Comparison of Polymerase Insertion and Extension Kinetics of a Series of O^2-Alkyldeoxythymidine Triphosphates and O^4-Methyldeoxythymidine Triphosphate†

B. Singer,*,‡ F. Chavez,‡ S. J. Spengler,‡ J. T. Kuśmierek,§ L. Mendelman,∥ and M. F. Goodman∥

Donner Laboratory, Lawrence Berkeley Laboratory, University of California, Berkeley, California 94720, Institute of Biochemistry and Biophysics, Polish Academy of Sciences, Warsaw 02089, Poland, and Department of Biological Sciences, Molecular Biology Section, University of Southern California, Los Angeles, California 90089-1340

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ABSTRACT: The effect of alkyl group size on ability to act as deoxythymidine triphosphate (dTTP) has been studied for the carcinogen products O^2-methyl-, O^2-ethyl-, and O^2-isopropyl-dTTP by using three types of nucleic acids as template and DNA polymerase I (Pol I) or Klenow fragment as the polymerizing enzymes. Apparent K_m and relative V_{max} values were determined in primer extension on M13 DNA at a single defined site, in poly[d(A-T)], and in nicked DNA. These data are the basis for calculation of the relative rate of insertion opposite A, relative to dTTP. The insertion rate for any O^2-alkyl-dTTP is much higher than for a mismatch between unmodified dNTPs. Unexpectedly, O^2-isopropyl-dTTP is more efficiently utilized than O^2-methyl-dTTP or O^2-ethyl-dTTP on each of the templates. O^2-Isopropyl-dTTP also substitutes for dTTP over extended times of DNA synthesis at a rate only slightly lower than that of dTTP. Parallel experiments using O^4-methyl-dTTP under the same conditions show that it is incorporated opposite A more frequently than is O^2-methyl-dTTP. Therefore, both the ring position and the size of the alkyl group influence polymerase recognition. Once formed, all O^2-alkyl-T·A termini permit elongation, as does O^4-methyl-T·A. In contrast to the relative difficulty of incorporating the O-alkyl-dTTPs, formation of the following normal base pair (C·G) occurs rapidly when dGTP is present. This indicates that a single O-alkyl-T·A pair does not confer significant structural distortion recognized by Pol I.

O^2-Alkylpyrimidines are formed in DNA treated in vivo with carcinogenic *N*-nitroso alkylating agents (Singer et al., 1978b, 1981; Steward et al., 1979). The biological significance of deoxythymidine alkylation is not yet clear although in vitro experiments indicate that the analogous ribo derivatives can act as either U or C (Singer et al., 1978a, 1979; Singer, 1982), similar to the changed coding found for O^4-alkyl-T or -U (Singer et al., 1978a, 1983, 1984, 1986b; Preston et al., 1987). O^2-Methyl-T and O^4-methyl-T in poly[d(A-T)] have been shown on replication with Pol I to lead to AT → GC transitions (Singer et al., 1983). The ability of Pol I to stably insert these derivatives was shown by HPLC analysis (Singer et al., 1983). Mutagenesis studies have not yet been performed with the larger analogues. The amount of O^2-ethyl-T formed in alkylated DNA is 50–100 times that of O^2-methyl-T (Singer & Grunberger, 1983; den Engelse et al., 1986) and is similar to the amount of O^6-ethyl-G or O^6-methyl-G, a well-known promutagen (Loechler et al., 1984; Chambers et al., 1985; Bhanot & Ray, 1986). However, repair of O^6-methyl- or O^6-ethyl-G in animal tissues, particularly liver, is extremely rapid compared to removal of O^2-methyl- or O^2-ethyl-T (Steward et al., 1979; Pegg, 1983; Yarosh, 1985; Brent et al., 1988). The persistence of O^2-alkyl-T may be biologically significant, since in *Salmonella typhimurium*, ethylation of T in DNA leads to AT → GC transitions. No differentiation has yet been made between the mutagenic contribution of O^2- compared to O^4-ethyl-T (Hu & Guttenplan, 1985; Richardson et al., 1987; Burns et al., 1988).

Previous kinetic studies of the ability of a series of O^4-alkyl-dTTPs[1] (methyl, ethyl, and isopropyl substituents) to substitute for dTTP in replication indicated that they could all be incorporated by polymerases. The relative incorporation decreased with increasing size of the alkyl group (Singer et al., 1986a). In in vitro site-directed mutagenesis of *am 3* ϕX174, the O^4-alkylated dTTPs and O^2-methyl-dTTP were added by polymerases to a 3′-primer terminus opposite template A in the amber codon (Preston et al., 1986). Evidence that newly inserted O-alkyl-T·A can serve as a primer for subsequent nucleotide extension is based on the infectivity of replicated phage DNA. Increased reversion of the ϕX174 amber codon, A → G transition, resulting from replacement of T by O^4-methyl- and O^4-ethyl-T derivatives was a measure of mutagenic potential (Preston et al., 1987). The apparent failure of O^2-methyl-T to increase reversion of the amber codon above background was attributed to repair of this derivative (Ahmmed & Laval, 1984; McCarthy et al., 1984) in the particular *Escherichia coli* strain used for phage replication. Experiments relating to this question are in progress.

