DNA Damage at Thymine N-3 Abolishes Base-pairing Capacity during DNA Synthesis*

(Received for publication, January 26, 1987)

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3-Methylthymine was synthesized into DNA copolymers and deoxynucleoside triphosphate to study its effect on DNA synthesis by the Klenow fragment of Escherichia coli polymerase I and avian myeloblastosis virus reverse transcriptase. Both polymerases were greatly inhibited by template 3-methylthymine. In response to 3-methylthymine, misincorporation of dTTP increased slightly, but occurred only at low levels consistent with spontaneous misincorporation in vitro. Surprisingly, template 3-methylthymine resulted in a striking decrease in background misincorporation, relative to normal incorporation by the Klenow fragment, of dGTP and, to a lesser extent, of dATP and dCTP.

The incorporation of 3-methyl-dTTP into DNA was studied using DNA sequencing technology. The Klenow fragment failed to incorporate 3-methyl-dTTP even at 1 mM. Reverse transcriptase incorporated 3-methyl-dTTP opposite adenine, cytosine, and thymine, but at only about 1/40,000th the efficiency of complementary deoxynucleoside triphosphate incorporation. Furthermore, synthesis generally stalled at sites of 3-methylthymine incorporation. From these results, we conclude that damage at the central hydrogen-bonding position of thymine abolishes its base-pairing capabilities during DNA synthesis.

DNA replication depends upon the faithful formation of Watson-Crick hydrogen-bonded base pairs. Modifications that block sites involved in hydrogen bonding might be expected to adversely affect base-pair formation. Alkylating agents, such as N-methyl-N-nitrosoarena, can alkylate the central hydrogen-bonding position of the nucleic acid bases whether in single- or double-stranded DNA (1). This damage may persist since repair activities for such damage have not been reported (2). Thus, it is important to know the consequences of this damage for DNA replication. A good model lesion for damage at the central hydrogen-bonding position is 3-methylthymine, which we found to be stable in solution; 1-

* This work was supported by United States Public Health Service Grants GM24798 and CA28632 (to M. D. T.) and National Research Service Traineeship 5T32 GM07092 (to A. C. H.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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MATERIALS AND METHODS

Nucleic Acids and Enzymes—Terminal deoxynucleotidyltransferase, poly(dC) (Sigma), ultrapure-grade dNTPs, and deoxyribonucleotide primers were purchased from Pharmacia P-L Biochemicals. Tritium-labeled dNTPs were purchased from ICN, and 32P-labeled and 35S-labeled dNTPs were purchased from Du Pont-New England Nuclear. The Klenow fragment was obtained from Bethesda Research Laboratories. AMV reverse transcriptase and Penicillium citrinum nuclease P1 were purchased from Boehringer Mannheim. E. coli alkaline phosphatase, snake venom 5'-nucleotidase, and yeast inorganic pyrophosphatase were obtained from Sigma.

Phage f-R206 DNA, an f-PBR8322 chimera (13), was isolated by standard procedures (14) from E. coli atrain K37 infected with phage 1. The abbreviations used are: dNTPs, deoxynucleoside triphosphates; AMV, avian myeloblastosis virus; HPLC, high pressure liquid chromatography.
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Mismatched DNA was resuspended in 38-44 μL of 0.125 M Tris-HCl buffer (pH 7.4, 1 mM MgCl₂, 1.2 mM β-mercaptoethanol, and 7.5 mM NaCl). Absorbance at 259 nm was measured by a Perkin-Elmer 320 analytical UV-visible spectrophotometer as the solution temperature was raised 1 °C/min from 4 to 43 °C by an automatic temperature programmer and digital controller (Perkin-Elmer).

DNA Sequencing Reactions—Minus sequencing reactions (27) were run essentially as described elsewhere (28). An array of asynchronously extended, labeled primers was produced using, as template-primer, 5'-R208 single-stranded DNA annealed with the synthetic dodecamer (26).

