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Kinetics of Extension of O^6-Methylguanine Paired with Cytosine or Thymine in Defined Oligonucleotide Sequences†

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ABSTRACT: The frequency of extending m^6G·C or m^6G·T pairs, when the 3′ and 5′ flanking neighbors of m^6G are either cytosines or thymines, was investigated using primed 25-base-long oligonucleotides and the Klenow fragment of *Escherichia coli* DNA polymerase I (Kf). The efficiency, V_{max}/K_m, of extension to the following normal base pair was up to 40-fold greater than for the formation of the m^6G·T or m^6G·C pair. The frequencies of inserting either dCMP or dTMP opposite these m^6G bases did not appear to be different in the two sequences, C-m^6G-C and T-m^6G-T, but extension was favored in the C-m^6G-C sequence. The m^6G·T pair extended to a C·G pair most efficiently, indicating that it was not a strong block to continued replication past the template lesion. Thus, m^6G·T flanked by cytosines replicates more readily than when flanked by thymines, increasing G → A transitions. These data lend further support to the importance of sequence context in mutagenesis.

The ability of modified bases in an oligonucleotide template to direct incorporation of normal dNTPs has been studied in several laboratories (Watanabe & Goodman, 1982; Eritja et al., 1986; Randall et al., 1987; Singer et al., 1989; Dosanjh et al., Shibutani et al., 1991). The emphasis has been placed on the specificity of the insertion step, using a variety of DNA polymerases. For the carcinogenic alkyl derivatives, O^6-methylguanine (m^6G) and O^4-methylthymine (m^4T), pairing with T and G, respectively, is favored in certain sequences. For example, when a 5′ C precedes a template m^6G, the preference for forming m^6G·T over m^6G·C is 6–7-fold greater than when there is a 5′ T (Singer et al., 1989).

Extension from a mismatch is an important factor in in vivo replication. Petruska et al. (1988), Perrino and Loeb (1989), Mendelman et al. (1990), and Shibutani et al. (1991) produced mismatched base termini by annealing an appropriate primer to template and then extending with different DNA polymerases. Extensions of purine–pyrimidine mismatches (e.g., G·T, A·C) were generally favored over the other mispairs.

In the present work, the kinetics of insertion and extension have been measured in the same experiment. These data allowed conclusions to be drawn concerning the relative ability of polymerases to perform the two steps consecutively. In these experiments, changes were made in the 3′ and 5′ bases flanking m^6G to allow measurements of insertion and extension individually and also simultaneously. The effects of sequence and nature of mismatch on mismatch extension efficiencies were measured by a primer extension kinetic assay. The sequences studied were C-G-C, C-m^6G-C, T-G-T, and T-m^6G-T. These sequences and the technique used allow comparison of any dNTP insertion of bases opposite G or m^6G and extension kinetics from newly formed termini to the following normal base pairs.

Experimental Procedures

Materials

The cloned Klenow fragment of *Escherichia coli* DNA polymerase I (Kf) and dNTP substrates (FPLC purified) were

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purchased from Pharmacia. In some experiments, exonuclease-free Klenow fragment (Kf, exo$^-$), a generous gift from Dr. Catherine Joyce of Yale University, was used (Joyce & Grindley, 1983; Derbyshire et al., 1988). Solvents and other reagents of the highest purity for the oligonucleotide deprotection were purchased from Aldrich. The [γ-^{32}P]ATP was obtained from Amersham, and the T4 polynucleotide kinase was obtained from U.S. Biochemicals.

Methods

Oligonucleotide Synthesis. Oligonucleotide synthesis, deprotection, and purification were as described by Singer et al. (1989). The following oligonucleotides were synthesized in which the underlined bases (position 19) were either G or m^{6}G:

25-mer: 5′-CCGCTCGCGGGTACCGAGCTCGAAT-3′

25-mer: 5′-CCGCTTGTGGGTACCGAGCTCGAAT-3′

The primers were as follows:

3′-GCCCATGGCTCGAGCTTA-5′ (18-mer-1)

3′-ACCCATGGCTCGAGCTTA-5′ (18-mer-2)

3′-CGCCCATGGCTCGAGCTTA-5′ (19-mer-1)

