Replication of O^6^-Methylguanaine-containing DNA by Repair and Replicative DNA Polymerases*

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The biological consequences of O^6^-methylguanamine (m6G) in DNA are well recognized. When template m6G is encountered by DNA polymerases, replication is hindered and trans-lesion replication results in the preferential incorporation of dTMP opposite template m6G. Thus, unrepaired m6G in DNA is both cytotoxic and mutagenic. Yet, cell lines tolerant to m6G in DNA have been isolated, which indicates that some cellular DNA polymerases may replicate m6G-containing DNA with reasonable efficiency. Previous reports suggested that mammalian pol β could not replicate m6G-containing DNA, but we find that pol β can catalyze trans-lesion replication; however, the lesion must reside in the optimal context for pol β activity, single- or short nucleotide gapped substrates. Primed single-stranded DNA templates, with or without template m6G, were poor substrates for pol β as reported in earlier studies. In contrast, trans-lesion replication by bacteriophage T4 DNA polymerase was observed for primed single-stranded DNA templates. Replication of m6G-containing DNA by T4 DNA polymerase required the gp45 accessory protein that clamps the polymerase to the DNA template. The rate-limiting step in replicating m6G-containing DNAs by both DNA polymerases tested was incorporation of dTMP across from the lesion.

O^6^-Methylguanamine (m6G)-DNA methyltransferase repairs m6G residues in DNA; however, 20–30% of human solid tumor cell lines do not express this repair activity (1). Exposure of cells lacking m6G-DNA methyltransferase to alkylating agents such as MNNG results in high levels of mutations, sister chromatid exchanges, and cell death (reviewed in Ref. 2). Yet, cells unable to repair the m6G damage but tolerant to the killing activities of MNNG have been isolated (reviewed in Refs. 2, 3). As these tolerant cells remain sensitive to the cytotoxic effects of alkylating agents, questions about the replication of m6G-containing DNA arise.

DNA polymerases are predicted to encounter m6G in three DNA environments: 1) in single- or short nucleotide gaps formed during short patch DNA repair, 2) in lengthy single-stranded regions formed by long patch repair, and 3) at replication forks. Single-nucleotide gaps may be produced by short patch mismatch repair activity that is normally directed to the repair of G:T mispairs that are the result of 5-methylcytosine (5mC) deamination (4). The enzyme that initiates this repair process is a DNA G:T mismatch-specific thymine-DNA glycosylase that removes the mispaired thymine from G:T DNA to generate an apyrimidinic site (5). A G:T thymine-DNA glycosylase also initiates the removal of thymine from m6G:T base pairs (6). The abasic site is further processed to generate a single-nucleotide gap across from guanine for G:T mismatches and across from m6G for m6G:T mismatches. While pol β can efficiently fill in single-nucleotide and small gaps across from undamaged DNA templates (7), pol β activity on single-nucleotide gaps across from m6G has not been reported. Pol β replication is blocked, however, by template m6G in long single-stranded DNA templates (8).

Long patch mismatch repair also appears to act on m6G:T base pairs. The model proposed by Karran and Marinos (9), Scudiero et al. (10), Goldmacher et al. (3) and others as reviewed by Karran and Biggini (2) is that mismatch repair enzymes recognize m6G base pairs in DNA as mismatches and produce long excision tracks in the non-m6G strand. Replication of the single-stranded region can result in incorporation of dTMP across from m6G to again produce the m6G:T base pair. Several studies demonstrate that m6G templates the preferential incorporation of dTMP in vivo (11–13) and in vitro (14–16). The resulting m6G:T mispair is again recognized by long patch repair enzymes, and the process of excision followed by replication is repeated. These futile cycles of excision and resynthesis are predicted to produce persistent discontinuities in the DNA that are thought to contribute to the cytotoxic effects of m6G in DNA. This model is supported by the observation that at least some cell lines that lack methyltransferase, but are tolerant to MNNG, also lack long patch mismatch repair (17, 18).

In addition to replication associated with the repair of m6G-containing DNA, tolerant cells must be able to replicate chromosomes in preparation for cell division. Although several DNA polymerases have been shown to have some bypass activity, trans-lesion replication is poor (14).

