Proc. Natl. Acad. Sci. USA
Vol. 79, pp. 2211–2215, April 1982
Biochemistry

DNA precursor pool: A significant target for *N*-methyl-*N*-nitrosourea in C3H/10T½ clone 8 cells

(chemical carcinogenesis/chemical mutagenesis/fidelity of DNA replication/cell cycle specificity)

MICHAEL D. TOPAL AND MARY S. BAKER

Department of Pathology and the Cancer Research Center, University of North Carolina Medical School, Chapel Hill, North Carolina 27514

Communicated by K. M. Brinkhous, January 4, 1982

ABSTRACT Synchronized C3H/10T½ clone 8 cells were treated *in vitro* with a nontoxic dose of *N*-methyl-*N*-nitrosourea during their S phase. Chromatographic isolation of the deoxyribonucleotide DNA precursor pool and measurement of the precursor content per cell showed that a nucleic acid residue in the precursor pool is 190–13,000 times more susceptible to methylation than a residue in the DNA duplex, depending on the site of methylation. This conclusion comes from measurements indicating that, for example, the N-1 position of adenine in dATP is 6.3 times more methylated than the same position in the DNA, even though the adenine content of the pool is only a fraction (0.0005) of the adenine content of the DNA helix. The comparative susceptibility between pool and DNA was found to vary with the site of methylation in the order the N-1 position of adenine > phosphate > the N-3 position of adenine > the O^6 position of guanine > the N-7 position of guanine. The significance of these results for chemical mutagenesis and carcinogenesis is discussed.

We recently proposed, from chemical considerations, that the pool of DNA precursor deoxyribonucleotides should be a significant target *in vivo* for chemical mutagens (1). Such chemical modification of the precursor pool could have significant biological consequences. For example, modified precursors might inhibit nucleotide binding enzymes and perturb DNA replication. Indeed, modification of precursors together with rapid turnover of the nucleotide pool (for discussion, see ref. 2) may offer a direct explanation for the S-phase dependence of alkylation-induced mutation (3) and neoplastic transformation (4, 5) of cells in culture and the chromosome replication point specificity of alkylating agents in bacteria (6, 7). In support of our proposal, dATP was shown to be a good target *in vitro* for *N*-methyl-*N*-nitrosourea (MNU), a potent methylating agent, mutagen, and carcinogen (1). Moreover, methylated nucleotide products from this reaction were found to incorporate into DNA during replication by bacteriophage T4 DNA polymerase *in vitro* (1). For these *in vitro* results to be applicable *in vivo*, however, the DNA precursor pool must be shown to be a significant target for MNU.

In this paper, we report that the DNA precursor pool in mouse embryo fibroblast C3H/10T½ clone 8 cells (10T½ cells) (8) is modified to a greater extent per cell than is the DNA. Furthermore, determination of the cellular content of deoxyribonucleotide residues in the precursor pool enables us to conclude that the purine nucleotide residues in the pool are 190–13,000 times more susceptible to modification by MNU than the same residues in the DNA helix, depending on the site being modified. The order of susceptibility was found to be the N-1 position of adenine > phosphate > the N-3 position of adenine > the O^6 position of guanine > the N-7 position of guanine.

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

MATERIALS AND METHODS

Deoxyribonucleotides, Deoxyribonucleosides, and Methylated Purine Standards. Purity of dNTPs and deoxyribonucleosides (Sigma) was determined by chromatography on polyethyleneimine-cellulose and ion exchange HPLC. Purity of 1-methyladenine (1MeAde), 3-methyladenine (3MeAde), 7-methyladenine (7MeAde), 1-methylguanine (1MeGua), 3-methylguanine (3MeGua), and 7-methylguanine (7MeGua) (Vega Biochemicals) and N^2-methylguanine (N^2MeGua) and N^2-dimethylguanine (N^2Me_2Gua), prepared from N^2-methylguanosine and N^2-dimethylguanosine (P.-L. Biochemicals) by acid hydrolysis, was determined by reverse-phase C_{18} HPLC and verified by their spectral characteristics (9, 10). O^6-Methylguanine (O^6MeGua) was synthesized from 2-amino-6-chloropurine (Aldrich) according to published procedures (11), purified by reverse-phase C_{18} HPLC, and identified by its spectral characteristics (11).