3′-TGCCCATGGCTCGAGCTTA-5′ (19-mer-2)

Primer End Labeling and Primer-Template Annealing. All primers used in the kinetic experiments were labeled at the 5′ terminus with ^{32}P and annealed to m^{6}G- or G-containing templates as described by Boosalis et al. (1987). Figure 1 shows the primer- templates used. The 18-mer-1 primer was used to obtain the kinetic parameters for (a) insertion of either C or T opposite G or m^{6}G (see Figure 1, C1) and (b) extension to the next base, C, either by simultaneous formation of the previous base pair or by allowing the polymerase to first form the previous base pair and then adding the next base, dGTP, in the sequence C-m^{6}G(G)-C. The 18-mer-2 was used in the sequence T-m^{6}G(G)-T to obtain the kinetic parameters for (a) insertion of C or T opposite G or m^{6}G (see Figure 1, T1) and (b) extension to the next base, T, with simultaneous formation of the previous base pair (see Figure 1, T2). The two 19-mers (i.e., 19-mer-1 and 19-mer-2) were annealed to C-m^{6}G(G)-C templates to form four different types of termini: G·C, G·T, m^{6}G·C, and m^{6}G·T. These primer-template complexes were used for the insertion opposite the next base, C, to form the following C-G pair (see Figure 1, C3) and for complete replication.

General Method of DNA Polymerase Reactions. Equal volumes of solution containing the enzyme primer-template complex and a solution containing either one or two dNTPs as substrates were mixed to start polymerization reactions for the kinetic studies as described by Randall et al. (1987) and modified by Singer et al. (1989). The main deviation from Randall et al. (1987) was the amount of Kf used. In order to calculate apparent second-order rate constants (V_{max}/K_m) for extension, no more than 20% of the original primer should be extended. Thus, in order to ensure these enzyme-limiting conditions, both commercial Kf (exo$^+$) and Kf (exo$^-$) were titrated to find an appropriate level of enzyme. This amount of enzyme was 1/100 of that previously used for measuring insertion (Singer et al., 1989). These conditions were then used for the determination of the kinetic parameters, both for extension and also for initial insertion. In addition, 10-fold and 100-fold of this polymerase level were used in order to compare insertion under enzyme-limiting and previous conditions. The 10-fold higher enzyme concentration was also used for replicating the entire length of the template containing the alkylated base pairs (m^{6}G·C, m^{6}G·T), the G·T base mispair, and the normal G·C base pair.

Insertion opposite G or m^{6}G. Templates were replicated at 37 °C for time periods of 1.5–10 min with an amount of enzyme which allowed about 20–25% extension depending on the sequence used and base pair formed. Reactions were terminated by adding EDTA in 95% formamide (Randall et al., 1987). Time course experiments were performed using the same procedure except that the time points were over a 0–30-min range.

Extension of Polymerase-Formed Termini. The extension of the polymerase-formed termini, G·C, G·T, m^{6}G·C, and m^{6}G·T, to the next base pairs, C·G or T·A, in the sequences C·m^{6}G(G)-C and T·m^{6}G(G)-T (Figure 1, C2 and T2) was carried out as described above. The reaction mixture was incubated at 37 °C for 10 min with a fixed high amount of dCTP or dTTP (approximately 10 times the K_m) and varying concentrations of dGTP or dATP. The reaction was terminated by adding EDTA in 95% formamide.

Complete Replication of 18-mer-1. The four termini, G·C, G·T, m^{6}G·C, and m^{6}G·T, formed by polymerase extension of the 18-mer primer were allowed to replicate using Kf (exo$^-$) equal to 10-fold the amount used for extension kinetics. These polymerase-formed termini were obtained by first adding either dCTP or dTTP at 10 times the molarity found for the K_m, replicating for 10 min prior to adding other dNTPs, and then incubating for a further 20 min. For the pair m^{6}G·T, made in the absence of dCTP, extension will terminate at position 23, a template G. For m^{6}G·C, the lack of dTTP will not hinder replication to position 25 since there are no template A's.