In view of these numerous reports that demonstrate that m6G in DNA inhibits replication by many DNA polymerases but that m6G-containing DNA is replicated, nevertheless, in tolerant cells, experiments were designed to measure the ability of repair and replicative DNA polymerases to replicate m6G-containing DNA. The two DNA polymerases studied were 1) a mammalian repair DNA polymerase, pol β, and 2) a replicative DNA polymerase, bacteriophage T4 DNA polymerase with its accessory protein, the product of gene 45 (gp45) which clamps the DNA polymerase to the DNA template. The T4 DNA polymerase-gp45 complex is functionally analogous to the hu-
man pol α-PCNA complex (19). Although replication of m6G-containing substrates by several DNA polymerases including pol β and the T4 DNA polymerase have been examined previously, studies presented here attempted to approximate in vivo conditions more closely. Thus, while pol β was reported to have no trans-lesion replication activity on a single-stranded m6G-containing DNA template (8), we observed pol β and m6G trans-lesion activity on short gapped templates, the optimal substrates for pol β replication activity (7). In contrast, T4 DNA polymerase trans-lesion replication was dependent upon association with the gp45 accessory protein and on DNA templates that resemble DNA replication forks or long, single-stranded repair tracks.

In addition to the highly purified recombinant pol β and T4 DNA polymerases, preparations of pol β from m6G-sensitive and m6G-tolerant cell lines were tested on the m6G-containing substrates in order to determine if tolerant cells encoded a mutant pol β with increased ability to replicate m6G-containing DNA. Mutant T4 DNA polymerases with reduced 3′ → 5′-exonuclease activity or increased intrinsic processivity were also examined for trans-lesion replication.

**EXPERIMENTAL PROCEDURES**

DNA Polymerases and Accessory Proteins—Recombinant rat pol β was provided by S. Wilson and purified as described (20). Human pol β was partially purified from human glioma cell line A1235 and A1235M4 (1, 21). Both A1235 cell lines lack O6-methylguanine-DNA methyltransferase activity, but the A1235M4 cells are tolerant to the cytotoxic effects of m6G in DNA, whereas the A1235 cells are hypersensitive. Extracts from 10 confluent 100-mm tissue culture plates were prepared as described (22, 23). The extracts were chromatographed on a Mono Q HR5/5 column (Pharmacia Biotech Inc.) in buffer containing 50–200 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 10 mM dithiothreitol (DTT), and 0.1 mM EDTA. Fractions eluting at 240 mM NaCl contained pol β activity as determined by several criteria: high activity on short gapped DNA substrates, but low activity on primed single-stranded DNA templates; and inhibition by dideoxy-NTPs, but resistance to aphidicolin. A second set of fractions containing DNA polymerase activity eluted at a higher salt concentration (≥ 0.4 M NaCl). This polymerase activity was different from pol β activity in the following ways: high activity on long, single-stranded DNA templates compared to short gapped DNA substrates, inhibition by ddNTPs, and sensitivity to aphidicolin. These properties are characteristic of eukaryotic replicative DNA polymerase, pol α, δ, and ε. The fractions containing apparent pol β activity were pooled and dialyzed against 20 mM sodium phosphate buffer (pH 7.0) containing 50% glycerol, 5 mM EDTA, and 0.1 mM DTT. The dialyzed fractions were stored at −80 °C. This partially purified pol β fraction was used in the assays described below.

Purification and characterization of wild type and mutant recombinant bacteriophage T4 DNA polymerases have been described (24–26). The T4 DNA polymerase accessory protein, the gene 45 protein (gp45), was purified from the expression vector constructed by Lin et al. (27) and purified by a modification of the procedure described by Morris et al. (28). A crude extract was prepared, and the extract was applied to a 75-ml, 5-cm diameter Q-Sepharose column. The gp45 bound to the column, and fractions containing gp45 eluted at 210 mM NaCl from a gradient of 50–500 mM NaCl in Ag buffer (40 mM Tris-HCl (pH 7.4), 0.1 mM EDTA, 10 mM β mercaptoethanol, and 10% glycerol). These fractions were pooled and further purified by chromatography through a 55-ml hydroxyapatite (Bio-Rad, HTP) 5-cm diameter column in buffer containing 50–200 mM potassium phosphate (pH 7.0), 0.1 mM EDTA, 10 mM β mercaptoethanol, and 10% glycerol. Fractions containing gp45 eluted at 135 mM potassium phosphate. The single major containing band was then removed by chromatography on a 1-ml Mono-Q column (Pharmacia) with a 20-ml linear gradient of 50–350 mM NaCl. The gp45 eluted at 200 mM NaCl as an apparently homogeneous protein.