For minus reactions catalyzed by the Klenow fragment, labeled DNA was resuspended in 38-44 μL of 0.125 M Tris-HCl buffer (pH 7.4 at 0 °C), 50 mM MgCl₂, and 50 mM β-mercaptoethanol. Five-μl reactions contained 2 μL of resuspended DNA, 2 μL of the appropriate minus mixture (25 μM concentration of each of three dNTPs plus 3-methyl-dTTP at various concentrations), and 0.8 unit of the Klenow fragment in 1 μL of dilution buffer. Reactions were incubated at 0 °C for 30 min and then stopped by addition of 5 μL of stop mixture (0.025% bromphenol blue, 0.025% xylene cyanol, and 10 mM EDTA in formamide).

For minus reactions catalyzed by AMV reverse transcriptase, labeled DNA was resuspended in 38-44 μL of 0.125 M Tris-HCl buffer (pH 7.4 at 0 °C), 15 mM MgCl₂, 25 mM dithiothreitol, and 0.25 mM NaCl. Five-μl reactions contained 2 μL of resuspended DNA, 2 μL of the appropriate minus mixture (12.5 μM concentration of each of three dNTPs plus 3-methyl-dTTP at various concentrations), and 5 units of reverse transcriptase in 1 μL of dilution buffer. Reactions were incubated at 37 °C for 1 h and then stopped by addition of 5 μL of stop mixture.

All stopped reaction mixtures were heated to 100 °C, cooled, and then electrophoresed as described (22). After electrophoresis, the gel was soaked for 15 min in 10% acetic acid, 10% methanol, dried at 80 °C under vacuum onto Whatman No. 3MM chromatography paper, and autoradiographed.

RESULTS

Polydeoxyribonucleotide Templates—The chemical nature of 3-methylthymine suggested that it would be relatively free of spontaneous chemical rearrangements. Therefore, we synthesized 3-methyldeoxythymine and confirmed its stability by reversed-phase HPLC after incubation at 37 °C for 18 h at pH 2.0, 7.4, and 12.0 (not shown). Polymers were synthesized using terminal deoxyribo nucleotidyltransferase, 3-methyl-dTTP, and purified dNTPs (Table 1). It is interesting to note that terminal deoxyribo nucleotidyltransferase was biased for or against 3-methyl-dTTP depending on the unmodified dNTP in the reaction (Table 1).

Inhibition of Replication by 3-Methyldeoxythymine in Template—3-Methylthymine in poly(dT,3-mdT) (Fig. 1) and in poly(dA,3-mdT) and poly(dC,3-mdT) (Fig. 2) copolymer templates inhibited DNA synthesis by the Klenow fragment. If 3-methyldeoxythymine acts as a strong block to strand elongation, then it should be a strong inhibitor of the Klenow fragment. However, 3-methyldeoxythymine was only a weak inhibitor of the Klenow fragment (Fig. 1B) and did not inhibit DNA synthesis by the Klenow fragment at 37 °C (Table 1).
plates were purified, analyzed for composition, and then sized on polyacrylamide slab gels as described under "Materials and Methods." The resulting template-primer complexes were assayed by gel electrophoresis as described under "Materials and Methods." Samples (3 µl) were taken after 5, 15, 30, and 45 min incubation, were analyzed as described (26). Values for present inhibition of replication were calculated relative to incorporation of dNTP opposite corresponding homopolymer controls. The amounts of poly(dA), poly(dT), and poly(dC) replicated by the Klenow fragment were 33.2 = 0.3, 29.8 = 1.5, and 46.3 = 0.3% of available template nucleotide, respectively. Reverse transcriptase replicated 18 = 2% of poly(dC). Values for reactions catalyzed by the Klenow fragment are the mean = S.D. (n = 3). Values for reactions catalyzed by reverse transcriptase are the mean of duplicate samples at range. Reactions catalyzed by the Klenow fragment contained poly(dA,3-mdT) (O), poly(dT,3-mdT) ( ), or poly(dC,3-mdT) ( ) templates. Reactions catalyzed by reverse transcriptase contained poly(dC,24.0% 3-mdT) ( ).

![Fig. 1. Kinetics of replication of poly(dT) and poly(dT,3-mdT) copolymers by the Klenow fragment.](image1)

![Fig. 2. Inhibition of replication by 3-methyldeoxythymine in template.](image2)

![Fig. 3. Effect of increasing temperature on the 259 nm UV absorbance of poly(dT)-oligo(dA) and poly(dT,17.3% 3-mdT)-oligo(dA).](image3)
bases (29). In addition, electrophoresis of the polymerization reaction products (on a DNA sequencing gel after incorporation of radiolabeled nucleotide) revealed a high molecular weight product in the absence of 3-methyldeoxythymine, but primarily low molecular weight product in the presence of 3-methyldeoxythymine (not shown).