In contrast to O^4-alkyl-dTTP kinetics of insertion opposite A, we find that the ability of a series of O^2-alkyl-dTTPs (methyl, ethyl, isopropyl) to substitute for dTTP in different DNA synthesis systems is not correlated with size; O^2-iso-

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‡ University of California.
§ Polish Academy of Sciences.
∥ University of Southern California.

[1] Deoxynucleoside triphosphates are abbreviated as dNTP or, when substituted, as, e.g., O^2-methyl-dTTP. The superscript identifies the position of substitution. When the nucleoside is in a polymer, a single-letter abbreviation is used.

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propyl-dTTP is incorporated opposite A with significantly higher efficiency than either O^2-methyl- or O^2-ethyl-dTTP. The efficiency of extending primers terminated by O^2-alkylated derivatives and O^4-methyl-T, compared to primers terminated by T, was also studied.

Materials and Methods

Poly[d(A-T)], bacterial alkaline phosphatase, dNTPs, and the Klenow fragment of Pol I were purchased from Pharmacia. ^{3}H- and ^{32}P-labeled dNTPs were from Amersham. Pol I was a generous gift from Dr. L. A. Loeb. The primer for the defined site incorporation and extension assay (5′-AA-ACGGGTAAAATACGT-3′) complements bases 1269–1285 in wild-type M13 DNA (van Wexenbeek et al., 1980). O^4-Methyldeoxythymidine triphosphate was prepared according to Singer et al. (1986a).

Phosphorylation of O^2-Alkylthymidines and Purification of the 5′-Triphosphates. Characterized O^2-methyl-, O^2-ethyl-, O^2-isopropyldeoxythymidines, prepared as described (Kuśmierek & Singer, 1976; Singer et al., 1983), were phosphorylated to 5′-phosphates by using crude wheat shoot extract as a source of phosphotransferase (Giziewicz & Shugar, 1975). The products from a typical reaction mixture after phosphorylation of 0.05–0.1 mmol of O^2-alkylthymidine were adsorbed to a 1.8 × 18 cm column packed with Dowex 1x4 200/400 (HCO_3^- form) and eluted with a linear gradient from 600 mL of H_2O to 600 mL of 0.6 M triethylammonium bicarbonate (TEAB) at a flow rate of 3.5 mL/min. The peak of 5′-nucleotide (yield 40–60%) appeared at 0.3 M TEAB and, in the case of each O^2-alkyl derivative, was contaminated with inorganic phosphate. O^2-Alkylthymidine 5′-phosphates were rechromatographed in a 1.25 × 15 cm column packed with XAD-4 resin (Serva), prewashed with 0.05 M TEAB. Inorganic phosphate was eluted with 0.05 M TEAB (100 mL). Nucleotides were subsequently eluted using 0.7 M TEAB containing 30% of isopropyl alcohol.

The synthesis of O^2-alkylthymidine 5′-triphosphates followed the procedure of Hoard and Ott (1965). The products from a typical reaction mixture after reaction of 0.02–0.1 mmol of O^2-alkylthymidine 5′-phosphate were separated on a 2 × 20 cm column packed with DEAE Sephadex A-25 (HCO_3^- form) with a linear gradient from 600 mL of H_2O to 600 mL of 0.6 M TEAB at 3.5 mL/min. The peak of 5′-monophosphate was at 0.20 M, 5′-diphosphate at 0.32 M, 5′-triphosphate at 0.42 M, inorganic phosphate at 0.25 M, and inorganic pyrophosphate at 0.40 M TEAB. The separation of O^2-alkylthymidine 5′-triphosphates (yield 30-40%) from inorganic pyrophosphate used XAD-4 resins and identical conditions to those for separation of O^2-alkylthymidine 5′-phosphates from inorganic phosphate.

Immediately before use, O^2-alkyl-dTTPs were again reisolated to maximize purity. Separation was by thin-layer chromatography (TLC) on cellulose plates developed in 1-propanol/NH_4OH/H_2O (55/10/35). In this system, the modified triphosphates have R_f's 2–3-fold greater than that of dTTP. Further verification of the base moiety was obtained by using bacterial alkaline phosphatase. HPLC analysis was performed (Singer et al., 1983) which resolved the O^2-alkylthymidines from the position of thymidine, which was not detectable to 1 part in 200 (>99.5% pure).