DNA Substrates—Gapped and single-stranded DNA templates were made using combinations of synthetic dinucleotides (Fig. 1). The 45-mer template strand had either a G or m6G residue as indicated. A three-nucleotide gapped substrate was formed by annealing a 24-mer that was complementary to the template DNA sequence upstream from the m6G residue plus an 18-mer primer that was complementary to template sequence downstream from the m6G residue (Fig. 1, panel A). A single-nucleotide gapped substrate was prepared by annealing a 20-mer primer (Fig. 2, panel B) in place of the 18-mer dinucleotide. A two-nucleotide gapped substrate that allowed measurement of incorporation was made from m6G-stable as well as extended was provided by annealing the 20-mer primer upstream and a 23-mer downstream of the template m6G (Fig. 1 panel C). “Gaps” were flanked by a 3′-OH group for extension of the 18- or 20-mer primers and a 5′-phosphate (P) in the upstream 23- or 24-mers. Primer extension DNA templates did not have a downstream oligomer (Fig. 1B).

The DNAs used were prepared by automated DNA synthesis. The m6G-containing DNA was synthesized by the Regional DNA Synthesis Laboratory at the University of Calgary as described by Sibghat-Ullah and Day (6). Approximately 65–70% of the m6G-containing template could be replicated and templated the preferential incorporation of dTMP in the complementary strand; the remainder of this template was refractory as a DNA template in any of the in vitro replication assays. We inferred from these observations that 65–70% of the template was authentic m6G-containing DNA.

The 18-nucleotide primer (Fig. 1, A and B) and the 20-nucleotide primer (Fig. 1, B and C) were labeled with 32P at the 5′-end using T4 polynucleotide kinase (Pharmacia) and standard reaction conditions (29). The labeled primers were annealed with the other dinucleotides illustrated in Fig. 1 by combining equal concentrations of each dinucleotide (45 nM) in 50 μl of 50 mM Tris-HCl (pH 8.0), 200 μM bovine serum albumin, 100 μM dNTPs, 1.5 mM DTT, 8.3% glycerol, 0.15 mM EDTA, 3.75 mM DNA substrate, and 6.25 mM T4 DNA polymerase. For reactions with gp45, the same reaction conditions were used with the addition of 7.5% polyethylene glycol (Carboxax PEG-8000, Fisher) and 8 μM gp45 monomer. The ratio of DNA substrate to DNA polymerase to gp45 hexamer was 0.6:1:0.213. The high concentrations of gp45 and polyethylene glycol allowed formation of the DNA polymerase-gp45 complex without the additional accessory proteins (30).

The second step was to start the reactions by the addition of Mg2+, or MgCl2 and heparin, to give a final concentration of 6.7 mM MgCl2. Heparin, when added, was at a final concentration of 0.1 mg/ml and was sufficient to trap all free DNA polymerase. The total reaction volume was 12 μl. The conditions for producing an effective heparin trap for T4 DNA polymerase were described previously (26). The reactions were incubated at 37 °C for the indicated times. The reactions were stopped with an equal volume of 12 μl of gel loading solution (92% deionized formamide, bromphenol blue, and xylene cyanol blue markers in TBE (10 mM Tris-HCl (pH 8.3), 100 mM boric acid, 2 mM EDTA)) and placed on ice.

Reaction products were separated by electrophoresis through 15% polyacrylamide gels with 8 M urea in TBE buffer using 45 watts constant power for 80 min. The 32P-labeled products were visualized by autoradiography with Kodak X-Omat AR film. Reaction products were quantitated by densitometry using a Bio-Rad model GS-700 imaging densitometer. Several film exposures were used to keep the signal within the linear range of the film. Analysis of image data was done using the Molecular Analyst software program (version 1.4) provided by Bio-Rad. The rate constant was determined as the slope of the intensity versus time.