Carcinogen Treatment and Fractionation of S-Phase 10T½ Cells. 10T½ cells growing in 100-mm tissue culture dishes were synchronized with respect to growth cycle by postconfluence inhibition of cell division (8). On entering S phase, cells on three or four dishes were washed twice with Tris-buffered saline (pH 7.6) and then treated for 30 min at 37°C with 2 ml of 0.04 mM [^{3}H]MNU (1–1.6 Ci/mmol; 1 Ci = 3.7×10^{10} becquerels; New England Nuclear) in the same saline. This dose kills <20% of these cells (unpublished results). After treatment with MNU, the cells were washed twice with Tris-buffered saline, and lysed by addition to each dish of 2 ml of 100 mM NaCl/10 mM EDTA/0.5% Triton X-100, pH 7 to 8. Lysis occurred within 2 min at room temperature. The lysis solution was carefully removed with a Pasteur pipette, leaving intact nuclei adhering to the dish. The nuclei were then washed twice with 100 mM NaCl/10 mM EDTA, pH 7 to 8. DNA was extracted for specific activity determination by lysing the nuclei with Sarkosyl/proteinase K (Worthington) and then banding the DNA in the lysates in a CsCl gradient. The DNA band was isolated, dialyzed, treated with RNase A, and then rebanded in CsCl. The amount of DNA isolated per nucleus was equivalent to that consistently isolated per intact cell, showing that nuclei were not lost during the procedure.

Isolation of the Deoxyribonucleotide Precursor Pool from Carcinogen-Treated Cells. To isolate deoxyribonucleotides, the Triton lysate (at 4°C) was filtered through a 0.45-μm Mil-

Abbreviations: MNU, *N*-methyl-*N*-nitrosourea; 10T½, C3H/10T½ clone 8; 1MeAde, 3MeAde, and 7MeAde, 1-, 3-, and 7-methyladenine, respectively; 1MeGua, 3MeGua, 7MeGua, O^6MeGua, and N^2MeGua, 1-, 3-, 7-, O^6-, and N^2-methylguanine, respectively; N^2Me_2Gua, N^2-dimethylguanine.

lipore filter and then extracted three times with phenol to remove protein and lipid. The aqueous phase was washed twice with ether to remove the remaining [^{3}H]MNU, lipid, and phenol and then adjusted to 0.5 M ammonium acetate/20 mM $MgCl_2$, pH 8.5, and passed through a 0.7 × 15.0 cm column of phenylboronic acid/polyacrylamide (Bio-Rad Affi-Gel 601) at a flow rate of 1 ml/min. These conditions completely retained ribose-containing molecules on the Affi-Gel while not retaining deoxyribose molecules (12), which eluted in the void volume. The column was washed with 3 to 4 vol of buffer and the unbound sample was pooled and diluted with water to 0.1 M ammonium acetate (pH 8.0). This was passed through a 0.7 × 30 cm column of QAE-Sephadex A-25 (Pharmacia). At pH 8.0 and 0.1 M ionic strength, deoxyribonucleotides are negatively charged and bind to this anion exchanger. After passing the sample through and washing the column with 100 ml of 0.1 M ammonium acetate to remove weakly charged molecules, bound deoxyribonucleotides were eluted in 50 ml of 1 M ammonium acetate (pH 8.5).

HPLC Analysis of Methylated Purines. The 50-ml sample was lyophilized to dryness, suspended in 6 M HCl (final pH, 1.0), and incubated at 37°C for 20 hr to yield free 1MeAde, 3MeAde, 7MeGua, and O^{6}MeGua with no loss of methyl group (10). One hundred microliters of the hydrolyzed sample was chromatographed on an MCH-10 octyldecylsilane reverse-phase high-pressure column (Varian). Elution was at a flow rate of 0.7 ml/min with, for the first 50 min, a mobile phase of 95% 0.01 M $NaPO_4$/5% methanol, pH 4.0, for the next 15 min, a 0–35% methanol/water gradient, and, for the last 10 min, 35% methanol. One-minute fractions were collected for monitoring of radioactivity by liquid scintillation counting.

