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Comparative Efficiency of Forming m^{4}T·G versus m^{4}T·A Base Pairs at a Unique Site by Use of *Escherichia coli* DNA Polymerase I (Klenow Fragment) and *Drosophila melanogaster* Polymerase α–Primase Complex†

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ABSTRACT: Synthesis of a 25-mer oligonucleotide template containing O^4-methylthymine (m^{4}T) at a unique site is reported. The sequence used is analogous to that studied previously to determine the mutation frequency of O^6-methylguanine in vitro and in vivo. The templates containing m^{4}T or unmodified T were used in a primer-extension gel assay to determine kinetic parameters for incorporation by DNA polymerases of dGTP and dATP opposite either m^{4}T or T. Both *Escherichia coli* DNA polymerase I (Klenow fragment, Kf) and *Drosophila melanogaster* polymerase α–primase complex (pol α) were used. On the basis of the V_{max}/K_m ratios, the pairing of m^{4}T·G was preferred over that of both m^{4}T·A and T·G by more than 10-fold. The two polymerases gave almost identical values for the frequency of formation of all pairs investigated including m^{4}T·G pairs, suggesting that the 3′→5′ exonuclease activity of the Klenow fragment does not efficiently edit such pairs. Extension beyond m^{4}T·G was demonstrated with both Klenow and pol α. In similar kinetic experiments, bacteriophage T4 DNA polymerase, which has a very high 3′→5′ exonuclease activity, allows stable incorporation of G opposite m^{4}T in contrast to G opposite T. This kinetic approach allows quantitation of the mutagenic potential in the absence of alkylation repair and additionally provides qualitative data on mutagenesis that are in accord with our previous in vivo studies showing that replication of m^{4}T causes T → C transitions.

The established role of O^6-alkyl-G in mutagenesis and initiation of carcinogenesis by alkylating agents has obscured the possible similar role of other alkyl derivatives. Over the last decade there has been increased attention focused on *O*-alkylpyrimidines as also having biological relevance. Several experiments indicated not only that O^4-alkyl-T was mutagenic (Singer et al., 1983, 1984, 1986; Preston et al., 1986, 1987) but that repair was very slow in mammalian cells and tissues (Swenberg et al., 1984; Richardson et al., 1985; Brent et al., 1988), so that this modified base persists for long periods. The presence of O^4-methyl-T and O^4-ethyl-T has been correlated with organ specificity of tumors resulting from *N*-nitroso alkylating agents (Singer et al., 1981; Swenberg et al., 1984; Dyroff et al., 1986; Belinsky et al., 1986; Huh & Rajewsky, 1988).

It is of interest to compare mutation frequency of O^6-alkyl-G with that of O^4-alkyl-T in the same system, using site-directed methods for insertion of the desired derivative. The lability of the O^4-alkyl group on thymine (Singer et al., 1978b) made the synthesis of deoxynucleoside triphosphates and of defined oligonucleotides difficult using established methodology. Using modified techniques, Singer et al. (1983, 1986) were able to demonstrate that O^4-methyl-dTTP could substitute for dTTP in poly[d(A-T)] synthesis by *Escherichia coli* DNA polymerase I. When the resulting polymer, poly[d(A-T,m^{4}T)], was replicated in vitro with the same polymerase, dGTP was incorporated, showing the likely pairing of m^{4}T with G as well as with A. Later, Preston et al. (1986, 1987) used this fact to design a ϕX174 *am*3 genome in which a single m^{4}T was placed opposite A at position 587. Upon replication of this genome in *E. coli* spheroplasts, mutants were generated, all of which contained C at this position, again indicating pairing of m^{4}T with G during replication. In these experiments, no synthetic m^{4}T oligonucleotide was necessary as the modified base was incorporated by polymerase insertion.

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It is well documented that there is asymmetry in the formation of a base pair. For example, polymerase insertion of dTTP opposite A does not show identical kinetic parameters with insertion of dATP opposite T (Kunkel & Bebenek, 1988; Perrino & Loeb, 1989; Mendelman et al., 1989). In the case of m⁴T, the methyl group is reported to be syn (Allore et al., 1983). When m⁴dTTP is placed opposite template A, the polymerase can reorient the methyl group to the more favorable anti conformation as found for m⁶dATP (Engel & von Hippel, 1978). In contrast, once the base is inserted into the template, it is likely to be syn–anti and be less favorable for the formation of a base pair. For these reasons, we wished to study the kinetics of base pair formation with m⁴T in the template.