Pol β Assay Conditions—DNA pol β reactions were done in two steps. Reaction components, 35 mM Tris-HCl (pH 8.0), 100 mM NaCl, 1.5 mM DTT, 200 μM bovine serum albumin, 100 μM dNTPs, 6.3% glycerol, 3.75 mM DNA substrate, and 6.25 mM pol β were preincubated for 5 min at 37 °C. The reactions were then started with either Mg2+ or a mixture of Mg2+ and heparin at the same concentrations used for T4 DNA polymerase. The conditions for producing an effective heparin trap were found identical to the conditions used for the T4 DNA polymerase. Reaction products were processed as described for the T4 DNA polymerase reactions. The concentration of pol β activity in preparations from human glioma cells was determined by comparing activity with the homogenously purified rat pol β and the nonmodified gapped DNA substrate.

**RESULTS**

Pol β Activity on m6G-containing DNA Substrates—In studies of short patch repair of m6G-T base pairs, we observed...
efficient replication of single-nucleotide gaps across from template m6G by partially purified pol β from human glioma cell lines (discussed below). This result was unexpected from earlier reports of pol β activity on an m6G-containing single-stranded DNA substrate that demonstrated that pol β could not incorporate nucleotides across from m6G (8). These earlier studies, however, did not use what is now known to be the optimal DNA substrate for pol β activity, substrates with small gaps (7). In order to verify that pol β can incorporate nucleotides across from m6G in gapped substrates but not on substrates with long single-stranded regions, a homogeneous preparation of recombinant rat pol β was tested with the DNA substrates shown in Fig. 1.

One of the gapped substrates used was the three-nucleotide gapped substrate illustrated in Fig. 1, panel A. With no m6G in the template, 44% of the primer was fully extended in 15 s to the +3 position, and significant displacement synthesis was observed to the +4 position (Fig. 2, panel A, lane 1). Heparin was used to measure the processivity of pol β DNA replication. When pol β dissociates from the DNA template, the enzyme is effectively trapped by heparin. A burst of replication was observed in the presence of the heparin trap; 12% of the primer was fully extended without dissociation (Fig. 2, panel A, lane2). When these experiments were repeated with the m6G-substrate (lanes 3 and 4), most of the primer extended terminated one nucleotide before the template m6G residue, with or without the heparin trap. Thus, the burst of processive DNA replication by pol β was limited primarily to a two-nucleotide extension in the presence of template m6G. Without the heparin trap, the enzyme could cycle back onto the +2 extended primer for repeated attempts at trans-lesion replication (Fig. 2, panel A, lane 3).

Similar results were observed with single- and double-nucleotide gapped substrates (data presented below). For all short gapped substrates, with or without m6G, efficient replication by pol β required a 5'-terminal phosphate on the downstream oligomer (data not shown). Requirement for the downstream 5'-phosphate for optimal pol β activity was reported previously (7).

Pol β activity was reduced considerably with single-stranded DNA templates (Fig. 1B). Reaction conditions were the same as those used for the gapped DNA substrates. With no m6G in the primer extension template, only 7% of the primer was extended (Fig. 2B, lane 1). Pol β replication on the single-stranded substrate was also dissociative as indicated by the "ladder" pattern of products (Fig. 2B, lane 1) and the absence of replication with the heparin trap (Fig. 2B, lane 2). With the m6G substrate (Fig. 2B, lane 3), 7% of the primer was again extended, but all of the products terminated one position before the template m6G residue. No trans-lesion replication was observed with the heparin trap (Fig. 2B, lane 4).

Because pol β first converted the three-nucleotide gapped substrate into a single-nucleotide gapped substrate before replication of template m6G (Fig. 2A, lane 4), the specificity of nucleotide incorporation across from m6G was investigated with a preformed single-nucleotide gap positioned directly across from either G or m6G. This substrate was formed by annealing the 24-mer plus the 20-mer primer to the template 45-mer (Fig. 1). The reactions contained either 100 μM dCTP, 100 μM dTTP, or both nucleotides each at 100 μM. The rate of incorporation of dTMP was 20-fold higher for the m6G template than for the nonmodified G template that verifies the mutagenic mistemplating of m6G (Table I and Fig. 3). Incorporation of dCMP across from m6G was also detected, but the rate was 5-fold less than that of dTMP.

