomal replication and repair, respectively (20,21). These two polymerases have been reported to exhibit similar kinetic constants for dTTP and dUTP (4). These results suggest that dUMP incorporation into nuclear DNA may directly reflect the abundance of dTTP and dUTP during DNA synthesis. DNA polymerase δ which may also participate in nuclear DNA replication (22, 23) and/or repair (24) has not been characterized in a similar respect. In mammalian cells, DNA replication also occurs within mitochondria which contain DNA polymerase γ as the principal replication enzyme (20). The extent which dUMP is incorporated into the mitochondrial genome has not been established. Several lines of evidence suggest that nuclear and mitochondrial dNTP pools are regulated by distinctly different mechanisms (25-27). Thus, it cannot be assumed that the frequency of dUMP incorporation in mitochondria parallels that of the nucleus. Unfortunately, estimates of the relative mitochondrial dUTP and dTTP pool sizes have not been determined. However, the observation that a distinct uracil-DNA glycosylase exists in this organelle, (28-30) suggests that uracil residues appear in mitochondrial DNA. These residues might be introduced during mitochondrial DNA replication. This has prompted the following study that characterizes the ability of porcine liver DNA polymerase γ to incorporate dUMP into DNA *in vitro*.

MATERIALS AND METHODS

Reagents and Chemicals

Unlabeled deoxyribonucleoside triphosphates, calf thymus DNA, purine and pyrimidine bases were purchased from Sigma; dideoxyribonucleoside triphosphates, poly (dA) • p(dT)₁₂₋₁₈, poly (rA) • p(dT)₁₂₋₁₈, poly (dA-dT) and poly (dG-dC) were from P-L Biochemicals. DEAE-Sephadex A-50 and Sephadex G-150 were purchased from Pharmacia; phosphocellulose P-11 from Whatman and double-stranded DNA-cellulose from Sigma. Heparin-agarose was prepared according to the method of Davison et al. (31). [³H]dTTP and [³²P]dTTP were obtained from New England Nuclear and [³H]dUTP was from Amersham.

Enzyme Preparations

Novikoff hepatoma DNA polymerase β (fraction VI) was purified as described previously (32). *E. coli* uracil-DNA glycosylase (fraction V) was isolated according to the procedure of Lindahl et al. (13).

DNA Polymerase Assay

Standard DNA polymerase γ reaction mixtures (100 μ L) contained 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 7.5 mM $MgCl_2$, 200 mM NaCl, 15 μ M each dATP, dGTP dCTP and [3H]dTTP (345 cpm/pmol) or [^{32}P]dTTP (1,680 cpm/pmol), 10 μ g activated calf thymus DNA and 0.005–0.5 units of DNA polymerase. When dUMP incorporation was measured, 15 μ M [3H]dUTP (350 cpm/pmol) was substituted for dTTP in the standard reaction mixture. In both cases, incubation was for 30 minutes at 37°C. Reactions were stopped on ice by addition of 200 μ l of 1 mg/ml of bovine serum albumin in 0.1 M sodium pyrophosphate prior to precipitation with 1 ml of 10% (saturation) trichloroacetic acid. Precipitates were collected on Schleicher & Schuell #30 glass filters, washed with 15 ml of 1 N HCl in 0.1 M sodium pyrophosphate and dried with 95% ethanol. Acid-insoluble radioactivity was measured using single or double isotope-counting techniques in a Beckman LS-6800 liquid scintillation spectrometer with 0.4% 2,5-bis-2-(5-tertbutylbenzoxazolyl)-thiophene (BBOT) in toluene being the scintillator. One unit of polymerase activity catalyzes incorporation of one nmol of total dNMP into DNA in one hour at 37° C.