Synthesis of oligonucleotides containing m⁴T have been recently reported (Li et al., 1987; Borowy-Borowski & Chambers, 1989). In the present paper we describe a new synthesis of a 25-base DNA oligomer containing m⁴T at a defined position, the purity of which permits fidelity assays. This oligomer was then used in a gel extension assay (Boosalis et al., 1987) to measure the efficiency of incorporation of dATP and dGTP opposite m⁴T, using the Klenow fragment of *E. coli* DNA polymerase I (Kf) and *Drosophila melanogaster* polymerase α–primase complex (pol α). With either of these polymerases or T4 polymerase, there was a clear preference for pairing with dGTP, indicating that the source of the enzyme (prokaryotic or eukaryotic) and the differential 3′→5′ exonuclease activity did not affect the formation of such pairs.

Materials and Methods

Cloned Klenow fragment of *E. coli* DNA polymerase I and dNTP substrates (HPLC purified) were purchased from Pharmacia. Purified *D. melanogaster* DNA polymerase α consisting of at least three polypeptide subunits, including primase (Kaguni et al., 1983), was a generous gift of Dr. I. R. Lehman (Stanford University).

Solvents and reagents of the highest purity for chemical synthesis were purchased from standard suppliers. Thymidine and snake venom phosphodiesterase were purchased from Sigma. Bacterial alkaline phosphatase was obtained from New England Biolabs, [γ-^{32}P]ATP was obtained from New England Nuclear, and the T4 polynucleotide kinase was from United States Biochemical.

TLC monitoring was done on silica gel IB-F plates (J. T. Baker). Absorption spectra were recorded on a Hitachi 557 UV–visible spectrophotometer. Proton and ^{31}P NMR were performed on a Bruker 250-MHz spectrometer. HPLC was performed on a Beckman Model 322 gradient liquid chromatograph equipped with a Hewlett-Packard 1040A diode array detector. Nucleoside composition analysis was conducted by reverse-phase HPLC on a 5-μm Ultraphere ODS column eluted with a gradient of 0–35% acetonitrile in 0.1 M ammonium acetate buffer (pH 6.8) over 45 min at 0.75 mL/min.

O⁴-Methylthymidine. Thymidine was treated with Ag_2O and isopropyl bromide in the dark at 37 °C. The isopropyl group was then substituted by a methyl group, and *O⁴*-methylthymidine was purified from the crude mixture by column chromatography on a silica gel eluted with methanol in chloroform (Singer et al., 1983). Fractions containing the product were combined, concentrated, and rechromatographed. The purified, dried material was identified by its UV spectra (Kušmierek & Singer, 1976).

5′-O-(Dimethoxytrityl)-O⁴-methylthymidine. *O⁴*-Methylthymidine (500 mg, 2 mmol) was dried twice by evaporation with pyridine (10 mL) and then resuspended in 10 mL of pyridine. 4,4′-Dimethoxytrityl chloride (800 mg, 1.2 equiv) and 400 μL (1.4 equiv) of triethylamine were added, followed by 12.2 mg (0.05 equiv) of 4-(dimethylamino)pyridine. The mixture was stirred for approximately 2 h until TLC monitoring using 5% methanol in dichloromethane as solvent indicated the disappearance of *O⁴*-methylthymidine and the appearance of higher mobility nonpolar trityl-positive material. An equal volume of water was added to stop the reaction, and the product was extracted into diethyl ether and purified by silica gel column. The yield was 65%, and the compound was shown by HPLC to have a purity of >90%. UV (λ_{max}, methanol) 278 nm (ε 10 200), 282 nm (ε 10 400); ^{1}H NMR (DMSO-d_6) δ 7.80 (s, 1, H_6), 7.40–6.88 (m, 13, Ar), 6.18 (t, 1, J = 6.4 Hz, $H_{1'}$), 5.32 (d, 1, J = 4.5 Hz, 3′-OH), 4.31 (m, 1, $H_{3'}$), 4.01 (m, 1, $H_{4'}$), 3.85 (s, 3, CH_3O–), 3.74 (s, 6, 2 CH_3O–), 3.20 (m, 2, $H_{5'5''}$), 2.22 (m, 2, $H_{2'2''}$), 1.55 (s, 3, 5-CH_3).