In the cell, however, both dCTP and dTTP as well as dATP and dGTP are present. The rate of nucleotide incorporation in reactions with both 100 μM dCTP and dTTP and the single-nucleotide gapped substrate was comparable with the rate of dTMP incorporation in reactions that contained only dTTP (Table I and Fig. 3). Whether dCMP or dTMP was incorporated can be determined by the mobility of the +1 product in DNA synthesis.
sequencing gels. The 3’-terminal nucleotide has a significant effect on mobility (31). The primer extended with dCMP has a greater mobility than the primer extended with dTMP (Fig. 3).

In reactions with both 100 μM dCTP and dTTP, primarily the slower moving 3’-dTMP-terminated primer was detected (Fig. 3). Replication of the two-nucleotide gapped substrate can be considered in two steps: 1) extension to the +1 position required incorporation of dCMP or dTMP opposite template m6G, and 2) replication to the +2 position required extension of the m6G-C or m6G-T primer template and incorporation of dGMP opposite template C (Fig. 1, panel C). In reactions with either dCTP or dTTP but not dGTP, the rates of either dCMP or dTMP incorporation across from m6G were slower with the two-nucleotide gapped substrate than for the single-nucleotide gapped substrate which suggests that the single-nucleotide gapped substrate may be the optimal substrate for pol β activity (Table I). The rate of dTMP incorporation opposite m6G was about 3-fold slower for the two-nucleotide gapped substrate (0.006 s⁻¹ compared with 0.02 s⁻¹). Incorporation of dCMP

**Fig. 2. DNA pol β activity on gapped and single-stranded DNA substrates.** Pol β was incubated with the three-nucleotide gapped substrate, with template G or m6G (panel A) or with the single-stranded DNA substrate, with template G or m6G (panel B). As in Fig. 1, m6G is indicated by an * over the G residue. All four dNTPs, each at 100 μM, were present. Reactions were incubated for 15 s, and the products were resolved by gel electrophoresis. The gels were dried and exposed to x-ray film. The amount of gap-filling or primer extension was quantitated by densitometer scanning. The values for extension are relative to the amount of labeled DNA. Reactions without heparin are indicated by a minus sign, whereas reactions with heparin are indicated by the plus sign. Detailed reaction conditions are described under “Experimental Procedures.”

**Table I**

| Nucleotide | Substrate* | Extension | Extension rate |
|------------|------------|-----------|---------------|
| dCTP       | G          | +1        | 1b            |
| dCTP       | m6G        | +1        | 0.001         |
| dTTP       | G          | +1        | 0.004         |
| dTTP       | m6G        | +1        | 0.02          |
| dCTP + dTTP| G          | +1        | 0.01          |
| dCTP       | m6G        | +1        | 0.001         |
| dTTP       | G          | +1        | 0.006         |
| dCTP + dGTPc| G          | +1        | 0.002         |
| dTTP + dGTP| G          | +1        | 0.0003        |
| dCTP + dGTP| m6G        | +1        | 0.004         |
| dTTP + dGTP| m6G        | +1        | 0.003         |

* The DNA substrates were the single nucleotide gapped substrate with either G or m6G in the template position across from the gap or the two-nucleotide gapped substrate illustrated in Fig. 1, panel C.

**Table I**

Replication of m6G DNA substrates by DNA pol β

Rates of nucleotide extension were determined as described under “Experimental Procedures.” Reactions were incubated for 15 s, 1, 10, and 20 min. Rates were determined from densitometric scanning of exposed x-ray films (note Fig. 3).

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**Table I**

| Nucleotide | Substrate* | Extension | Extension rate |
|------------|------------|-----------|---------------|
| dCTP       | G          | +1        | 1b            |
| dCTP       | m6G        | +1        | 0.001         |
| dTTP       | G          | +1        | 0.004         |
| dTTP       | m6G        | +1        | 0.02          |
| dCTP + dTTP| G          | +1        | 0.01          |
| dCTP       | m6G        | +1        | 0.001         |
| dTTP       | G          | +1        | 0.006         |
| dCTP + dGTPc| G          | +1        | 0.002         |
| dTTP + dGTP| G          | +1        | 0.0003        |
| dCTP + dGTP| m6G        | +1        | 0.004         |
| dTTP + dGTP| m6G        | +1        | 0.003         |

* The DNA substrates were the single nucleotide gapped substrate with either G or m6G in the template position across from the gap or the two-nucleotide gapped substrate illustrated in Fig. 1, panel C.