Extension of Annealed Termini. Primers in Figure 1, C3, are 19-mers complementary to G or m^{6}G. When annealed, the same pairs are formed as when the polymerase extends the corresponding 18-mers (see Methods, above). Complete replication from these annealed termini was performed using the same 10-fold Kf used for polymerase-formed termini. All necessary dNTPs were present, and incubation was for 30 min at 37 °C.

Calculation of Approximate V_{max}, K_m, and Frequency (f) of Incorporation. The gel electrophoresis, autoradiography, densitometry, and data analysis were done as described by Boosalis et al. (1987) and Mendelman et al. (1989). The calculation of the apparent kinetic parameters, K_m and V_{max}, for extension with a running start were described by Petruska et al. (1988) and for insertion with a standing start by Mendelman et al. (1990) and were followed. For C1, T1, and C3 primer-templates, the standing start calculation was used, while for C2, C3, and T2, the extension to normal base pairs, C·G or T·A, used the running start calculation. In each case, the relative frequencies for incorporation (f) of G·T, m^{6}G·C, and m^{6}G·T pairs for both insertion and extension were compared to the normal Watson–Crick base pair.

It should be noted that the relative extension efficiency for two template-primer termini (e.g., matched versus mismatched) depends explicitly on the absolute concentration of the next correct dNTP substrate to be added. The V_{max}/K_m ratio measures the "intrinsic" efficiency of primer extension, which compares the relative rates to extend matched versus mismatched termini at low [dNTP] (see Mendelman et al., 1990). In contrast, nucleotide misinsertion rates are independent of absolute dNTP levels but depend only on the ratios of dNTPs competing for insertion, i.e., nucleotide "pool bias" ratios [see, e.g., Boosalis et al. (1987)].

Results

Kinetics of Insertion of dCMP and dTMP opposite m^{6}G or G. The mutagenic potential of m^{6}G in the two sequences, C-m^{6}G(G)-C and T-m^{6}G(G)-T, was determined using a po-

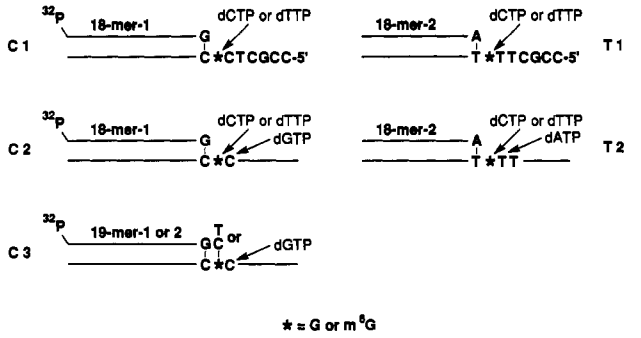

FIGURE 1: Schematic representation of the primer-templates used.

Table I: Kinetics of Incorporation of dTTP or dCTP opposite m^6G or G with either C or T at both the 3′ and 5′ Positions[a]

dCTP or dTTP

3′ G ——— 18-mer-5′-^{32}P

5′ —— C - m^6G(G) - C ——— 25-mer-3′

pair	K_m^{app} (μM)	V_{max}^{rel}	V_{max}/K_m	f
G·C (5)	0.04 ± 0.03	12 ± 4	300	1
G·T (4)	56 ± 20	2.4 ± 1.5	0.04	1.3×10^{-4}
m^6G·C (5)	84 ± 12	3.2 ± 1.8	0.04	1.3×10^{-4}
m^6G·T (5)	26 ± 4	2.7 ± 1.7	0.1	3.3×10^{-4}

dCTP or dTTP

3′ A ——— 18-mer-5′-^{32}P

5′ —— T - m^6G(G) - T ——— 25-mer-3′

pair	K_m^{app} (μM)	V_{max}^{rel}	V_{max}/K_m	f
G·C (5)	0.02 ± 0.008	3.8 ± 1.6	190	1
G·T (5)	37 ± 22	2.2 ± 0.2	0.06	3.2×10^{-4}
m^6G·C (5)	52 ± 18	2.9 ± 0.5	0.06	3.2×10^{-4}
m^6G·T (5)	45 ± 12	2.9 ± 0.6	0.06	3.2×10^{-4}

[a] See Experimental Procedures for the experimental conditions. The number of independent determinations is shown in parentheses. The standard deviation for the mean is given for each value; f is the ratio of each misincorporation efficiency compared to that for formation of the normal base pair G·C. The kinetic constants were derived from incubation times which fulfilled the requirements of a steady state.

lyacrylamide 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 G or m^6G at a unique site (position 19) (Figure 1). Either dCTP or dTTP was inserted opposite G and m^6G, and the apparent second-order rate constants (V_{max}/K_m) were calculated. Initial velocities and kinetic parameters for insertion and extension were determined using enzyme concentration and incubation times where less than 20% of the input primers were extended.