3-Methyldeoxythymine-directed Incorporation by the Klenow Fragment—To determine if inhibition of replication by 3-methyldeoxythymine in template resulted from lack of incorporation opposite 3-methyldeoxythymine, incorporation of dATP and dTTP was studied during replication of poly(dC,3-mdT). Similarly, incorporation of dCTP and dGTP was studied during replication of either poly(dA,3-mdT) or poly(dT,3-mdT). Incorporation of dNTPs complementary to the primer dNTP could not be determined due to possible nucleotide addition to the template terminus at overlapping ends.

Only dTTP incorporation increased detectably in response to 3-methyldeoxythymine (Table II), whereas, surprisingly, incorporation of the other dNTPs generally decreased more than the decrease in DNA synthesis in response to 3-methyldeoxythymine. For example, noncomplementary (radiolabeled) dGTP incorporation decreased 100-fold opposite poly(dA,5.8% 3-mdT) relative to that opposite poly(dA) (19.4 ± 22 versus 2000 ± 22 dpm, respectively; each value is the result of three determinations). In contrast, dTTP incorporation opposite poly(dA,5.8% 3-mdT) decreased only 2.5-fold (Fig. 2) relative to that opposite poly(dA) (1.5 × 10⁶ versus 3.8 × 10⁶ dpm, respectively).

3-Methyldeoxythymine-directed Incorporation by AMV Reverse Transcriptase—Since the Klenow fragment apparently only incorporated a small amount of dTTP opposite 3-methyldeoxythymine, we tested the error-prone AMV reverse transcriptase. Incorporation of noncomplementary dNTP was tested opposite poly(dC) and poly(dC,24.0% 3-mdT) since only poly(dC) is used efficiently as template by AMV reverse transcriptase (30). The nucleotide pool was biased 20-fold in favor of the noncomplementary dNTP; and, as with the Klenow fragment, only stimulation of dTTP incorporation (10-fold) was detected (Table III). A small increase in dCTP incorporation was noted, but it was within experimental error.

### Table II

| Noncomple- | Ratio of noncomplementary dNTP to total dNTP incorporation (×10⁶) |
| mentary | | |
| dNTP | Poly(dA) | Poly(dA,3.0% 3-mdT) | Poly(dA,5.8% 3-mdT) |
| dCTP | 1.44 ± 0.01 | 1.3 ± 0.4 | 1.0 ± 0.1 |
| dGTP | 5.66 ± 0.08 | 0.59 ± 0.08 | <0.1 ± 0.2 |
| poly(dC) | Poly(dC,14.3% 3-mdT) | Poly(dC,24.0% 3-mdT) |
| dATP | 8 ± 1 | 5.0 ± 0.4 | 5.5 ± 0.4 |
| dTTP | 0.43 ± 0.03 | 1.39 ± 0.09 | 0.8 ± 0.1 |
| poly(dT) | Poly(dT,8.8% 3-mdT) | Poly(dT,17.3% 3-mdT) |
| dCTP | 0.4 ± 0.35 | 0.35 ± 0.02 | <0.1 ± 0.1 |
| dGTP | 2.0 ± 0.1 | 0.8 ± 0.1 | <0.5 ± 1 |

### Table III

<table>
<thead>
<tr>
<th>Incorporation of noncomplementary dNTP by AMV reverse transcriptase</th>
</tr>
</thead>
<tbody>
<tr>
<td>Noncomplementary dNTP</td>
</tr>
<tr>
<td>dATP</td>
</tr>
<tr>
<td>dCTP</td>
</tr>
<tr>
<td>dTTP</td>
</tr>
</tbody>
</table>

### Turnover of Noncomplementary dNTP by the Klenow Fragment—Noncomplementary dNTPs could have been incorporated by the Klenow fragment, but then removed as dNMPs (turnover) by the polymerase's proofreading 3'- to 5'-exonuclease. Therefore, turnover was measured concomitant with incorporation. Only dGTP opposite poly(dA) gave measurable levels of turnover; 3.1 ± 0.02 bases turned over per 1000 bases incorporated (not shown). This result agrees with results from incorporation studies (Table II), in which the highest level of misincorporation was seen with dGTP during replication of poly(dA). Turnover of dGTP was decreased to undetectable levels by 3-methyldeoxythymine in template, which suggests that the dramatic decrease in dGTP misincorporation in response to template 3-methyldeoxythymine resulted from selectivity in the incorporation step rather than in an exonucleolytic proofreading step.