**Full extension was observed by the earliest time point in reactions with the nonmodified template and the correct nucleotide, dCTP. The extension rate of 1 s⁻¹ on nonmodified templates is from published reports and is included as a reference point to compare reactions with the m6G template (8).**

**For the two-nucleotide gapped substrate, dCTP and dGTP are the correct nucleotides for incorporation (Fig. 1, panel C).**
across from m6G was slowed from 0.004 s⁻¹ to 0.001 s⁻¹ for the two-nucleotide gapped substrate (Table I). Adding the next correct nucleotide, dGTP, in reactions with dTTP did not appreciably affect the rate of trans-lesion replication (0.004 s⁻¹ compared with 0.006 s⁻¹), but the addition of dGTP to reactions with dCTP slowed the rate of trans-lesion replication by about 3-fold (0.0003 s⁻¹ compared with 0.001 s⁻¹).

The rate-limiting step in filling the two-nucleotide gapped m6G substrate was incorporation of dTTP or dCMP across from m6G and not extension of either the T:m6G or C:m6G base pair since the rates of +1 and +2 extension were similar, 0.0003 and 0.0002 s⁻¹ for the dCTP/dGTP reaction and 0.004 and 0.003 s⁻¹ for the dTTP/dGTP reaction. Thus, although incorporation of a nucleotide opposite m6G was slow, most of this product was extended relatively rapidly. This result was in contrast to replication of the nonmodified G template with dTTP and dGTP. Both misincorporation of a dTMP opposite template G and extension of the T:G mispair were kinetically slow steps.

Pol β from Human Cancer Cell Lines—Having confirmed that recombinant rat pol β can replicate m6G residues located in short gapped DNA substrates, albeit slowly and requiring recycling of the enzyme on and off the primer template, we re-examined pol β purified from human glioma cell lines. Both cell lines were deficient in m6G methyltransferase activity, but while the parental cell line was highly sensitive to the cytotoxic effects of alkylating agents (cell line A1235), a cell line isolated from the parent (A1235MR4) was tolerant to alkylating agents. Two hypotheses to explain the tolerance were tested: 1) to determine if long patch mismatch repair was defective in the tolerant cell line as observed by Branch et al. (17) and Kat et al. (18) in studies of other m6G-tolerant cell lines, and 2) to determine if some aspect of short patch repair was altered in the tolerant cell line. We report here on studies designed to test the second hypothesis.

Both the A1235 m6G-sensitive cell line and the A1235MR4 m6G-tolerant cell line catalyze removal of thymine from m6G:T base pairs (6). Thus, single-nucleotide gaps are expected to occur in both sensitive and tolerant cell lines in response to attempted correction of m6G:T base pairs. Tolerance could arise if gap-filling activity by pol β and subsequent ligation were more effective in tolerant cells than in sensitive cells. Partially purified preparations of pol β from sensitive and tolerant cell lines were tested on the three-nucleotide gapped substrate (Fig. 4). When similar amounts of the partially purified human pol β (lanes 1 and 2) and recombinant rat pol β (lanes 3 and 4) were tested on the m6G-containing three-nucleotide gapped substrate, good gap-filling activity was detected for both pol β preparations. In comparison to the 15-s reactions shown in Fig. 2, panel A, more gap-filling activity was observed in the longer 1- and 10-min reactions. Similar results were observed with the single-nucleotide gapped substrate. No differences were detected between pol β preparations from the m6G-sensitive or -tolerant cell lines, but less displacement synthesis to the +4 position was observed for the partially purified pol β preparations than for the highly purified rat pol β (Figure 4, lane 2 and 4).

Bacteriophage T4 DNA Polymerase Activity on m6G-containing DNA Substrates—Strong gap-filling activity on the three-nucleotide nonmodified gapped substrate was observed for wild type T4 DNA polymerase, but T4 DNA polymerase was less processive than pol β. In the presence of the heparin trap, just 3% of the primer was extended to the +3 and +4 positions by T4 DNA polymerase (Fig. 5, panel A), whereas 13% of the primer was extended by pol β (Fig. 2A, lane 2). High processivity was observed, however, in reactions with the T4 DNA polymerase-gp45 complex (Fig. 5, panel A). Products longer than three nucleotides were produced, particularly in reactions with the T4 DNA polymerase-gp45 complex. In separate reactions, no degradation of the downstream oligonucleotide was observed. Thus, the T4 DNA polymerase-gp45 complex can catalyze significant displacement replication. Only limited gap filling was observed with the m6G-gapped substrate, and this activity required gp45 (Fig. 5, panel B). As observed for pol β (Fig. 2), most replication terminated one nucleotide before template m6G at the +2 position.