2′-Deoxy-5′-O-(4,4′-dimethoxytrityl)-O⁴-methylthymidine 3′-(2-Cyanoethyl N,N-diisopropylphosphoramidite). The 5′-*O*-(dimethoxytrityl)-*O⁴*-methylthymidine (0.25 mmol) was dissolved in dichloromethane (1.5 mL) with diisopropylamine hydrotetrazolide (20 mg, 0.12 mmol) and purged with argon. To this, 0.5 mL of a 1.1 M solution of bis(diisopropylamino)cyanoethoxyphosphine in ether was added, and the mixture was shaken until the solid dissolved. The reaction was checked for completion by TLC on plates developed with 20% hexane in ethyl acetate. The reaction was stopped by an addition of ~2 volumes of 5% aqueous sodium bicarbonate, and the organic layer was then washed sequentially with 5% sodium bicarbonate and with saturated sodium chloride solution and then dried over sodium sulfate. The solution was filtered, concentrated, and purified over a silica column with 20% hexane in ethyl acetate and finally precipitated into hexane. The yield was 200 mg, 21%. The phosphoramidite was shown to be >99% pure by HPLC. ^{31}P NMR ($CDCl_3$) δ 149.50, 149.17; ^{1}H NMR ($CDCl_3$) (two diastereomers) δ 7.92 (s, 0.5, H_6, isomer I), 7.85 (s, 0.5, H_6, isomer II), 7.43–6.80 (m, 13, Ar), 6.42 (t, 0.5, $H_{1'}$, isomer II), 4.61 (m, 1, $H_{4'}$), 4.16 and 3.77 (m, 2, $H_{5'5''}$), 3.97 (s, 3, CH_3O–), 3.79 (s, 6, 2 CH_3O–), 3.57 and 3.32 (m, 1, $H_{2'}$), 2.62 and 2.40 (2 t, 4, CH_2–CH_2–CN), 2.27 (m, 1, $H_{2''}$), 1.68 (s, 3, CH_3), 1.50–1.02 (m, 14, *i*-Pr).

Oligonucleotide Synthesis. Oligonucleotides were synthesized on an Applied Biosystems Model 381A automated DNA synthesizer by the cyanoethylphosphoramidite method (Gait, 1984; Sinha et al., 1984). A few modifications were made owing to the lability of the alkyl group of *O⁴*-methylthymidine. These included addition of the *O⁴*-methylthymidine-protected monomer manually and, after its addition, discontinuation of the capping reactions. The latter modification avoided the breakdown of the modified nucleoside by the capping reagents. The oligonucleotide at the end of the synthesis was deprotected under nonaqueous conditions with 1,8-diazobicyclo[5.4.0]undec-7-ene in tetrahydrofuran and methanol (Kuzmich et al., 1983; Basu et al., 1987). The oligonucleotides synthesized were

25-mer: 5′-CCGCTAGCGGGTACCGAGCTCGAAT-3′
25-mer: 5′-CCGC(m⁴T)AGCGGGTACCGAGCTCG AAT-3′
19-mer: 3′-CGCCCATGGCTCGAGCTTA-5′
6-mer: 5′-GC(m⁴T)AGC-3′

It is difficult to determine the optimal conditions for deprotection of long oligonucleotides containing modified bases and to assess the levels of impurities generated during synthesis. These potential problems were addressed by preparing, de-

Table I: Kinetics of Incorporation of dATP and dGTP Opposite m^4T in Position 21 of a 25-mer Oligonucleotide[a]

	Kf[b]			pol α[b]		
pair	K_m^{app} (μM)	V_{max}^{rel}	f[c]	K_m^{app} (μM)	V_{max}^{rel}	f[c]
T·A	0.19 ± 0.07	4.7 ± 0.9	1	0.1 ● 0.05	5.6 ± 1.5	1
T·G	38 ± 16	0.5 ± 0.2	5×10^{-4}	117 ± 25	2.2 ± 0.8	3.3×10^{-4}
m^4T·A	35 ● 15	0.4 ± 0.1	4×10^{-4}	55 ± 12	0.9 ± 0.1	2.9×10^{-4}
m^4T·G	29 ● 7	3.6 ± 0.6	50×10^{-4}	25 ± 8	6.7 ● 1.6	48×10^{-4}