Primer Synthesis and Purification. The primer (Figure 1) was synthesized on an Applied Biosystems (ABI) 381A DNA synthesizer. The tritylated primer was deprotected by exposure to thiophenol and ammonium hydroxide, as recommended by ABI. It was lyophilized and resuspended in 995 μL of 0.01 M TEAB, pH 7.0, and 5 μL of triethylamine.

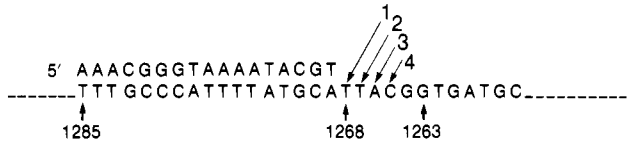

FIGURE 1: System used to measure kinetics of incorporation of modified dTTPs opposite A in the template. The synthetic primer strand (17 nucleotides) is labeled at the 5′-end with ^{32}P and annealed to complementary bases 1285–1267 of the circular M13 DNA template. A saturating level of dATP is added to allow primer extension from site 0 to site 2 by insertion of A opposite T. Details of the experimental system are under Materials and Methods.

HPLC was performed on a Vydac C4 semipreparative column (10 mm × 23 cm) using a linear gradient of 0.1 M triethylammonium acetate (TEAA), pH 7/acetonitrile (85/15), to 0.1 M TEAA/acetonitrile (75/25) over 20 min at a flow rate of 3 mL/min. The entire 1-mL sample was injected. The major peak was completely eluted in 25 min and lyophilized. The trityl groups were removed by exposure of lyophilized primer to 80% acetic acid, as recommended by ABI. Detritylated primer was resuspended in 1 mL of H_2O.

For purification on gels, five A_{260} units were lyophilized and resuspended in 50% glycerol. The sample was loaded onto a 20% acrylamide gel (0.5 × 20 × 40 cm) and electrophoresed at 20 V/cm until the marker dye, bromphenol blue, migrated off the bottom of the gel. The gel was wrapped in Saran Wrap and placed on a TLC plate with a fluorescence indicator. The primer was visualized with short-wave UV light, cut out, placed in a dialysis membrane with electrophoresis buffer (MW cutoff = 3500, and electroeluted in a minigel apparatus according to Maniatis et al. (1982) for 3 h at 200 V. The DNA was detached from the dialysis membrane by reversing the current for 2 min. The DNA was dialyzed against water, transferred to a sterile Eppendorf tube, and kinased to check for purity (Boosalis et al., 1987).

Incorporation and Extension Using DNA Polymerase I or the Klenow Fragment. Three template-primers were used: poly[d(A-T)], nicked salmon sperm DNA, and primed single-stranded (ss) M13 DNA.

For the determination of K_m^{app} and V_{max}^{app} using poly[d(A-T)], the reaction mixture (100 μL) contained 200 mM tris(hydroxymethyl)aminomethane hydrochloride (Tris-HCl), pH 7.8, 2 mM $MgCl_2$, 100 μM [^{32}P]dATP (0.15 μCi/nmol), varying levels (1–30 μM) of dTTP or modified dTTPs, 0.15 A_{260} unit of polymer, and 2 units of Pol I (L. A. Loeb). When the Klenow fragment was used, 0.4 unit (Pharmacia) were added. These units represent the equivalent molarity (0.6 nM) of the two enzymes, which differ in specific activity and molecular weight. Reaction mixtures were incubated at 37 °C for 30 and 60 s, and aliquots were removed, spotted on Whatman DEAE disks, washed, and counted as previously described (Singer et al., 1983).

Controls were carried out in an identical manner under conditions of DNA saturation. One set lacked enzyme and the other set contained enzyme but lacked dTTP or analogues. The incorporated radioactivity in this latter control, which allowed extension of all primer-templates to the first A residue, was subtracted from the measured incorporation prior to calculation of K_m^{app}, V_{max}, or extent of incorporation. The K_m^{app} for the low dATP insertion opposite template A was measured and is >1 mM. The control lacking enzyme was significantly lower since only nonspecific retention of radioactivity could occur. Calculation of data was according to Singer et al. (1986a).