Two-mutant T4 DNA polymerases were also tested with the three-nucleotide gapped substrates, the D112A + E114A-T4
DNA polymerase which lacks 3'→5'-exonuclease activity (25) and the L412M-T4 DNA polymerase which has increased intrinsic processivity (26). The mutants were no more active under these conditions than the wild type enzyme, even in the presence of gp45 (data not shown). The wild type and mutant T4 DNA polymerases were also inactive on single-nucleotide gapped substrates with template m6G.

In contrast to the poor primer extension activity observed for pol β on the single-stranded DNA template (Fig. 2, panel B), T4 DNA polymerase was highly active and replicated the full-length (+27) product even in the presence of the heparin trap (Fig. 5, panel C). As expected, the T4 DNA polymerase-gp45 complex was more processive than T4 DNA polymerase alone (Fig. 5, panel C), and this processivity was required for bypass replication of template m6G (Fig. 5, panel D). In the absence of gp45 most primer extension stopped at position +27, one position before template m6G.

The exonuclease-deficient D112A + E114A-DNA polymerase and the L412M-DNA polymerase which has increased intrinsic processivity were also tested with the single-stranded m6G DNA substrate. Neither of the mutant DNA polymerases were able to produce any more full-length product than detected for the wild type enzyme during the 15-s incubation or with the heparin trap (Fig. 5). As observed for wild type T4 DNA polymerase, the T:m6G primer terminus may be extended to produce full-length product or degraded to produce the 18-mer product. For the exonuclease-deficient D112A + E114A-DNA polymerase, degradation was not a possibility, but the same amounts of 18-mer and full-length products were produced as observed for the wild type T4 DNA polymerase-gp45 complex (Fig. 5). Thus, incorporation of a nucleotide opposite m6G is coupled to the subsequent extension reaction, at least under these reactions conditions with 100 μM dNTPs.

The exonuclease-deficient D112A + E114A-DNA polymerase was used to measure specificity of nucleotide incorporation.
Replication of m6G-Containing DNA

**DISCUSSION**

Previous experiments indicated that DNA polymerases replicated m6G-containing substrates with difficulty (Escherichia coli DNA pol I, T4 DNA polymerase) or not at all (mammalian pol β). Since certain cells are tolerant to considerable amounts of m6G modified DNA, some cellular DNA polymerases must be able to replicate m6G-containing DNA reasonably well, or tolerant cells have one or more mutant DNA polymerases with enhanced m6G trans-lesion replication activity.

Experiments presented here confirmed that pol β cannot replicate past an m6G residue located in a single-stranded region of DNA (Fig. 2, panel B) as would occur at a replication fork or in a long patch mismatch repair track (8). The optimal DNA substrates for pol β, however, are gapped substrates with single-stranded regions from one to six nucleotides (7). With small gapped DNA substrates, pol β incorporated dTMP across from m6G if the DNA on the 5′-side of the gap was phosphorylated (Fig. 2, panel A). With prolonged incubation, significant gap-filling replication was observed (Fig. 2). T4 DNA polymerase also catalyzed an efficient gap-filling reaction on the single-nucleotide gapped substrate, but gap filling was generally not processive (Fig. 5A). The T4 DNA polymerase-gp45 complex was highly processive, but on the gapped substrate only a low level of nucleotide incorporation across from m6G was detected (Fig. 5, panel B). The T4 DNA polymerase-gp45 complex was even less active in m6G-trans-lesion replication on single-nucleotide gapped substrates which indicates that pol β is better suited than T4 DNA polymerase for gap-filling replication with template m6G.

Unlike pol β, the phage T4 DNA polymerase alone, and particularly when complexed with gp45, catalyzed efficient replication of the primed single-stranded DNA substrate (Fig. 5, panel C). The gp45 protein clamps the T4 DNA polymerase to DNA and increases the processivity of DNA replication as demonstrated in reactions with the heparin trap (Fig. 5, panel C). Trans-lesion replication was observed on the single-stranded DNA substrate and was dependent on the gp45 accessory protein (Fig. 5, panel D).