Exonuclease Assay

Exonuclease activity was measured in reaction mixtures (100 μ l) containing 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 0.5 mM EDTA, 7.5 mM $MgCl_2$, 150 mM NaCl, 10 nmol of T7 [3H]DNA (18,000 cpm/nmol) and 0.6 units of DNA polymerase γ . Incubation was for 60 minutes at 37° C. Then samples were chilled, adjusted to 50 μ g/ml of calf thymus DNA, and precipitated with the addition of 220 μ l of 170 mM perchloric acid. After 30 minutes on ice, the samples were centrifuged for 5 minutes in an Eppendorf model 5414 centrifuge, and a sample (150 μ l) of the supernatant was removed, added to 350 μ l of water and counted for radioactivity with 5 ml of Ready Protein + (Beckman) as scintillator.

Endonuclease Assay

Endonuclease activity was measured in reaction mixtures (50 μ l) containing identical components as described for the exonuclease assay except that 10 nmol of PM2 DNA (>90% form I) was substituted as the DNA substrate. Incubation was for one hour at 37° C and the conversion of form I to form II was monitored after electrophoresis in an 0.8% agarose gel with 40 mM Tris-acetate and 2 mM EDTA buffer, (pH 8.2).

Deoxyuridine Triphosphatase Assay

Reaction mixtures (50 μ l) included 20 mM potassium phosphate (pH 7.5), 2 mM dithiothreitol, 5 mM $MgCl_2$, 100 mg/ml bovine serum albumin, 10 μ M [3H]dUTP (1460 cpm/pmol) and 0.17 units of DNA polymerase γ . Samples were incubated at 37°C for 90 minutes before 10 μ l of the reaction mixture was removed, chilled on ice and adjusted to 67 mM EDTA. Reaction products

were spotted (20 μ l) on a polyethylenimine (PEI) thin layer chromatographic sheet along with 7.5 μ g each of dUTP, dUDP and dUMP as internal markers. Ascending chromatography was performed using 1 M LiCl as solvent. The chromatogram was removed after the solvent front migrated 15 cm, dried and examined under a Mineralight Model UVGLJ-25 (254 nm) to locate internal markers. Three spots corresponding to dUTP, dUDP and dUMP were cut from the sheet and counted for radioactivity using 5 ml of Ready Protein⁺.

Analysis of DNA Polymerase γ Reaction Products by Paper Chromatography.

Standard DNA polymerase γ reactions (300 μ l) but containing [³H]dUTP and/or [³²P]dTTP were incubated for 1 hour at 37° C. The reactions were placed on ice, then 60 μ l of 0.1 mM EDTA was added and the samples were diluted ten-fold with buffer A containing 100 mM Tris-HCl (pH 7.5) and 1 mM EDTA. DNA products were then purified by chromatography on a NENSORB (DuPont) column which was activated with methanol, equilibrated with buffer A, sample applied and eluted with 1 ml of 50% (v/v) methanol. Three fractions (325 μ l) were collected and evaporated to dryness. The DNA was resuspended with 100 μ l of buffer containing 10 mM Tris-HCl (pH 7.5) and 1 mM EDTA and then digested with *E. coli* uracil-DNA glycosylase. The glycosylase reactions (150 μ l) contained 70 mM Hepes-KOH (pH 8.0), 1 mM EDTA, 1 mM dithiothreitol, 30 μ g DNA and 260 units of *E. coli* uracil-DNA glycosylase. Incubation was for 1 hour at 37° C and then the product (120 μ l) was spotted onto Whatman No. 1 (12.5 x 45 cm) chromatographic paper along with 50 nmol each of the five bases (T, U, A, G, C). Descending chromatography was performed for 21 hours at 25° C using isopropanol, H₂O and HCl (65:18.3:16.7) as the solvent. Internal standards were located using UV light. The chromatograms were dried, sliced at 1.3-cm intervals, placed in 50 μ l H₂O for 1 hour and then counted for radioactivity as described above using Ready Protein⁺.

Gel Filtration Chromatography.

DNA polymerase γ (fraction IV, 5.8 units) was chromatographed on a Sephadex G-150 column (5.3 cm² x 63 cm) equilibrated in TMEG buffer [50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, and 10% (w/v) glycerol] containing 100 mM NaCl. Fractions (3 ml) were collected at a flow rate of 12.5 min/hr. The column was calibrated using ovalbumin (Mr 45,000), bovine serum albumin (Mr 66,000), alkaline phosphatase (Mr 80,000), *E. coli* DNA polymerase I (Mr 109,000) bovine serum albumin dimer (Mr 135,000) and the void volume (121 ml) was determined using blue dextran 2000.