[a] Each value represents five independent experiments with the SEM given. See Figure 2 for representative Hanes–Woolf plots used to determine these data. The complete protocol is given under Materials and Methods and shown schematically in Figure 1. [b] Kf is the Klenow fragment of *E. coli* DNA polymerase I; pol α is the intact polymerase–primase from *D. melanogaster*. [c] f is the ratio of each misincorporation efficiency compared to that for formation of the normal base pair T·A. See eq 1.

protecting, and purifying the hexanucleotide containing m^4T, shown above, by the same methods as the other oligonucleotides and assessing its purity. The oligonucleotides after deprotection were desalted on a Sephadex G-10 column (2.5 × 40 cm) eluted with water. The oligonucleotides were purified by gel electrophoresis on a 40 × 0.15 cm, 20% polyacrylamide gel electrophoresed at 20 V/cm until the marker dye, bromphenol blue, migrated off the bottom of the gel. The gel was wrapped with polyethylene and placed on a TLC plate with a fluorescent indicator; the DNA was visualized by a short-wavelength UV shadowing, excised, and eluted into water. The oligonucleotides were then desalted by gel filtration chromatography as above and 5′-phosphorylated by use of polynucleotide kinase and [γ-^{32}P]ATP, and the purity (>95%) was checked by gel electrophoresis.

The presence of the modified base was assessed by HPLC analysis and UV diode array detection of the snake venom phosphodiesterase and bacterial alkaline phosphatase digestion products of the oligonucleotide. The nucleoside composition of the oligonucleotides was found to be within experimental error of the theoretical values. All UV spectra of the nucleosides had the correct λ_{max}, λ_{min}, and UV ratios. No other UV-absorbing components were detectable at the high sensitivity of the diode array detector.

Incorporation and Extension Using the Klenow Fragment or pol α. The ^{32}P 5′-end-labeled primer annealed to either of the two 25-mer templates was used in a gel assay, following procedures described by Boosalis et al. (1987). Reactions for kinetic data were carried out at 37 °C for 90 s with Kf and 4 min with pol α. Gel electrophoresis, autoradiography, densitometry, and data analysis were done as described by Boosalis et al. (1987).

Results and Discussion

O^4-Methyl-T is a potent mutagen and likely initiator of carcinogenesis, but its base pairing properties have not been rigorously studied. Thus, there were two aims in this work. The first was to determine which dNTP pairings, if any, occurred upon replication opposite a single m^4T situated at a unique template site and with what relative frequency compared to normal Watson–Crick base pairs. The second aim was to determine if there were substantive differences in the kinetics of replication of the primed template when two different polymerases were used, Kf and pol α.

In order to address these questions, it was first necessary to prepare an oligonucleotide containing m^4T. This was accomplished with a modified machine synthesis but by changing the protocol for deprotection so that the m^4T was not demethylated, as judged by the most sensitive techniques available to us (see Materials and Methods). On the basis of the kinetic data for base insertion, described below, the level of possible contamination with T was <1/2500 m^4T. This value is derived from the m^4T·A frequency, which is 4×10^{-4} compared to that of T·A, defined as 1 (Table I). With this purity, kinetic

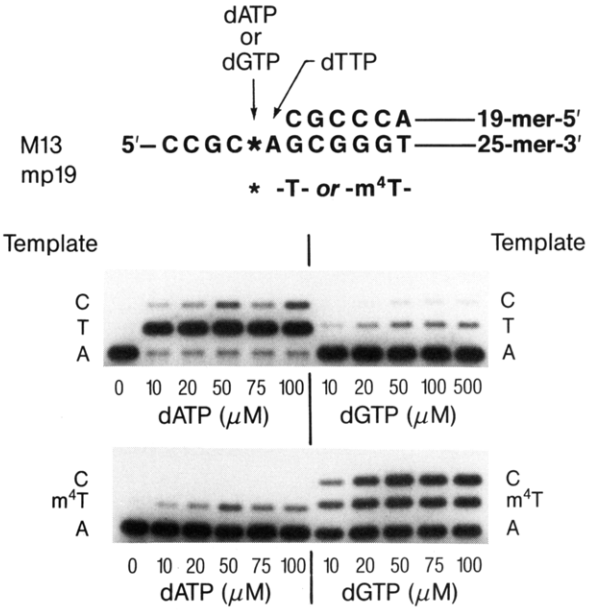

FIGURE 1: Illustration of the PAGE method for obtaining kinetic data when the oligomers containing T or m^4T were primed with the complementary 19-mer and the Klenow fragment was used. All reactions contained 10 μM dTTP with varying dATP and dGTP concentrations and were for 90 s. The primer band is not shown. The T-containing oligomer (top sections) illustrates the extension of T·A to a C-A pair at the dATP concentrations used, and the lack of extension of T·G to a C-G even at 500 μM dGTP. The m^4T-containing oligomer (bottom sections) illustrates the relatively low probability of formation of m^4T·A pair and the facile formation of a m^4T·G pair, which easily extends to a C·G pair.