For nicked DNA, the assay mixture also contained 100 μM dCTP and dGTP. In addition, dTTP or modified dTTP was

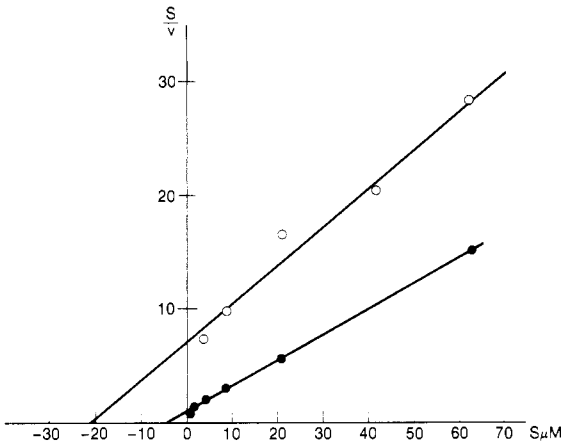

FIGURE 2: Hanes–Woolf plot for K_m^{app} of O^2-methyl-dTTP (○) and O^2-isopropyl-dTTP (●) using the same substrate concentrations and poly[d(A-T)] template described under Materials and Methods. Table I lists the K_m^{app} and V_{max} of these and O^2-ethyl-dTTP and dTTP.

present at the same molarity, and 0.1 A_{260} unit of DNA was used with 2 units of Pol I. The ^{32}P-end-labeled primer annealed to ssM13 DNA (Figure 1) was used in a gel assay, following the procedure of Boosalis et al. (1987). Extension was from position 1269 (Figure 1). Reaction mixtures (6 μL) contained 50 mM Tris-HCl, pH 7.5, 8 mM $MgCl_2$, 1 mM β-mercaptoethanol, 10 μM dATP, varying amounts of dTTP or modified dTTPs (1–500 μM), 37 nM primer-template, and 0.1 unit of Klenow fragment (0.15 nM). Reaction mixtures were incubated at 37 °C for 90 s and terminated with 12 μL of 95% formamide and 20 mM EDTA, pH 8. Ninety seconds was within the linear range of reaction for all dNTP and *O*-alkyl-dTTP substrates.

In order to measure kinetics of extension to position 1265 (Figure 1, site 4), the reaction mixture also contained dGTP at three concentrations (0.4, 19, and 283 μM). The dTTP or modified dTTP concentration was constant (0.5 μM dTTP, 31 μM O^2-methyl-dTTP, 63 μM O^2-ethyl-dTTP, 25 μM O^2-isopropyl-dTTP, and 8 μM O^4-methyl-dTTP). Reactions were carried out for 90 s and terminated as above.

The gel electrophoresis, autoradiography, densitometry, and data analysis were as described by Boosalis et al. (1987).

Use of O^2-Alkyl-dTTPs To Replace dTTP in Extended DNA Synthesis. The reaction mixtures (200 μL) contained 100 μM each of [^{32}P]dATP, dCTP, dGTP, and dTTP or alkylated dTTP, 0.1 A_{260} unit of nicked salmon sperm DNA, and 2 units of Pol I under the same conditions as used for kinetic measurements. Aliquots were taken over a 90-min time period and incorporated radioactivity determined on disks as described above. Controls included an enzyme blank and reactions with three dNTPs only. The latter incorporation has been subtracted from each time point. The ratio of enzyme/DNA was chosen to be the same as used for K_m determination on poly[d(A-T)], although it is less favorable for extended synthesis on nicked DNA than higher ratios (Singer et al., 1986a).

RESULTS

Determination of Apparent K_m and V_{max} Using Poly[d(A-T)] as Template-Primer. Under the same conditions previously used for determining kinetic parameters of O^4-alkyl-dTTPs substituting for dTTP (Singer et al., 1986a), values for apparent K_m and V_{max} of O^2-methyl-, O^2-ethyl-, and O^2-isopropyl-dTTP were determined by using two different preparations of modified triphosphates. A Hanes–Woolf plot for determining the K_m of O^2-methyl-dTTP and O^2-isopropyl-dTTP is shown in Figure 2.

Table I: K_m^{app} and Relative V_{max} Obtained from Replication by Pol I and the Klenow Fragment by Using a Series of O^2-Alkyl-dTTPs as Analogues of dTTP for Insertion Opposite A in Poly[d(A-T)]

substrate[a]	K_m^{app} (μM)	V_{max}(rel)[b]	V_{max}/K_m^{app} (M^{-1})	f_T[c]	f_{m^2T}[d]
dTTP	0.7	1	1.4×10^6	1	
m^2dTTP	21	0.42	2.0×10^4	1.4×10^{-2}	1
e^2dTTP	16	0.23	1.4×10^4	1.0×10^{-2}	0.7
ip^2dTTP	4	0.57	1.4×10^5	1.0×10^{-1}	7

[a] Abbreviations: m^2, methyl; e^2, ethyl; ip^2, isopropyl. [b] V_{max}(rel) is the relative velocity compared to that using dTTP, indicated as 1. [c] $f_T = (V_{max}/K_m^{app})_{O^2T}/(V_{max}/K_m^{app})_T$. [d] f_{m^2T} = (f_T for ethyl or isopropyl)/(f_T for methyl).