### Incorporation and Turnover of Complementary dNTP by the Klenow Fragment—The Klenow fragment did not turn over any noncomplementary dNTP in response to 3-methyldeoxythymine in template. However, "idiing" of polymerase at a 3-methyldeoxythymine block to elongation would result in increased turnover of the complementary dNTP. Therefore, turnover and incorporation of complementary dNTP were determined during replication of copolymers containing 3-methyldeoxythymine (Table IV).

Turnover of each complementary dNTP increased in response to 3-methyldeoxythymine in template (Table IV), whereas incorporation of each complementary dNTP decreased, in agreement with results in Figs. 1 and 2. Turnover of dATP and dGTP was low relative to that of dTTP, which probably reflects the relative stacking interactions on the nascent strand at the growing point (26), coupled with weaker A-T base pairing. The increase of complementary dNTP turnover in response to 3-methyldeoxythymine is further evidence that 3-methyldeoxythymine is affecting the polymerase rather than primer-template annealing.

### Incorporation of Precursor 3-Methyl-dTTP by the Klenow Fragment—Besides the base-pairing capacity of template 3-methyldeoxythymine, the base-pairing capacity of precursor 3-methyl-dTTP was also determined during DNA synthesis. DNA sequencing technology enables characterization of modified precursor incorporation by DNA polymerases over a wide range of sequence (28). Using this technology, we determined that 3-methyl-dTTP (0.25–1 mM) was not incorporated.
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**TABLE IV**

Incorporation and turnover of complementary dNTP by the Klenow fragment

Reactions included a 33 μM concentration of the complementary dNTP and an equimolar concentration of the companion dNTP listed. Samples (3 μl) were taken after 60 min and analyzed by thin-layer chromatography (see "Materials and Methods"). Values in the final column are multiplied by 10^3 and represent the ratio of complementary dNTP turned over to the sum of all complementary dNTP, either turned over or incorporated into polymer. All values are given as mean ± S.D. (n = 3).

<table>
<thead>
<tr>
<th>Complementary dNTP</th>
<th>Companion dNTP</th>
<th>Template</th>
<th>Complementary dNTP incorporated</th>
<th>Complementary dNTP turned over</th>
<th>Turnover rate (×10^3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>dATP</td>
<td>dCTP</td>
<td>Poly(dT)</td>
<td>89 ± 1</td>
<td>&lt;0.11 ± 0.03</td>
<td>12.2 ± 0.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(dT, 17.3% 3-mdT)*</td>
<td>17.9 ± 0.9</td>
<td>0.67 ± 0.09</td>
<td>37 ± 5</td>
</tr>
<tr>
<td>dGTP</td>
<td>dATP</td>
<td>Poly(dC)</td>
<td>98 ± 1</td>
<td>&lt;0.14 ± 0.08</td>
<td>2.4 ± 0.2</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(dC, 24.0% 3-mdT)</td>
<td>14.8 ± 0.2</td>
<td>0.5 ± 0.2</td>
<td>34 ± 14</td>
</tr>
<tr>
<td>dGTP</td>
<td>dTTP</td>
<td>Poly(dC)</td>
<td>112 ± 1</td>
<td>&lt;0.15 ± 0.06</td>
<td>&lt;1.3 ± 0.5</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(dC, 24.0% 3-mdT)</td>
<td>24 ± 3</td>
<td>0.2 ± 0.3</td>
<td>3.3 ± 12.5</td>
</tr>
<tr>
<td>dTTP</td>
<td>dGTP</td>
<td>Poly(dA)</td>
<td>62 ± 2</td>
<td>0.4 ± 0.2</td>
<td>6.5 ± 3.3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Poly(dA, 5.8% 3-mdT)</td>
<td>35 ± 1</td>
<td>0.57 ± 0.08</td>
<td>16 ± 2</td>
</tr>
</tbody>
</table>

*3-mdT, 3-methyldeoxythymine.

by the Klenow fragment at any of the 38 sites tested during replication of primed φ1-R208 single-stranded DNA (not shown).