Quantitation of the Concentration of Cellular Deoxyribonucleotides in the DNA and in the Precursor Pool. The nucleotide content of the DNA per 10^6 cells was determined from the UV absorbance of the extracted DNA preparation assuming an extinction coefficient of 6.5×10^3. Intracellular dNTP pools were measured enzymatically as described by Skoog (13). The dependence of "elongation" of DNase-activated calf thymus DNA by *Escherichia coli* DNA polymerase I (Boehringer-Mannheim) on the concentration of dNTP was measured by using one radioactive dNTP at several rate-limiting concentrations while keeping the three remaining dNTPs present in excess. These measurements were used to determine the dNTP concentrations in perchloric acid-soluble cellular extracts that served as the rate-limiting substituent in the same reaction.

RESULTS

Distribution of Radioactivity During Isolation of Deoxyribonucleotides from Cells Treated with [^{3}H]MNU. The procedure for isolating deoxyribonucleotide precursors described in *Materials and Methods* is summarized in Fig. 1. To determine whether the isolate of methyl-adducted deoxyribonucleotide precursors was free of other possible methylated molecules, radiolabelled compounds representative of those molecules likely to contaminate our final isolate were carried through all or part of this procedure. These included [^{14}C]methyl-labeled proteins (bovine serum albumin, carbonic anhydrase, lysozyme, myosin, ovalbumin, and phosphorylase-b), [^{3}H]leucine, nonradiolabeled glutamic acid (a negatively charged amino acid at neutral pH), [^{3}H]ATP, and [^{3}H]TTP. The results indicated that 99.5% of the protein was removed during phenol extraction (also expected to remove lipids during the ether wash) at step 4 (Fig. 1). Of the 0.5% left, 0.46% bound to the anion exchange column at step 6. Only 0.2% of the initial protein eluted from the column at the pH and ionic strength used to elute deoxyribonucleotides at step 7. Thus, at most, 0.2% of the radioactivity removed by the phenol step will contaminate the radioactivity eluted as deoxyribonucleotides. [^{3}H]Leucine was tested for binding to this column and no detectable radioactivity was found. Also, no glutamic acid was detected by UV absorbance. [^{3}H]ATP was completely retained by the phenylboronic acid affinity column (at step 5 of the procedure). This matrix has an exclusion limit of 6,000 daltons and a high capacity for binding compounds that have coplanar *cis*-diol groups such as ribonucleotides and -sides, sugars, catecholamines, and coenzymes. [^{3}H]TTP was used to test for deoxyribonucleotide loss at each step. Approximately 15% loss occurred during phenol extraction. This figure varied with the experiment but never exceeded 30%. No [^{3}H]TTP was lost during phenylboronic acid affinity chromatography, and recovery by the anion exchange steps (6 and 7) was always >95%. The final isolate of methylated deoxyribonucleotide, therefore, is probably contaminated only by the amounts of protein discussed below. Its measured concentration, however, may be underestimated due to losses during the procedure.

To determine the effect of MNU on the precursor pool, 10T½ cells were treated with [^{3}H]MNU as described and then carried through the deoxyribonucleotide isolation procedure. Total radioactivity remaining in the sample after each step is shown in Table 1. The largest amount of radioactivity was removed by phenol extraction. This is consistent with extensive protein methylation by MNU. Methyl groups associated with deoxyribonucleotide pools represent ≈2% of the methyl groups present in the total cell lysate. Although small, this amount of methylation is greater than that found in the cell DNA. The 0.2% of radiolabel associated with protein that may contaminate the radiolabel associated with presumed deoxyribonucleotides amounted to <6.3%, 6.6%, and 67.5% of the radiolabel eluted from QAE-Sephadex (step 7) in experiments 1, 2, and 3, respectively. Thus, contamination cannot account for the elution products in experiments 1 and 2.

Susceptibility of Pool and DNA Deoxyribonucleotide to Methylation by MNU. The radioactivity eluted from QAE-Sephadex (step 7) and the radioactivity found in DNA were converted to picomoles of methyl group by using the specific activity of the [^{3}H]MNU. More variability occurs in the data for pool methylation shown in Table 1 than in those for DNA methylation. This is probably due to the smaller amount of material and greater number of manipulations involved in purification of precursors than in purification of DNA. Despite such experimental error, the results of three independent determinations indicate 1.2- to 7.6-fold greater methylation of pool deoxyribonucleotide residues than of DNA residues.