Table II: K_m^{app} and Relative V_{max} Obtained from Replication by Pol I and the Klenow Fragment by Using a Series of O^4-Alkyl-dTTPs as Analogues of dTTP for Insertion Opposite A in Poly[d(A-T)]

substrate[a]	K_m^{app} [b] (μM)	V_{max}(rel)	V_{max}/K_m^{app} (M^{-1})	f[c]	f_{m^4T}[d]
dTTP	0.7	1	1.4×10^6	1	
m^4dTTP	5	0.51	1.0×10^5	7.1×10^{-2}	1
e^4dTTP	13	0.47	3.6×10^4	2.6×10^{-2}	0.36
ip^4dTTP	30	0.45	1.5×10^4	1.1×10^{-2}	0.15

[a] See Table I, footnote *a*, for the abbreviation system. The superscript indicates the position of alkylation. [b] Values taken from Singer et al. (1986a). [c] See Table I, footnote *c*. [d] See Table I, footnote *d*, for method of calculation.

Apparent K_m and V_{max} data are listed in Table I. The apparent K_m's for dTTP measured under saturating DNA concentrations are roughly comparable to the true K_m of 2.1 μM reported by McClure and Jovin (1975), in which data obtained with poly[d(A-T)] were extrapolated to infinite DNA concentration. The ratio of V_{max}/K_m measures the nucleotide incorporation efficiency (Fersht, 1977; Boosalis et al., 1987). The ratio of incorporating O^2-alkylated dTTP relative to dTTP is termed *f* (Table I). O^2-Methyl- and O^2-ethyl-dTTP appear to be incorporated with a 50–60-fold lower efficiency than dTTP. Unexpectedly, we observe that the analogue containing the bulkiest substituent, O^2-isopropyl-dTTP, is preferred by about a factor of 7 over O^2-methyl- or O^2-ethyl-dTTP. Thus, the efficiency of O^2-isopropyl-dTTP substituting for dTTP is about 10%.

In contrast to the data given in Table I for the O^2-alkyl derivatives, K_m^{app} values for O^4-alkyl-dTTPs follow the order of methyl < ethyl < isopropyl (Table II). The relative incorporation frequencies for the O^4-alkyl-dTTPs are also inversely related to adduct size, since there is only a 2-fold change in apparent V_{max} between the O^4-alkyl adducts and dTTP. Incorporation is most efficient in the case of O^4-methyl-dTTP (14-fold lower compared to dTTP) and least efficient for O^4-isopropyl-dTTP (95-fold lower compared to dTTP).

Ability of O^2-Alkyl-dTTPs To Substitute for dTTP in DNA Synthesis. The apparent K_m and V_{max} values in the previous section were calculated at time points within a linear range of reaction. Experiments were also performed to assess the ability of the analogues to support extended synthesis. Over a 90-min period, in a reaction containing nicked salmon sperm DNA and equal concentrations of [^{32}P]dATP, dCTP, dGTP, and O^2-alkyl-dTTP or dTTP, synthesis continued to increase only when dTTP or O^2-isopropyl-dTTP were present (Figure 3). No increase in synthesis occurred with O^2-methyl- or O^2-ethyl-dTTP after 30 min. The extent of synthesis at 30 min with each O^2-alkyl-dTTP is in general agreement with the data for poly[d(A-T)] (Table I), i.e., O^2-isopropyl-dTTP

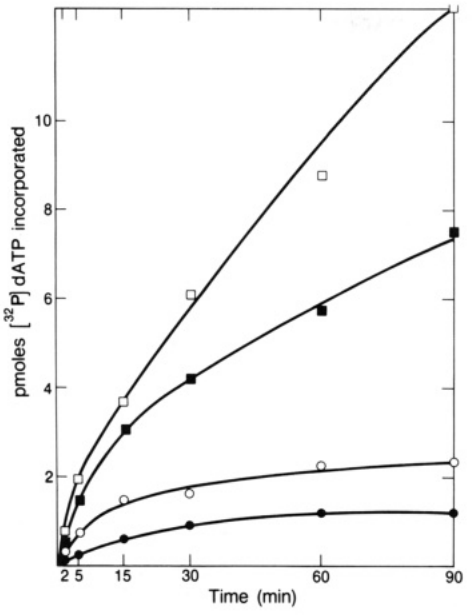

FIGURE 3: Time course of DNA synthesis using dTTP or O^2-alkyl-dTTPs as the sole source of T. [^{32}P]dATP, dCTP, and dGTP were present, all at 100 μM. (□) dTTP; (■) O^2-isopropyl-dTTP; (○) O^2-methyl-dTTP; (●) O^2-ethyl-dTTP. Points were quantitated by measuring incorporation of ^{32}P into the nicked DNA and subtracting synthesis in reactions where dTTP was omitted.