In both sequences, C-m^6G(G)-C and T-m^6G(G)-T, all four base pairs (G·C, G·T, m^6G·C, and m^6G·T) were formed with the insertion frequencies shown in Table I (illustrated in Figure 2). Data in Table I indicate there was no discernible difference in the incorporation of T opposite G and C opposite m^6G. However, there was a reproducible, 2–3-fold, preference for the formation of an m^6G·T compared with an m^6G·C pair in the C-m^6G-C sequence (Table I). This differential, while relatively small, was consistent throughout this work. In contrast, in the sequence T-m^6G(G)-T, m^6G·C and m^6G·T were formed with similar insertion frequencies, again reproducibly.

The values in Table I are for the limited enzyme concentration. For purposes of comparison with previous work (Singer et al., 1989), the relative frequency for the sequence C-m^6G(G)-C of m^6G·T/m^6G·C using 10–100-fold more enzyme is in agreement with the present data obtained using

Table II: Kinetics of Extension of m^6G·T or m^6G·C to the Following 5′ C·G or T·A[a]

dCTP or dTTP

dGTP

3′ G ——— 18-mer-5′-^{32}P

5′ —— C - m^6G(G) - C ——— 25-mer-3′

template-primer terminus	K_m^{app} (μM)	V_{max}^{rel}	V_{max}/K_m	f
G·C (6)	0.26 ± 0.04	3.6 ± 0.6	14	1
G·T (4)	6.3 ± 2.4	0.32 ± 0.1	0.05	3.6×10^{-3}
m^6G·C (7)	20 ± 8	1.1 ± 0.1	0.06	4.3×10^{-3}
m^6G·T (7)	15 ± 6	2.7 ± 0.3	0.18	12.9×10^{-3}

dCTP or dTTP

dATP

3′ A ——— 18-mer-5′-^{32}P

5′ —— T - T - m^6G(G) - T ——— 25-mer-3′

template-primer terminus	K_m^{app} (μM)	V_{max}^{rel}	V_{max}/K_m	f
G·C (6)	0.05 ± 0.08	1.5 ± 0.5	30	1
G·T (5)	20 ± 15	0.5 ● 0.01	0.03	1.0×10^{-3}
m^6G·C (6)	9 ± 3.8	1.0 ± 0.5	0.1	3.7×10^{-3}
m^6G·T (6)	14 ± 4.5	1.5 ± 0.9	0.1	3.7×10^{-3}

[a] The complete protocol and the method of calculation are given under Experimental Procedures. Each value represents four to seven independent experiments with the standard deviation of the mean being given; f is the ratio of incorporation efficiency compared to that for formation of the normal base pair C·G (top) or T·A (bottom). The kinetic constants were derived under enzyme-limiting conditions using a running start.

Table III: Comparison of Frequency of Polymerase Insertion of dCTP or dTTP opposite m^6G or G with Extension To Form the Following Watson–Crick Base Pair, C·G or T·A[a]

insertion	frequency (f_1)	extension	frequency (f_2)	$f_1 \times f_2$ ($\times 10^7$)
primer-template C1[b]		primer-template C2[b]		
C → G	1	G·C → G·C	1	
T → G	1.3×10^{-4}	T·G → G·C	3.6×10^{-3}	4.7
C → m^6G	1.3×10^{-4}	C·m^6G → G·C	4.3×10^{-3}	5.6
T → m^6G	3.3×10^{-4}	T·m^6G → G·C	12.9×10^{-3}	42.6
primer-template T1[b]		primer-template T2[b]		
C → G	1	G·C → A·T	1	
T → G	3.2×10^{-4}	T·G → A·T	1.0×10^{-3}	3.2
C → m^6G	3.2×10^{-4}	C·m^6G → A·T	3.7×10^{-3}	11.8
T → m^6G	3.2×10^{-4}	T·m^6G → A·T	3.7×10^{-3}	11.8