parameters could be obtained that supported the mutagenesis data earlier reported for replication of poly[d(A-T,m^4T)] and ϕX174 *am3* with m^4T at genome position 587 (Preston et al., 1986, 1987). In these earlier experiments $m^4T \rightarrow C$ transitions were measured, but the experimental design did not permit any comparison of the frequency of pairing of m^4T with A versus that with G. Kinetic data were obtained separately for the efficiency of forming m^4T·A pairs, which is not a mutagenic event (Singer et al., 1989a).

Site-Specific Nucleotide Insertion Kinetics Involving m^4T on the Template Strand. The mutagenic potential of m^4T was investigated with a polyacrylamide gel fidelity assay (Randall et al., 1987; Boosalis et al., 1987). In this assay, DNA polymerase elongates a ^{32}P-labeled primer molecule annealed to a DNA template containing either normal T or modified m^4T located at a unique site (Figure 1, top). A direct example of the ability of m^4T to form mutagenic base pairs with incoming dGTP in preference to nonmutagenic base pairs with dATP can be seen by inspection of the gel band intensities, using *E. coli* Klenow fragment (Figure 1, bottom).

At relatively low dGTP substrate concentrations (~10–20 μM), the level of G incorporated opposite m^4T is significantly

greater than that of A opposite m⁴T over the range of [dATP] = 20–100 μM. Once incorporated opposite m⁴T, G is easily extended by addition of another G opposite C at an adjacent template site. The relative ease with which the m⁴G·T mispair can be extended suggests that it is not a good substrate for proofreading by the "weak" 3′-exonuclease activity of the Klenow fragment. In the absence of any dNTP the exonuclease activity of the Klenow fragment did not excise even m⁴T·A pairs (Singer, 1986). For the case of another modified base pair, m⁶G·T, we also found that it was extended much more rapidly to the next template site than the parent G·T base pair (Singer et al., 1989b).

The corresponding incorporation of A opposite the normal base T shows, as expected, the highest efficiency (Figure 1, middle left). Incorporation of G opposite T (Figure 1, middle right) occurs much less efficiently and is comparable to the incorporation of A opposite m⁴T (Figure 1, bottom left). The relative incorporation efficiencies dAMP·T > dGMP·m⁴T > dAMP·m⁴T ~ dGMP·T appear to reflect the relative rates of base pair formation (nucleotide insertion) rather than the effects of proofreading because the relative incorporation efficiencies using Klenow fragment are similar to those using pol α (Table I), containing no detectible exonuclease activity (Reyland et al., 1988). In the case of T4 polymerase, which contains a highly active proofreading exonuclease, incorporation of A opposite T and G opposite m⁴T was observed, but there appeared to be no stable incorporation of A opposite m⁴T or G opposite T, probably because of rapid exonucleolytic degradation (data not shown).

Quantitation of the relative base pairing efficiencies involving T and m⁴T can be achieved by integrating gel band intensities measured within a linear intensity range. (Several of the bands shown in Figure 1 are deliberately overexposed and not suitable for analysis.) The ratio of integrated gel band intensities, I, at the target template site (T or m⁴T) and at the previous template site (A) was used to determine the relative nucleotide incorporation velocity at the target site, $v \sim I_2(\text{T or m}^4\text{T})/I_1(\text{A})$. See, e.g., Boosalis et al. (1987).

The velocity of the incorporation of A or G opposite T or m⁴T, measured as a function of [dNTP], was used to obtain a linear least-squares estimate for the ratio of V_{max}/K_m. Accurate estimates of this ratio were obtained by measuring the y intercept on a Hanes–Woolf plot, $[\text{dNTP}]/v$ versus [dNTP] (Figure 2). The ratio of the kinetic constants for the two dNTP substrates, f (eq 1), obtained by measuring the

$$f = (V_{max}/K_m)_1/(V_{max}/K_m)_2 \quad (1)$$

incorporation kinetics for each substrate separately, is equal to the ratio of nucleotides incorporated when both substrates are present simultaneously, at equimolar concentrations, to compete for incorporation at a specific template site (Fersht, 1977; Boosalis et al., 1987; Randall et al., 1987).