Table III: Kinetics of Nucleotide Insertion Using O-Alkyl-dTTPs as Substrates for Insertion Opposite A in the M13 DNA Sequence 5′-TTACG-3′[a]

substrate	K_m^{app} (μM)	V_{max}(rel)	V_{max}/K_m^{app} (M^{-1})	f	f_{m^2T}
dTTP	0.4 (0.1)[b]	1	2.5×10^6	1	
m^{2}dTTP	227 (13.8)	0.17	7.5×10^2	3.0×10^{-4}	1
e^{2}dTTP	280 (95.4)	0.10	3.6×10^2	1.4×10^{-4}	0.46
ip^{2}dTTP	181 (7.1)	0.72	4.0×10^3	1.6×10^{-3}	5.3
m^{4}dTTP	24 (1.3)	0.62	2.5×10^4	1.0×10^{-2}	33

[a] See footnotes to Tables I and II for abbreviations and definitions. [b] The numbers in parentheses are the estimated standard deviation for K_m^{app} obtained from a linear least-squares fit to a plot of S/V versus S (Hanes–Woolf plot).

supports synthesis better than the O^2-dTTPs with smaller substituents.

Apparent values of K_m and V_{max} were determined for O^2-methyl-dTTP and O^2-isopropyl-dTTP with nicked DNA as template. O^2-Isopropyl-dTTP is preferred 2.5-fold over O^2-methyl-dTTP (data not shown). These data agree with the extent of incorporation seen in Figure 3.

Single-Site Incorporation Kinetics for O^2-Alkyl-dTTPs Using Primed M13 DNA. The experiments using poly[d(A-T)] or nicked DNA contain two factors that complicate the interpretation of kinetic data. First, incorporation of the alkylated dTTP analogue is not observed directly since the radioactive label is contained only in the natural dNTP substrate (dATP) required to sustain DNA synthesis. Second, incorporation of the analogue at multiple sites along the primer strand may result in significant reduction in priming efficiency for subsequent nucleotide incorporation events (Singer et al., 1986a).

To obtain a more direct measurement of incorporation, a methodology developed by Randall et al. (1987) and Boosalis et al. (1987) was used to compare the kinetics of incorporating O^2-methyl-, O^2-ethyl-, and O^2-isopropyl-dTTP and dTTP opposite a single A site on a primed ssM13 DNA template

Table IV: Fraction of Radioactivity Incorporated into C·G after Formation of A·T or A·O-Alkyl-T Termini in M13 DNA[a]

	% of M13 primer extended using O-alkyl-dTTPs				
	deoxynucleoside triphosphate				
position[b]	T	m^{2}T	e^{2}T	ip^{2}T	m^{4}T
2 T·A	21	83	85	57	55
3 A·T, T*	8	6	5	9	7
4 C·G	71	11	10	34	38

[a] Ninety-second reaction with dATP, dGTP, and dTTP or O-alkyl-dTTP all present. Percent radioactivity excludes the primer and position 1. [b] The percent of oligomer at position 2 is taken as 100% before elongation to positions 3 and 4. The total radioactivity in positions 2–4 was approximately equivalent in all samples. [c] Molarities of indicated dTTPs are based on K_m data. Abbreviations are as in Table I, footnote *a*.

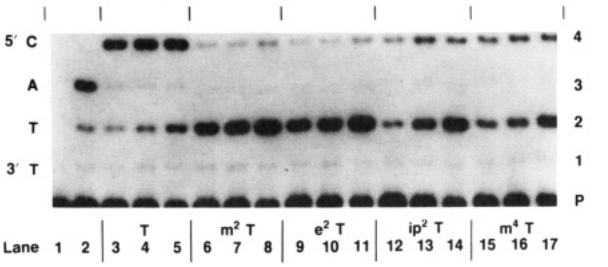

FIGURE 4: Gel radioautogram showing band intensities as a function of the presence of dGTP and dTTP or modified dTTP. The 17-nucleotide primer (Figure 1) (lane 1), annealed to ssM13 DNA, is extended to position 3 (lane 2) with dATP and dTTP alone. Lanes 3–17 contain primer-template, dATP, dTTP or the indicated O-alkyl-dTTP, and three increasing concentrations of dGTP, as described under Materials and Methods. For each group of three lanes, the dGTP concentrations are 0.4, 19, and 283 μM. The dTTP substrate is indicated with an abbreviation: m^2, O^2-methyl; e^2, O^2-ethyl; ip^2, O^2-isopropyl; m^4, O^4-methyl. Extension to position 4 indicates that after insertion of O-alkyl-T opposite A (position 3) the succeeding base pair, C·G, can be formed at position 4. Quantitation of the bands was by densitometry (Table IV).