[a] Data from Tables I and II. See Figures 2 and 3 for illustrations of insertion and extension of sequence C1. $f_1 \times f_2$ is the overall frequency for insertion followed by extension. All values shown in the final column are thus $\times 10^{-7}$, as indicated. [b] See Figure 1 for the sequences used and the general scheme for insertion followed by extension.

much less enzyme and longer times (data not shown). Thus, within the wide range of enzyme used, the kinetics of insertion appear unaffected.

Kinetics of Extension of G·C, G·T, m^6G·C, and m^6G·T Termini to the Next Normal Base Pair, C·G or T·A. The extension kinetics of the four pairs, G·C, G·T, m^6G·C, and m^6G·T, to the next base pair, C·G or T·A, in the sequences C-m^6G(G)-C and T-m^6G(G)-T were determined. Both enzyme-limiting and 10-fold enzyme gave similar qualitative results. Quantitative extension data are given only under enzyme-limiting conditions (Table II). Both m^6G·C or m^6G·T termini are easily extended to the following base pair with extension frequencies up to 40-fold better than the corresponding insertion frequencies (Table III).

In the sequence T-m^6G(G)-T, extension data for formation of the next base pairs, T·A and T·A, could not be obtained independently since formation of the second T·A pair occurred

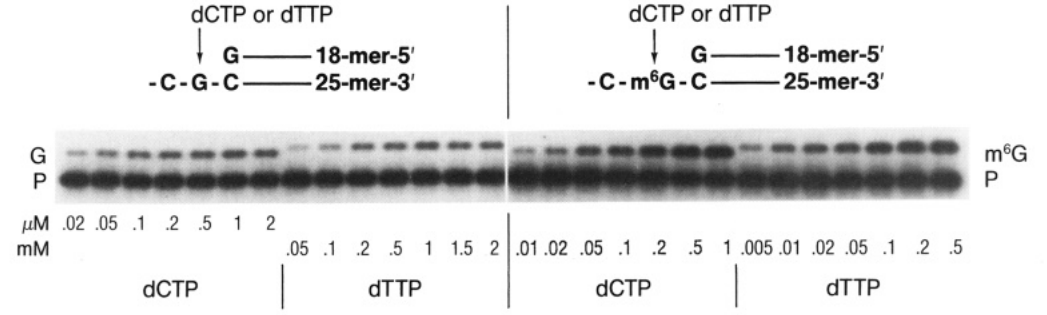

FIGURE 2: Illustration of the PAGE method for obtaining kinetic data when the oligomers containing G or m^{6}G with flanking cytosines were primed with the complementary 18-mer-1 (Figure 1, C1) and replicated with Kf. Reactions were for 1.5 min for forming G·C (lanes 1–7) and 10 min for forming G·T, m^{6}G·C, and m^{6}G·T. Increasing molarities of dCTP or dTTP were used to obtain kinetic parameters for the insertion step to form G·C, G·T, m^{6}G·C, and m^{6}G·T (Table I). Note the difference in mobilities when a T, as compared to a C, is at the primer 3′ terminus. The concentration of the dCTP or dTTP used is given below the primer band. It should be noted that the concentration of the dCTP opposite G (left panel) is given in micromolar and the rest are in millimolar. P denotes the position of the primer band.

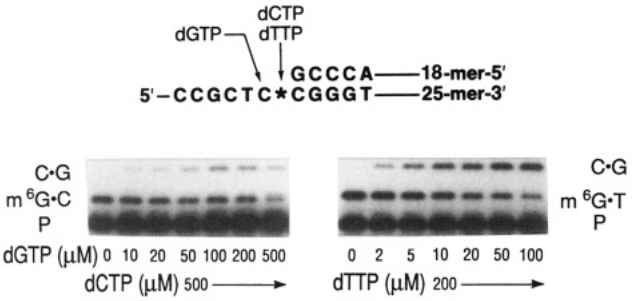

FIGURE 3: Illustration of the gel autoradiogram showing primer extension. The template used contained m^{6}G with flanking cytosines and was primed with 18-mer (Figure 1, C1). The original primer band is shown (P). Note the two different levels of the dCTP and dTTP necessary to get approximately 20% insertion, forming a new primer for determining extension. The new termini, m^{6}G·C (left) and m^{6}G·T (right), were extended by polymerase. Replication was for 10 min for forming these pairs using the Kf. Increasing amounts of dGTP in the presence of dCTP or dTTP were used to determine the kinetics for formation of C·G (Table II).