RESULTS

Purification of Porcine Liver DNA Polymerase γ .

All operations were performed at 0-4° C unless otherwise indicated. Typically, 1200 gm (wet weight) of fresh pig liver, temporarily stored in 0.25 M sucrose, was used to isolate mitochondria, according to the procedure of Meyer and Simpson (33) except that the isolation buffer contained 0.25 M sucrose, 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 5 mM

MgCl₂ and 25 mM KCl. Each mitochondria preparation was frozen in liquid nitrogen and stored at -75° C until needed.

Preparation of Mitochondrial Extract.

Isolated mitochondria (125-170 gm) were disrupted by grinding with levigated alumina as described by Domena and Mosbaugh (29). The mitochondrial paste was extracted with 200 ml of buffer [50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 50 mM MgCl₂ and 1 M NaCl] and the alumina was removed by centrifugation at 500 xg for 10 minutes. The supernatant solution was then centrifuged at 125,000 xg for 1 hour in a Beckman 50 Ti rotor and the supernatant fraction was dialysed against TMEG buffer containing 50 mM NaCl. The dialysed extract constituted fraction I.

DEAE-Sephadex Chromatography.

Fraction I was loaded onto a DEAE-Sephadex A-50 column (4.9 cm² x 65 cm) equilibrated with TMEG buffer containing 50 mM NaCl. The column was washed with 200 ml of equilibration buffer and then a linear gradient (1000 ml) from 0 to 500 mM NaCl was applied at a flow rate of 25 ml/hr using the same buffer system. The DNA polymerase activity eluted as a single peak at approximately 120 mM NaCl. All active fractions were pooled which contained >25% the peak activity and were designated fraction II.

Phosphocellulose Chromatography.

Fraction II was dialysed extensively against 50 mM Tris-HCl (pH 7.5), 5 mM 2-mercaptoethanol, 1 mM EDTA, 10% (w/v) glycerol, 150 mM NaCl and then loaded onto a phosphocellulose column (4.9 cm² x 30 cm) equilibrated in the same buffer. The column was washed with 200 ml of equilibration buffer and the enzyme was eluted with a 500 ml linear gradient from 0 to 1000 mM NaCl also in the same buffer. The flow rate was about 20 ml/hr and DNA polymerase activity eluted as a symmetrical peak at about 420 mM NaCl. The vast majority of the protein (>95%) did not bind to the resin under these conditions and eluted in the wash. Each fraction containing >20% the peak activity was pooled as fraction III.

Heparin Agarose Chromatography.

After dialyzing fraction III against TMEG buffer containing 100 mM NaCl, the sample was loaded onto a heparin agarose column (1.8 cm² x 8.5 cm) equilibrated in this buffer. The column was washed with 25 ml of equilibration buffer and a 150 ml linear gradient was applied from 0 to 800 mM NaCl also including equilibration buffer. Two major protein peaks eluted from the column, one (96% recovered protein) at about 275 mM NaCl and the other (4% recovered protein) at about 500 mM NaCl. The latter peak contained the polymerase activity which was pooled to contain fractions with >35% the maximum activity and designated fraction IV.

The mitochondrial DNA polymerase (fraction IV) was purified 9500-fold with a yield of about 60% relative to the crude mitochondrial extract. A typical purification is summarized in Table I. Additional purification was attempted on hydroxylapatite and double-stranded DNA-

Table I. Purification of Porcine Liver DNA Polymerase γ^a

Fraction	Total Protein (mg) ^b	Total Activity		dTMP/dUMP Incorp. (ratio)	Specific Activity (units/mg) ^c	Purification (xfold)
		dTMP Incorp. (units)	dUMP Incorp. (units)			
I. Mitochondrial Extract ^d	4460	1050	641	1.6	0.24	1.0
II. DEAE-Sephadex	1670	1340	1090	1.2	0.80	3.3
III. Phosphocellulose	12.8	756	582	1.3	59.1	246
IV. Heparin-Agarose	0.28	637	420	1.5	2280	9500

^aStandard DNA polymerase γ assays were performed as described under Materials and Methods with [³H]dTTP and [³H]dUTP being introduced into separate reactions.