A minimum estimate was made of the difference in abilities of 3-methyl-dTTP and dTTP to be incorporated opposite adenine by the Klenow fragment. The highest concentration (1 mM) of 3-methyl-dTTP tested showed no sign of incorporation, whereas dTTP at 250 nM gave significant replication (not shown). The results demonstrate, under our conditions, at least a 4000-fold preference for dTTP over 3-methyl-dTTP by the Klenow fragment.

**Incorporation of 3-Methyl-dTTP by AMV Reverse Transcriptase**—Similar to the lack of 3-methyl-dTTP incorporation by the Klenow fragment, AMV reverse transcriptase incorporated 3-methyl-dTTP, but only inefficiently even at 1 mM. Incorporation occurred opposite adenine, cytosine, and thymine template residues, but not opposite guanosine residues. Examples of 3-methyl-dTTP incorporation, demonstrated by a decrease in band intensity relative to control, can be seen in minus adenine reactions at positions 3669 and 3698 (Fig. 4a), in minus guanosine reactions at positions 3672 and 3700 (Fig. 4b), and in minus thymine reactions at positions 3664 and 3674 (Fig. 4c), but not in minus cytosine reactions (Fig. 4d).

To determine the relative abilities of 3-methyl-dTTP and dTTP to be incorporated opposite adenine by AMV reverse transcriptase, concentrations of the two dNTPs required for similar extension of DNA strands past blocked thymine sites were compared. One mM 3-methyl-dTTP (Fig. 4c) was required to produce effects similar to those seen with 25 nM dTTP (Fig. 5). This suggested that the AMV reverse transcriptase incorporation rate for 3-methyl-dTTP was about 1/40,000th that of dTTP under our conditions.

**3-Methyl-dTTP Incorporation Stalls DNA Synthesis**—3-Methyl-dTTP was incorporated by AMV reverse transcriptase in place of three of the four normal dNTPs, although with low efficiency. However, strand elongation frequently did not continue beyond the incorporation site, as seen in autoradiograms where intensity lost from one band due to 3-methyl-dTTP incorporation accumulated in the following band (Fig. 4).

3-Methyl-dTTP incorporated primarily either at single sites (i.e. at nonrepetitive sequences) or at the first position in a repeat of identical bases. AMV reverse transcriptase failed to incorporate past doublet AA at positions 3676-3677 (Fig. 4a), GG at 3662-3663, and 3678-3679 (Fig. 4b), TT at 3664-3665, 3684-3685, and 3701-3702 (Fig. 4c). At two adenine doublets (positions 3669-3670 and 3707-3708, Fig. 4a), 3-methyl-dTTP incorporated sequentially, although at low levels. Lack of incorporation may be due to steric effects or the instability of two sequential base pairs containing 3-methyl-dTTP. Alternatively, stalling after the incorporation of a single 3-methyl-dTTP may occur regardless of the dNTP that is to be incorporated next since stalling occurred at almost all single sites in which 3-methyl-dTTP was incorporated.

**DISCUSSION**

DNA replication in vitro was used to study the response of the Klenow fragment of E. coli DNA polymerase I and AMV reverse transcriptase to deoxyribonucleotide templates containing 3-methyldeoxythymine. DNA replication by both polymerases was inhibited by these templates. For the Klenow fragment, the extent of inhibition appeared to be dependent upon the amount of template 3-methyldeoxythymine, but not upon the identity of the unmodified template residue. This relationship was not examined for AMV reverse transcriptase. Replication by these two polymerases is also inhibited by noncoding apurinic sites, with replication by AMV reverse transcriptase less inhibited than replication by the Klenow fragment (31).