A comparison of relative base pairing efficiencies (Table I) shows that m⁴T·G base pairs are made with a 10-fold higher efficiency than T·G and with an efficiency of about 1/200 compared to T·A base pairs. Formation of m⁴T·G would result in T → C transitions, in accordance with in vivo data (Preston et al., 1986, 1987). The efficiency of forming m⁴T·A is 10-fold less than that of m⁴T·G (about 1/2000 of T·A), which is similar to that of making T·G base pairs.

Base Pairing Rationale for m⁴T·G. Base pairs between thymine and guanine in their favored tautomeric states can exist in a wobble configuration in which two H-bonds are present at the 3–6 and 2–1 positions coupling T to G (Figure 3A). Attachment of a methyl group to the oxygen at the 4-position of T causes a loss of the proton at the N-3 position

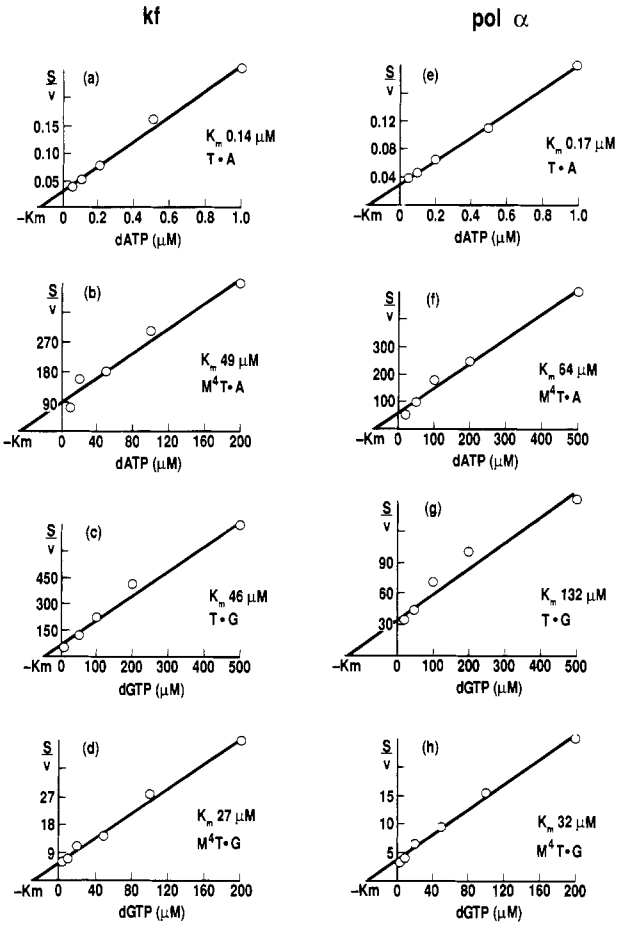

FIGURE 2: Representative Hanes–Woolf plots from a single experiment. The left side (a–d) is data obtained with the Klenow fragment (Kf), and the right side (e–h) shows data with pol α. Such plots $[S]/v$ versus [S] can be used to determine K_m^{app} and V_{max}^{rel} for nucleotide insertions.

of T, thus precluding formation of an uncharged m⁴T·G wobble pair. However, in the absence of a proton at the N-3 position of T, it would then be possible to form a two-hydrogen-bonded Watson–Crick m⁴T·G structure in which both protons were donated by G (Figure 3B).

Therefore, we suggest that an m⁴T·G base pair might occur as a neutral species, in which both bases are present in their favored tautomeric states containing two hydrogen bonds linking m⁴T with G at the 1–3 and 2–2 positions, respectively (Figure 3B). This base pairing scheme has been proposed by Singer (1980) for m⁴U/T·G formed during transcription. A similar Watson–Crick-type structure was proposed for 2-aminopurine–cytosine base pairs on the basis of proton NMR data (Sowers et al., 1986). Here, the 1–3 hydrogen bond results from protonation at the 3-position of cytosine, with both bases retaining their favored amino tautomeric forms.