^bProtein concentrations were determined by the method of Bradford (34) with bovine serum albumin as the standard.

^cSpecific activity was calculated based on the incorporation observed in the reactions containing [³H]dTTP.

^dFraction I was prepared from 168 gm of isolated mitochondria.

cellulose columns; however, the recovered polymerase was found to be very unstable. In contrast, fraction IV enzyme was stable for 6-10 weeks at 4° C. Fraction IV DNA polymerase γ was

examined for dUTPase and endonuclease but lacked both activities. The limit of detection of dUTPase and endonuclease activity was less than 0.05% and 0.07% of the polymerase activity, respectively. However, a low but detectable level of exonuclease was found which constituted about 4% the rate of the polymerase activity. The turnover rate of dTMP versus dUMP due to this minor activity was measured during DNA polymerase reactions and was found to be very similar (data not shown).

Incorporation of dUMP by DNA Polymerase γ .

During the purification of porcine liver DNA polymerase γ the incorporation rate of [³H]dTTP and [³H]dUTP into activated DNA was measured. At each step, polymerase γ efficiently utilized either dTTP or dUTP as a precursor for DNA synthesis (Table I). In these reactions where dTTP or dUTP was added separately along with the other three complementary deoxyribonucleoside triphosphates, a slight preference (1.2 to 1.6-fold) for utilizing dTTP was consistently observed. To determine if this result accurately reflected differential incorporation of thymine versus uracil residue into DNA, a second, more direct approach was taken which analysed the product of DNA synthesis. Three identical DNA polymerase reactions were prepared, as above, except that either [³²P]dTTP, [³H]dUTP or [³²P]dTTP and [³H]dUTP were added as radioactive precursors. After DNA synthesis, the products were isolated and treated with an excess of *E. coli* uracil-DNA glycosylase. This treatment was expected to release free uracil from DNA. Once separated from the polymer by paper chromatography, uracil residues that had been incorporated into DNA could be specifically identified and quantitated. As expected, the

Figure 1. Analysis of [uracil- ^3H , ^{32}P] DNA Synthesized *In Vitro* by DNA Polymerase γ . Three standard DNA polymerase γ reactions (300 μl) containing [^{32}P]dTTP (A), [^3H]dUTP (B) or [^{32}P]dTTP plus [^3H]dUTP (C) were prepared as described under Materials and Methods. Following DNA synthesis, the DNA products were purified by chromatography on a NENSORB column to remove deoxyribonucleoside triphosphates. Purified DNA (30 μg) was then treated with 260 units of *E. coli* uracil-DNA glycosylase and free uracil separated from DNA by descending paper chromatography using an isopropanol: H_2O :HCl (65:18.3:16.7) solvent system. Internal standards (guanine, adenine, cytosine, uracil and thymine) were spotted along with the samples and located (arrows) using a UV light following chromatography. The products [^3H]uracil (●) and [^{32}P]DNA (O) were identified by double isotope detection.

Table II. Incorporation of dTMP versus dUMP During *In Vitro* DNA Synthesis by DNA Polymerase γ .^a

dTTP (μ M)	dUTP (μ M)	Rate of Incorporation into DNA		
		[³² P]dTMP (pmol/hr)	[³ H]dUMP (pmol/hr)	dTMP + dUMP (pmol/hr)
15	—	29.8	—	29.8
—	15	—	22.1	22.1
7.5	7.5	22.4	7.7	30.1
15	15	21.8	5.8	27.6
15	0.5	30.2	<0.3	30.5
0.5	15	4.0	22.6	26.6

^aStandard DNA polymerase γ reactions conditions were as described under Materials and Methods. Six (1 ml) reaction mixtures were prepared containing 1 unit of DNA polymerase γ with [³²P]dTTP and/or [³H]dUTP added as indicated above. The rate of incorporation was measured from duplicate samples taken at 0, 15, 30 and 45 minutes following incubation at 37° C.