(Figure 1, position 3). In this assay, O^2-isopropyl-dTTP is also incorporated more efficiently than either O^2-methyl- or O^2-ethyl-dTTP (Table III), in qualitative agreement with the data using an alternating copolymer as template (Table I). O^2-Isopropyl-dTTP is inserted five times as frequently as O^2-methyl-dTTP. However, O^2-isopropyl-dTTP is only one-sixtieth as efficient as dTTP.

A measurement of the kinetics of incorporating O^4-methyl-dTTP was also carried out by the gel assay so that another comparison could be made between the two types of assays and substrates (Tables II and III). Although there are quantitative differences in apparent K_m values obtained by the two types of assays, there is general agreement that O^4-methyl-dTTP is utilized more efficiently than O^2-methyl-dTTP for incorporation opposite A by Pol I (Table III).

Use of the site-directed gel assay also allowed an estimate to be made of the efficiency of adding a normal nucleotide onto the newly incorporated analogue (Figure 1, position 4). The most efficiently incorporated analogue, O^4-methyl-dTTP, also acts as the most efficient primer terminus for extension to position 4 (Table IV). When the three O^2-alkyl derivatives are at 3′-primer termini, they are extended with no less than 20% the efficiency of a T·A terminus. The O^2 analogue with the highest efficiency for extension, isopropyl, is extended about 40% as well as the T·A end. Figure 4 illustrates the use of gel assay to measure extension (Petruska et al., 1988).

Significant primer extension is inhibited at position T (2) at the highest dGTP concentration. This we interpret as reflecting the higher probability of a G·T wobble when the

dGTP concentration is 28 times higher than the dATP concentration.

Discussion

There are three points made in these experiments. (1) The size of the O^2-alkyl group does not directly correlate with the ability of these derivatives to substitute for dTTP opposite A in any of the three systems used for kinetic measurement. (2) None of the O^2-alkyl-dTTPs is as acceptable as O^4-methyl-dTTP opposite template A in the M13 assay. (3) However, once formed, an O^2-alkyl-T·A or O^4-methyl-T·A terminus does not appear to present a significant block to further elongation.

The O^4-alkyl-dTTP kinetic data showed that the apparent K_m increased with size of the alkyl group (Singer et al., 1986a); the O^2-alkyl series differed markedly. In the present series, O^2-isopropyl-dTTP is both incorporated more efficiently (Tables I and III) and extended more easily (Table IV) than either O^2-methyl- or O^2-ethyl-dTTP. O^2-Isopropyl-dTTP is incorporated about 5–10 times more readily than are the analogues with smaller groups. However, this incorporation is still only about 20% that of O^4-methyl-dTTP (Tables I and III).

The differences in the apparent K_m and V_{max} values reported in Tables I and III reflect differences in the two assay systems used. Nevertheless, the same relative data for frequency of incorporation are found in both assays (Tables I and III, f_{m^2T}). Similarly, the ability of the O^2-alkyl-dTTPs to support synthesis of nicked DNA for extended times shows the same high acceptability of O^2-isopropyl-dTTP compared to O^2-methyl-dTTP or O^2-ethyl-dTTP. Preston et al. (1986) had previously found, using primed ϕX174, that after similar times of synthesis, O^4-methyl-dTTP was incorporated four times more frequently than was O^2-methyl-dTTP. This preference by Pol I for O^4-methyl-dTTP compared to O^2-methyl-dTTP is also seen in the kinetic data in this present series of experiments (Tables III and IV).

All of the O-alkylated derivatives used in the gel assay have f values in the range of 10^{-2}–10^{-4} for incorporation opposite template A sites. Although these values are likely to be sequence-dependent, they still indicate relative rates of incorporation 10–100-fold greater than those for formation of mismatches with unmodified bases (A·A, A·G, A·C, etc.) (Loeb & Kunkel, 1982; Boosalis et al., 1987).

Figure 4 shows the individual steps to incorporate a dTTP derivative opposite A (position 3) and, with the addition of dGTP, to position 4. Note that the dense bands at position 2 of lanes 6–17 reflect the difficulty in inserting the O-alkyl-dTTP compared to dTTP (lanes 2–5). However, the absence of bands in position 3, even at low dGTP concentrations (lanes 6, 9, 12, 15), reflects the relative ease of extension of an O-alkyl-T·A primer terminus by Pol I (Klenow).

Alkylation of template strand T's would not necessarily produce the same base-paired structure as the terminus before extension that is generated in this assay. However, it would appear from the ease of extending an O-alkyl-T·A terminus, particularly O^4-methyl-T·A, that these lesions would not be a significant block to replication and, being poorly repaired (Brent et al., 1988), would remain in the template. In further rounds of replication, the alkyl T can pair with G, a mutagenic event. Such pairing has been observed to occur frequently in vitro (Singer et al., 1984; Singer et al., 1986b) as well as in vivo (Preston et al., 1986).