rapidly. Both m^{6}G·T extended to the next two T·A pairs equally well, with extension frequencies about 10 times better than the corresponding insertion frequencies. There was, however, a difference in all extensions when C-G(m^{6}G)-C was the template, as illustrated in Figure 3. Note the two different levels of dCTP and dTTP necessary to get approximately 20% insertion, forming a new primer for determining extension. Figure 3 shows that the m^{6}G·T pair both forms and extends better than the nonmutagenic m^{6}G·C pair in this C-G(m^{6}G)-C sequence. All pairs in this sequence extended 30–40-fold better than insertion frequencies. In contrast, in the T-m^{6}G(G)-T-T sequence, extension was 3–12-fold more efficient than insertion.

Elongation of Polymerase-Formed or Annealed Base Pairs under Nonenzyme-Limiting Conditions. The ability of exonuclease-free Kf to form G·C, G·T, m^{6}G·C, and m^{6}G·T at position 19 and further extend to the limit of available dNTPs was tested using 19-mer-1 (Figure 1, C1 and C3) annealed to C-m^{6}G(G)-C templates. Extension was maximized by using exonuclease-free Kf, high levels of dNTPs, and a long replication time (see Methods above). In contrast to kinetic studies where primer extension is deliberately limited, in replication experiments testing whether various termini are hindrances to complete replication, a large amount of dTTP (in the absence of dCTP) or dCTP (in the absence of dTTP) was used to create the single desired termini at position 19.

Figure 4 illustrates that replication of both the polymerase formed and annealed pairs can proceed to completion, with the given dNTPs. Lane A shows the unextended primer; lanes B and C illustrate extension of G·C to the 25-mer and G·T to the expected 23-mer. Lane D is similar to lane C but with 10 times the amount of enzyme as for lane C. Lanes E and G show the extension of the m^{6}G·C base pair and lanes F and