DNA products isolated from the reaction containing [³²P]dTTP were unaffected by uracil-DNA glycosylase; all the ³²P-label remained at the origin (Figure 1A). In this reaction 139 pmol of [³²P]dTMP was incorporated into DNA. The same result was also obtained when [³H]dTTP was

Table III. Incorporation of dTMP and dUMP by DNA Polymerase γ on Various DNA Substrates.^a

Primer/Template	Rate of Incorporation into DNA		
	[³² P]dTMP (pmol/hr)	[³ H]dUMP (pmol/hr)	dTMP/dUMP (ratio)
Activated DNA	20.2	5.6	3.6
Poly (dA) • p(dT) ₁₂₋₁₈	335	131	2.6
Poly(rA) • p(dT) ₁₂₋₁₈	195	25.9	7.5
Poly (dA-dT)	8.2	2.5	3.3
Poly (dG-dC)	<0.3	<0.3	—

^aStandard DNA polymerase γ reactions were prepared as described in Table II except that 15 μ M [³²P]dTTP and 15 μ M [³H]dUTP was included in each reaction and 100 μ M of synthetic polymer added as indicated. The ratio of oligo p(dT)₁₂₋₁₈ to poly(dA) or poly(rA) was 1:10 and reactions were carried out at 30° C.

substituted in the DNA synthesis reaction as the label (data not shown). In contrast, greater than 95% of the ^3H -label incorporated into DNA as [^3H]dUMP was released as [^3H]uracil ($R_f=0.7$) when treated with uracil-DNA glycosylase (Figure 1B). Under this condition, only 96.3 pmol of [^3H]uracil was recovered as being incorporated into DNA. A sample which omitted uracil-DNA glycosylase showed no [^3H]uracil release from DNA (data not shown). These results establish that the reduced preference of polymerase γ for dUTP over dTTP truly reflects a decreased efficiency (1.4-fold) for incorporating uracil residues into DNA. Surprisingly, when [^{32}P]dTTP and [^3H]dUTP were added to the same polymerase reaction the preferential utilization of dTTP over dUTP was even more profound (Figure 1C). DNA polymerase γ incorporated 102 pmol of [^{32}P]dTTP whereas only 39 pmol of [^3H]dUMP was introduced into DNA. As before, virtually all the [^3H]dUMP in DNA was susceptible to hydrolysis by uracil-DNA glycosylase. Together, the total amount of dTMP plus dUMP incorporated (141 pmol) approximately equaled that of the reaction containing [^{32}P]dTTP alone. Thus, dTMP and dUMP were not equally incorporated into DNA, though about the same extent of DNA synthesis occurred as above. Under this condition where the two precursors competed, DNA polymerase γ selectively incorporated about 2.6 thymines per uracil residue into DNA.

Competition Between dTMP and dUMP Incorporation on Various DNA Substrates

Taken together, the experiments above indicate that the extent of [^{32}P]dTTP and [^3H]dUMP incorporation differed when both precursors competed for DNA synthesis. When both were incorporated separately into DNA, dTMP incorporation was still favored but to a lesser degree. This difference reflected a variance in the rate of incorporation of dTMP versus dUMP (Table II). The relative incorporation rate of dTMP (29.8 pmol/hr) was significantly greater than dUMP (22.1 pmol/hr) when introduced individually into separate reactions. When dTTP and dUTP competed, at an equal molar ratio, the dUMP incorporation rate was less than 25% that observed for dTMP. In reactions containing 7.5 μM or 15 μM of both dTTP and dUTP, it was calculated that polymerase γ incorporated 1 uracil residue for each 2.9 and 3.8 thymines introduced into DNA, respectively. This discrimination against dUMP incorporation was even more apparent in reactions with substantial pool bias. When dTTP and dUTP were mixed to give 15 μM and 0.5 μM respectively, less than 1 uracil residue was incorporated into DNA per 100 thymine residues polymerized. Conversely, when the reaction contained 0.5 μM dTTP and 15 μM dUTP, both precursors were introduced into DNA but with a frequency of 1 thymine for each 5.7 uracil residue. One interpretation of these results would be that DNA polymerase γ preferentially recognizes dTTP over dUTP during DNA synthesis. If this represented differential base selection by the polymerase, similar selectivity might be expected using other DNA substrates. When several synthetic polymers were tested, those containing adenosine residues in the template strand supported dTMP incorporation between 2.6-7.5 times more efficiently than that of dUMP (Table III).