Extension from an O-alkyl-T·A M13 DNA primer terminus follows the same trend as that for incorporation of the analogues among the O^2 derivatives; it is easier to extend from O^2-isopropyl-T·A than from either an O^2-methyl- or O^2-ethyl-T·A terminus. Among these analogues the most efficient extension occurs from an O^4-methyl-T·A terminus. Saturating levels of O^4-methyl-dTTP are at least 16 times higher than those for dTTP, suggesting that the misinsertion step for the analogues may be slower than the extension step, as has recently been observed for DNA polymerase α for normal base mispairs (Petruska et al., 1988).

Excision of inserted alkyl-dTTPs by the Pol I 3′→5′ proofreading exonuclease was not measured. However, the data provide indirect evidence that significant removal of the alkylated analogues did not occur. Efficient extension from a newly inserted analogue at a 3′-primer terminus (Figure 4) would not have been observed if proofreading were extensive.

The kinetic data for both poly[d(A-T)] (Table I) and the site-specific assay using M13 DNA (Table III) indicate a similar principle of selection of nucleotides by Pol I. Much of the discrimination against incorporation of O^2-alkylated dTTP substrates resides in increased K_m^{app} values. For the gel assay using M13 DNA, K_m^{app} values are 2–3 orders of magnitude larger for O^2-methyl- or O^2-ethyl-dTTP derivatives compared to that for dTTP, while apparent V_{max} values are only about 5–10-fold lower (Table III). In contrast, the O^2-isopropyl derivative has a large apparent K_m, but the V_{max} is higher than that of O^2-methyl- or O^2-ethyl-dTTP. In both assays, kinetic data suggest that once the alkylated analogues are bound in the polymerase active cleft (Ollis et al., 1985), the efficiency of phosphodiester bond formation may be relatively insensitive to the bulkiness of the adduct in either the O^2 or O^4 ring position.

Recent NMR and X-ray crystallographic studies of the nucleoside O^2-isopropyl-dT indicate a conformation similar to that of O^4-ethyl-dT (Lafortune, 1986; Birnbaum et al., 1988). However, preliminary modeling studies of the O^2-alkyl-dT series in the M13 DNA sequence and poly[d(A-T)] (E. Loechler, personal communication) suggest an unusual hydrophobic pocket resulting from the branched isopropyl group extending to the opposite A and interacting with the C2 of A. This type of interaction does not appear possible for methyl or ethyl groups on the O^2 of T. This unusual bonding, which increases hydrophobicity, may contribute to the relative acceptability by Pol I of O^2-isopropyl-dTTP.

Registry No. Pol I, 9012-90-2; dTTP, 365-08-2; m^2dTTP, 83133-65-7; e^2dTTP, 96027-04-2; ip^2dTTP, 118354-90-8; m^4dTTP, 83133-66-8; e^4dTTP, 96027-03-1; ip^4dTTP, 100515-08-0.

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Complete Assignment of the Deoxyribose 5′/5″ Proton Resonances of the *Eco*RI DNA Sequence Using Isotropic Mixing†

Steffen J. Glaser, M. Lyndsay Remerowski, and Gary P. Drobny*
Department of Chemistry, University of Washington, Seattle, Washington 98195
Received July 12, 1988; Revised Manuscript Received October 3, 1988

ABSTRACT: Using two-dimensional isotropic mixing spectroscopy all 5′/5″ proton resonances of the *Eco*RI restriction site DNA dodecamer $[d(CGCGAATTCGCG)]_2$ have been assigned. This completes the previous assignments of 1′H to 4′H resonances of the deoxyribose spin systems (Hare et al., 1983). With mixing times of up to 500 ms, many of these resonances showed connectivities of 5′/5″ protons in the two-dimensional isotropic mixing spectrum. Relying only on through-bond connectivities makes these assignments independent of assumptions about the conformation of the DNA oligonucleotide. The assignment of the 5′H/5″H resonances will allow the interpretation of intra- and interresidue NOEs to these protons, providing information about the DNA backbone conformation.

Nuclear magnetic resonance is the only method that is able to yield detailed information about the conformation of biopolymers in solution. Structural information is obtained by measuring the distance-dependent nuclear Overhauser effect (NOE) between assigned resonances (Wüthrich, 1986). As only distances smaller than 5 Å can be determined, the accumulation of small errors can potentially result in a distorted overall structure obtained from these data. This problem is especially severe for molecules with extended, nonglobular structures like DNA. Therefore, it is essential to take as many distance constraints into account as possible, and this requires

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