Only dTTP incorporation was detected in response to template 3-methyldeoxythymine. To evaluate the significance of this incorporation, the amount of incorporation possible opposite 3-methyldeoxythymine encountered by the Klenow fragment and AMV reverse transcriptase while replicating poly(dC, 24.0% 3-mdT) was determined. 3-Methyldeoxythymine was randomly distributed in the copolymers (32), so the number of 3-methyldeoxythymine residues encountered by polymerase given the percent of DNA replicated was calculated using a hypergeometric distribution (33). The result was 2,500 3-methyldeoxythymine residues encountered by AMV polymerase and 2,537 3-methyldeoxythymine residues encountered by the Klenow fragment per 10,000 bases replicated. These expected incorporation rates exceeded the observed dTTP incorporation rates opposite 3-methyldeoxythymine using AMV reverse transcriptase (Table III) by 2,155-fold and using the Klenow fragment (Table II) by 3,171-fold.
Thus, the overall incorporation of dNTP in response to 3-methyldeoxythymine in template was similar to that usually reported for background polymerase infidelity in vitro; about one misincorporation/10^3-10^4 template residues tested (see the background levels of misincorporation reported in this study; and for a review, see Table I in Ref. 34).

Turnover studies confirmed that the Klenow fragment did not efficiently incorporate any dNTP opposite template 3-methyldeoxythymine. Whereas no efficient incorporation or turnover of noncomplementary dNTPs occurred in response to 3-methyldeoxythymine residues, increased turnover of complementary dNTPs did occur. Thus, the polymerase was blocked by its inability to replicate past 3-methyldeoxythymine template residues. Consequently, the Klenow fragment idled, as previously reported for E. coli DNA polymerase I in response to pyrimidine dimers in template (35).

Surprisingly, introduction of 3-methyldeoxythymine into template apparently increased the overall fidelity of the Klenow polymerase without a concomitant increase in turnover of noncomplementary dNTPs. In fact, both noncomplementary dNTP turnover and incorporation decreased in response to 3-methyldeoxythymine relative to complementary dNTP incorporation, indicating that the effect was in the polymerization step of DNA synthesis. This response was most evident for dGTP misincorporation, which was the highest of any noncomplementary dNTP. This apparent increase in fidelity would appear to lend support to the notion that polymerase proofreading of errors can also occur in the polymerization step, independent of the proofreading exonuclease, through release of dNTP (36, 37).

It is not clear to us why a stuttering (progress impeded by 3-methyldeoxythymine) polymerase should be more accurate than a processive polymerase. Possibly, the stuttering polymerase can take greater advantage of the difference in off rates between complementary and noncomplementary dNTPs (36, 37) to enhance its incorporation accuracy.

DNA sequencing technology demonstrated that methylation at N-3 abolishes dTTP's base-pairing ability during DNA synthesis. Whereas the Klenow fragment did not substitute 3-methyl-dTTP, even at 1 mM, for any of the four normal dNTPs, unmodified dNTPs significantly incorporated at 250 nM (this study and Footnote 2), demonstrating a greater than 4,000-fold difference between the incorporation abilities of normal dNTPs and 3-methyl-dTTP. AMV reverse transcriptase substituted 3-methyl-dTTP only at high concentration for dATP, dGTP, and dTTP, but not for dCTP and not at every site. Comparison of 3-methyl-dTTP incorporation with the efficient levels of incorporation of the complementary unmodified dNTPs indicated a 40,000-fold difference in incorporation efficiency.

2 R. Reid and M. D. Topal, unpublished results.
suggestions that modified precursor incorporation during DNA replication may contribute to genomic damage. O\textsuperscript{6}-Methyl-dGTP (22, 40–42) and O\textsuperscript{4}-methyl-dTTP (43, 44) may be considered attractive candidates for incorporation into DNA during DNA synthesis because of their favorable incorporation properties during DNA synthesis; their apparent K\textsubscript{m} values are only about 10-fold higher than for unmodified, complementary dNTP. Indeed, O\textsuperscript{6}-methylthymine, supplied in culture medium, is incorporated in amounts rivaling amounts produced by direct alkylation (45). Our results, however, indicate that 3-methyl-dTTP and other dNTPs damaged at the central hydrogen-bonding position are unlikely to contribute to mutagenesis by this route, based on an apparent insertion rate for 3-methyl-dTTP that is greater than 4,000–40,000-fold below that of dTTP.

REFERENCES

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