While it is obvious that enzyme kinetic data cannot be used to define the nature of hydrogen-bonding interactions, measurements of relative rates of base pair formation can be used to suggest plausible models for further testing. In a recent NMR study of m⁴T·G in a synthetic DNA oligomer (Kalnik et al., 1988), it was proposed that the base pairing interaction may be limited to the presence of just a single "short" 2–2 H-bond coupling m⁴T to G (Figure 3C). However, the most stable base pairing structure in the final DNA product is not necessarily the one that is most favored during the polymerization step. For example, it has been shown with several different polymerases that base pairs that can be accommodated in a Watson–Crick structure are strongly favored over

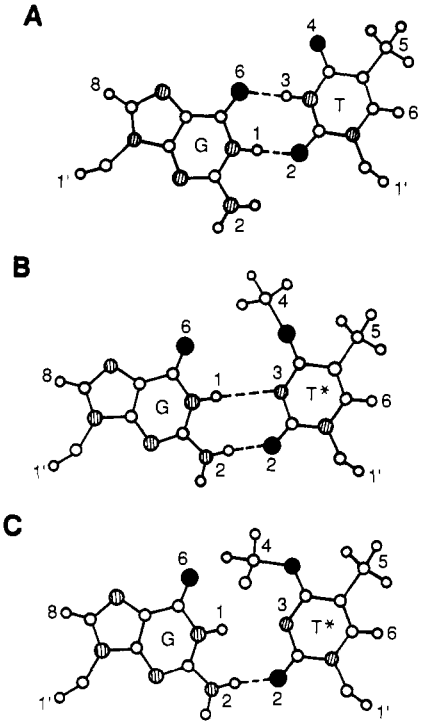

FIGURE 3: (A) Schematic drawing of the T·G wobble pair. The two hydrogen bonds are as shown. (B) Postulated base pairing between m^4T and G, a Watson–Crick structure containing two hydrogen bonds (Singer, 1980). The methyl group is not fixed in the syn position. T* denotes m^4T. (C) Base pairing scheme proposed by Kalnik et al. (1988) on the basis of 2D NMR solution data, containing a single hydrogen bond. Note that the methyl group on the O^4 of T is shown as syn. T* denotes m^4T.

those that are more likely to exist predominantly in a wobble conformation, even though the wobble may result in a more stable final DNA product (Eritja et al., 1986). Some examples include the more efficient formation of 2-aminopurine·C over A·C (Watanabe & Goodman, 1981; Mhaskar & Goodman, 1984), 2-aminopurine·C over 2-aminopurine·A (Mhasker & Goodman, 1984; Fazakarley et al., 1987), 5-bromouracil·G over T·G (Lasken & Goodman, 1984, 1985; Sowers et al., 1989), and xanthine·T and xanthine·C over xanthine·A and xanthine·G (Eritja et al., 1986).

The present data showing a 10-fold higher rate of formation of m^4T·G over T-G and m^4T·G over m^4T·A appear consistent with these other examples of nucleotide analogue incorporation kinetics. Note that in the case of m^4T·A the presence of the methyl group on T precludes formation of both 6–4 and 1–3 H-bonds. There is a growing body of enzyme kinetic data suggesting that Watson–Crick base pairing structures are formed more efficiently by polymerases than alternative structures having greater stability in the product DNA. We suggest that the relatively efficient formation of m^4T·G base pairs (Table I) indicates that they are also likely to be incorporated as a Watson–Crick base pair involving two H-bonds. Alternatively, it is possible that the 4-methyl group on thymine, when oriented syn (Kalnick et al., 1988), may confer greater stacking stability, thereby increasing the likelihood of the formation of the m^4T·G base pair containing just a single 2–2 H-bond (Figure 3C).

Concluding Remarks. In earlier work, using two m^6G-containing templates, one sequence gave a small difference between G·T and m^6G·T frequencies (Singer et al., 1989b). However, when the 3′ flanking base was changed from T to C, there was a dramatic increase in the frequency of m^6G·T pairing. Unpublished data for the m^6G-containing templates replicated with pol α confirmed the neighboring base effect. Thus, different neighboring sequences can exert a major effect on nucleotide insertion fidelity for base pairs involving an analogue as well as common nucleotides (Mendelman et al., 1989). Both m^6G and m^4T preferentially form a type of G·T pair, thus leading to transitions. However, in contrast to m^6G, which is a good substrate for repair in vivo and in vitro [reviewed by Pegg (1983) and Yarosh (1985)], m^4T is poorly repaired (Dolan et al., 1984, 1988; Brent et al., 1988; Graves et al., 1989) and thus may result in being the more efficient mutagenic lesion.

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