In reactions with exonuclease-proficient and -deficient T4 DNA polymerase-gp45 complexes, there was no accumulation of product that terminated at the lesion (+3) (Fig. 6). Primers terminated one position before the template m6G (+2) or full-length (+27) product was observed (Fig. 5, panel D). This observation indicates that once a nucleotide was incorporated opposite m6G, the DNA polymerase was committed to extension of the primer terminus. Thus, the rate-limiting step in trans-lesion replication was incorporation of a nucleotide across from m6G. Incorporation of a nucleotide opposite template m6G was also the rate-limiting step for pol β, but pol β produced a small amount of product that terminated opposite template m6G (Table I). Both pol β and T4 DNA polymerase incorporated dTMP preferentially across from template m6G (Tables I and II and Fig. 3).

Replication bypass of template m6G by either pol β or the T4 DNA polymerase-gp45 complex was reduced under reaction conditions containing the heparin trap, which indicates that the DNA polymerases frequently dissociated from the template when template m6G residues were encountered (Figs. 2, 5, and 6). Dissociation was observed even when the T4 DNA polymerase was clamped to the DNA by the gp45 protein (Fig. 5, panel D, and Fig. 6). Together, these observations suggest that m6G residues in various DNA contexts are not efficiently replicated. The reduced need to replicate m6G DNA, as would occur in long patch mismatch repair defective cells, may then contribute to tolerance of this type of DNA damage (17, 18).
There is still a question, however, as to how chromosomes containing large quantities of m6G are replicated even if cells lack long patch mismatch repair. Although we report here replication of template m6G residing in short gaps by pol β and in single-stranded DNAs by the T4 DNA polymerase-gp45 complex, replication is not robust. Mutant T4 DNA polymerases with reduced exonuclease activity or increased processivity may assist trans-lesion replication by allowing repeated attempts at nucleotide incorporation across from m6G without concurrent primer degradation. When the wild type T4 DNA polymerase-gp45 complex encountered template m6G, bypass was observed about 25% of the time and dissociation about 75%. (Compare 32% of +2 product formed with 10% full-length product in the reaction with heparin in Fig. 5, panel D.) If the T4 DNA polymerase was allowed to reassociate with the primer terminus, which occurred in reactions without the heparin trap, primer degradation was observed. About 44% of the primers were degraded to lengths shorter than the starting 18-mer (Fig. 5, panel D). In reactions with the mutant T4 DNA polymerases complexes with gp45, again most primer extension stopped at +2, one position before template m6G; however, the primers were not degraded. The absence of primer degradation allows more opportunity by the mutant DNA polymerases to attempt trans-lesion replication.

Although pol β from sensitive and tolerant human glioma cell lines were equally proficient in gap replication with template m6G, we did not measure another activity of pol β and the ability to excise 5′-terminal deoxyribose phosphate residues (32). The association of 5′-DNA-deoxyribophosphodiesterase activity with DNA polymerase activity makes pol β an ideal enzyme for short patch DNA repair, and recent studies demonstrate that pol β functions specifically in base-excision repair in vivo (33). It would be informative to test if 5′-DNA-deoxyribophosphodiesterase activity is affected by m6G.

There may be other factors in the cell besides DNA polymerases and mismatch repair that may contribute to the tolerance of m6G in DNA. Because the “tolerant” cells studied are sensitive to alkylating agents in culture and are also frequently derived from malignant or tumor cells, several differences compared to primary “normal” cells are expected. Extension of the S phase of the cell cycle, for example, could contribute to tolerance by allowing a longer time for chromosome replication; hence, a longer time for trans-lesion replication. Along with an extended S phase, apoptosis would also need to be prevented or the onset delayed. Another possibility is that standard methods of chromosome replication in tolerant cells may be supplemented by other mechanisms such as replication restart and recombinational repair that may assist replication of damaged chromosomes. Because of the use of alkylating agents in chemotherapeutic regimes, it is necessary to continue to pursue the molecular basis of tolerance to m6G. These studies, in turn, will provide important insights into differences between normal and cancer cells.