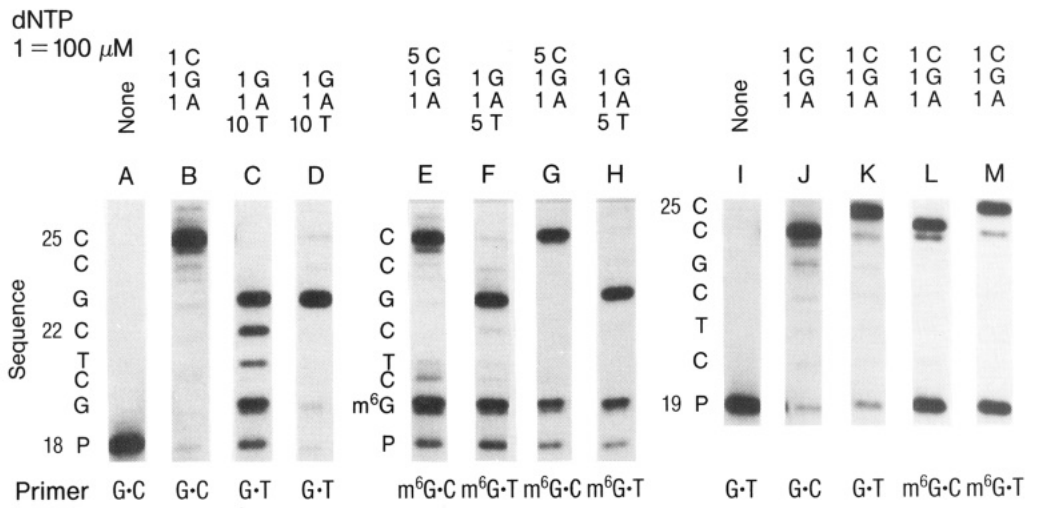

FIGURE 4: Illustration of replication beyond G·C (lane B), G·T (lanes C and D) (lane D contains 10-fold Kf), m^{6}G·C (lanes E and G), and m^{6}G·T (lanes F and H) formed by Kf (Figure 1, C2) and by annealing (lanes I–M) (Figure 1, C3). In lanes E and F the m^{6}G·C and m^{6}G·T termini were formed in the presence of 500 μM dCTP or dTTP, respectively. For extension of these termini, 100 μM dATP and dGTP were added and replication was continued for 30 min. In lanes J–M, all four dNTPs were present at 100 μM for 30 min. Lanes A and I are unextended controls. In lane F, replication cannot continue beyond the position shown since dCTP is absent. In all other cases, replication without stops can be clearly seen. The differences in mobility between lanes E, G, J, L and K, M are a result of sequence composition.

H that of m^{6}G·T pairs. In all cases, the base pairs were formed by polymerase incorporation.

All four termini formed by annealing were also used as primers for complete replication. Since the termini are preformed by annealing, no necessary dNTP is omitted. Thus replication can be demonstrated to the full 25-mer oligonucleotide in all four cases (lanes J–M). Lane I shows the unextended primer as a control.

Discussion

We earlier reported that a single m^{6}G in a defined template preferred to pair with T rather than C when the 3′ neighbor base was C, but when T flanked m^{6}G, the preference was absent (Singer et al., 1989). This illustrated that polymerase can incorporate both C and T opposite m^{6}G but sequence context plays a significant role in influencing misincorporation frequency. If a mismatch occurs and is neither proofread nor extended, genetic information would not be transmitted, resulting in lethality. Therefore, it is desirable to design experiments in order to study both insertion and extension independently as well as the ability to replicate the template to completion.

In our present experiments, we had three major aims: (1) to assess, by kinetic measurement, the effect of 5′ and 3′ neighbors on the ability of m^{6}G or G to direct incorporation of C or T (Figure 1, C1 and T1), (2) to determine the relative frequency of forming a normal Watson–Crick base pair following m^{6}G or G paired with C or T (Figure 1, C2 and T2), and (3) to replicate fully under saturating conditions the template-primers containing the same base pairs discussed above, formed by polymerase insertion of dCTP or dTTP (Figure 1, C2).

In each of the sequences used in the earlier work and the present experiments, reproducible differences ranging up to 6–7-fold could be found favoring formation of the m^{6}G·T base pair over the m^{6}G·C. m^{6}G·C would not be mutagenic, while m^{6}G·T is mutagenic, yielding G → A transitions in vitro (Snow et al., 1984; Singer et al., 1989) and in vivo (Loechler et al., 1984; Chambers et al., 1985; Bhanot & Ray, 1986; Dosanjh et al., 1991). For such potential mutations to be expressed, the original m^{6}G·T pair must be extended in order to result in a viable genome.

m^{6}G·T when flanked by cytosines is extended much more efficiently than all other pairs in both sequences used. This flanking sequence is the same as used by Patel and co-workers for structural studies, using 2D NMR (Patel et al., 1986a,b). They concluded that m^{6}G·C and m^{6}G·T were both destabilizing, but observations of phosphorus chemical shifts indicate that there are three shifted phosphorus resonances with the m^{6}G·C oligonucleotides but only one shift for m^{6}G·T, which would cause less backbone distortion. The role of polymerases in nucleotide insertion would indicate that T is inserted opposite m^{6}G to an equal or greater extent than C in the four sequences we have studied.

In vitro data on relative efficiency of incorporation of either dCMP or dTMP opposite the modified base, m^{6}G, show that the overall efficiency is low, ~1/100% that of the correct base. This is consistent with previous data obtained by Snow et al. (1984a), which showed that the presence of m^{6}G in the DNA template slows down the rate of replication. Hence, these data would suggest that m^{6}G would present a significant block to replication and could be lethal as well as mutagenic. However, in vivo data obtained from the single-stranded M13mp19 genome containing a site specifically placed m^{6}G showed that the survival rate is relatively high (50–70%; Dosanjh et al., 1991). Thus, even though the relative rate of extension at this lesion is low, given sufficient time for extension and/or repair, m^{6}G does not appear to be lethal.

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