Experiments were performed to determine the kinetic constants for dTMP and dUMP

Figure 2. Kinetic Parameters of dTMP and dUMP Incorporation by Novikoff hepatoma DNA Polymerase β and Porcine Liver DNA Polymerase γ . A Lineweaver-Burk analysis was performed and the K_m and V_{max} determined for incorporation of dTMP and dUMP using DNA polymerase β (A) or polymerase γ (B). DNA polymerase β reactions (100 μ l) contained 50 mM Tris-HCl (pH 8.0), 5 mM 2-mercaptoethanol, 1 mM EDTA, 7.5 mM MgCl_2 , 50 mM NaCl, 15 μ M each dATP, dGTP and dCTP, 10 μ g calf thymus activated DNA and 0.09 units of Novikoff hepatoma DNA polymerase β . Each reaction also contained either $[^3\text{H}]\text{dTTP}$ (●) or $[^3\text{H}]\text{dUTP}$ (○). Similarly, DNA polymerase γ activity was measured using the reaction conditions described under Materials and Methods.

Figure 3. Effect of Dideoxyribonucleoside Triphosphates on *In Vitro* Incorporation of dTMP and dUTP by DNA Polymerase γ . Standard DNA polymerase γ reactions were prepared as described under Materials and Methods except that either [^3H]dTMP (○) or [^3H]dUTP (●) was included. In some cases ddTTP (A), ddCTP (B), ddATP (C) and ddGTP (D) was added as indicated to reactions that contained 0.025 units of DNA polymerase γ . Incubation was for 30 minutes at 37° C and the acid-precipitable radioactivity was compared to a control lacking dideoxyribonucleoside triphosphates.

incorporation. For a comparison, Novikoff hepatoma DNA polymerase β was examined. This enzyme did not significantly discriminate between incorporation of dTMP and dUMP (Figure 2A). In agreement with previous studies (4), DNA polymerase β had a V_{max} (20 pmol dTMP/hr compared to 25 pmol dUMP/hr) and K_m (1.9 μM dTTP versus 2.0 μM dUTP) which were similar for both reactions. In contrast, DNA polymerase γ exhibited a significantly lower K_m for dTTP (0.4 μM) than for dUTP (1.1 μM); though the V_{max} values (23 pmol dTMP/hr and 19.5 pmol dUMP/hr) were similar (Figure 2B). Hence, these results offer an explanation for the observations above. When supplied separately at saturating concentrations, DNA polymerase γ

Figure 4. Effect of NaCl and N-ethylmaleimide on the Incorporation of dTMP and dUTP by DNA Polymerase γ . Standard DNA polymerase γ reactions were prepared as described under Materials and Methods except that they contained either $[^3\text{H}]\text{dTMP}$ (O) or $[^3\text{H}]\text{dUTP}$ (●). (A) Sodium chloride was added to each reaction as indicated and incubation was for 30 minutes at 37° C. (B) Similar reactions were prepared except that 2-mercaptoethanol was omitted from the mixture and N-ethylmaleimide was added at the concentrations shown. Each reaction contained 0.08 units of porcine DNA polymerase γ .

discriminates only slightly between dTTP and dUTP since the V_{max} values are only slightly different. However, during competition reactions the two precursors are selected differentially based on a difference in K_m that favors dTMP incorporation.

To further test whether discrimination occurred at the level of base selection, the effect of dideoxynucleoside triphosphates on inhibiting dTMP and dUMP incorporation was studied. When DNA polymerase γ was exposed to ddTTP, inhibition of dUMP incorporation was more apparent than that of dTMP (Figure 3A). However, similar preferential inhibition was not observed when ddCTP, ddATP or ddGTP were added to the polymerase reactions (Figure 3B-D). Thus, it appeared that ddTTP was incorporated more readily in reactions containing dUTP than those containing dTTP. Consequently, chain termination occurred more frequently when ddTTP competed with dUTP than dTTP.

Characterization of dUMP Incorporation by DNA Polymerase γ .

In an effort to define the properties that influence dUMP incorporation and to illustrate that DNA polymerase γ was directly responsible for this activity, the polymerization reaction was characterized. Several lines of evidence indicate that DNA polymerase γ and not another enzyme incorporated both dTMP and dUMP in the above reactions. (I) The ratio of incorporation of dTMP and dUMP was relatively constant throughout the polymerase purification (Table I). (II) Both activities resided in a polypeptide with a native molecular weight of $M_r=140,000$, as determined by gel filtration chromatography. Furthermore, the incorporation ratio using dTTP and dUTP was relatively constant across the activity peak of this and other columns during purification (data not shown). (III) The incorporation of both dTMP and dUMP were similarly affected by NaCl, showing an optimum of about 250 mM (Figure 4A). (IV) Both activities were equally sensitive to inhibition by the sulfhydryl blocking agent, N-ethylmaleimide (Figure 4B). Approximately 90% inhibition occurred at 1 mM N-ethylmaleimide for both activities.

DISCUSSION

Porcine liver DNA polymerase γ was purified 9,500-fold from isolated mitochondria and characterized with respect to its catalytic properties. Like other γ -polymerases, it is inhibited by both N-ethylmaleimide and ddTTP, is stimulated (up to 250 mM) by NaCl, utilizes poly (rA) • oligo p(dT) as substrate, exhibits a low (0.4 μM) K_m for dTTP and has a high native molecular weight ($M_r=140,000$). These properties clearly distinguish it from DNA polymerase α , β and δ (20).

Unlike other prokaryotic and eukaryotic DNA polymerases (3, 4, 11), polymerase γ preferentially incorporated dTMP over dUMP during *in vitro* DNA synthesis on several primer-templates. This discrimination against dUTP was not explained by contaminating dUTPase or exonuclease activity, but was attributed to a ~3-fold lower K_m value for dTTP than for dUTP. In contrast to DNA polymerase α and β (4) which incorporate both dTMP and dUMP with about equal efficiency, DNA polymerase γ did not utilize dUTP in equal proportion to the ratio of dTTP to

dUTP. Since the concentration of both mitochondrial dTTP and dUTP pools has not been established, the biological significance of this phenomenon remains unclear. However, it is curious that polymerase γ has evolved a K_m value for the other four deoxyribonucleoside triphosphates which is 5 to 50-fold lower than either DNA polymerase α or β (20, 35) but retained a higher K_m value for dUTP. This suggests that some advantage may be gained by reducing or excluding the incorporation of dUMP into the mitochondrial genome.

Mammalian mitochondria do not appear to possess a proficient nucleotide excision-repair system (36). Consequently, it has been proposed that mitochondria avoid mutagenesis by selectively removing DNA damage by destroying the genome (37, 38). If this were the case, it might be disadvantageous to introduce uracil residues at high frequency into mitochondrial DNA during DNA synthesis. Unlike uracil-DNA formed by deamination of cytosine, incorporated uracil residues, which are not particularly mutagenic, might trigger genomic destruction. This type of selective degradation mechanism would require a protein to identify the DNA adduct (uracil) and initiate DNA destruction. Mitochondria contain both a uracil-DNA glycosylase (28-30) and AP endonucleases (39) which could act sequentially to remove uracil residues and initiate DNA degradation. Alternatively, these two enzymes could act to initiate base-excision repair without destroying the genome. Clearly, additional experiments are required to elucidate the mechanisms by which mitochondria process DNA damage.

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