To compare methylation per nucleotide residue in the pool with that in the DNA it is necessary to know the nucleotide content per cell in both. The results of three independent measurements of pool content in S-phase 10T½ cells are given in Table 2. Each dNTP was separately measured for a total dNTP content of 76 pmol per 10^6 cells. The relative sizes of the individual pools agree with measurements from other mouse embryo cell lines (for review, see ref. 2), in which dCTP is generally the largest pool and dGTP is the smallest. We did not resolve dUTP, the spontaneous deamination product of dCTP, from dTTP in our measurements. Thus, dTTP may be slightly overestimated and dCTP may be slightly underestimated. However, this does not affect the final determination of total deoxyribonucleotide content in the DNA precursor pool.

The DNA was shown, from UV absorbance measurements, to contain 85,900 pmol of nucleotide per 10^6 cells.

HPLC Analysis of Methylated DNA Precursors. To study the nature of the products formed by interaction of [^{3}H]MNU

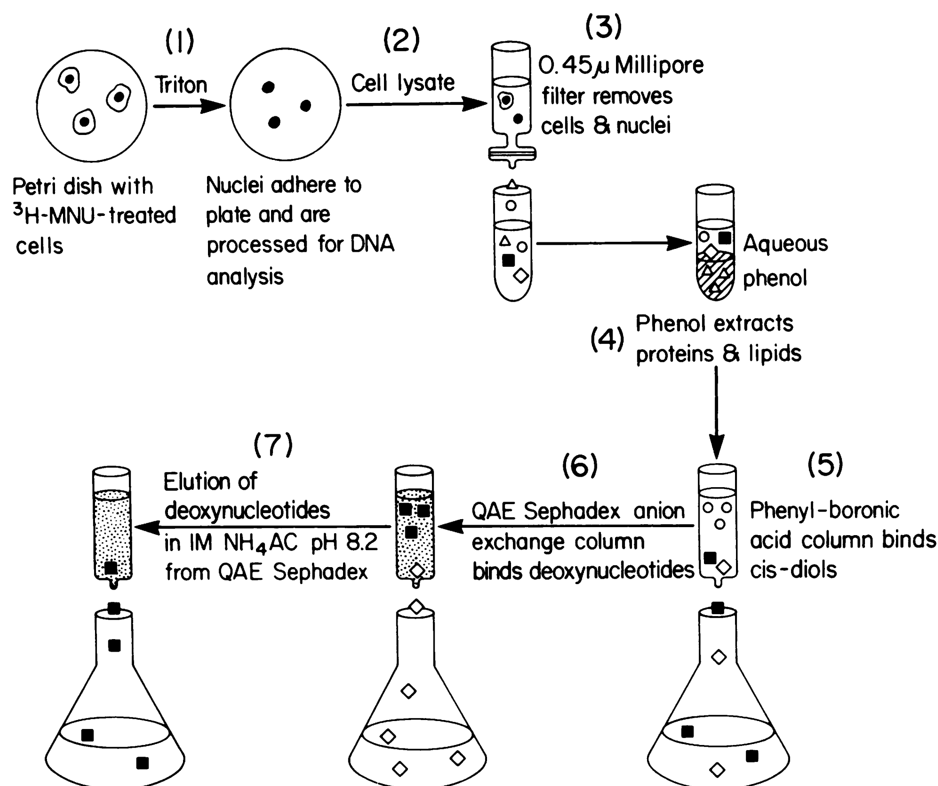

FIG. 1. Purification of cellular deoxyribonucleotide precursors. △, Protein and lipid; ○, molecule with *cis*-diol groups; ■, deoxynucleotide; ◇, deoxynucleosides and amino acids.

with deoxyribonucleotide and to further purify the pool residues, the eluate from QAE-Sephadex chromatography (step 7) in experiment 3 was acid hydrolyzed to release purines and analyzed by reverse-phase HPLC. The elution pattern when the radioactive sample was cochromatographed with methylated purine standards is shown in Fig. 2. The majority of radioactivity (retained <10 min) contained unhydrolyzed purine and pyrimidine nucleotides, methylated sugar phosphate moieties, and free methylated phosphate. Later eluting peaks cochromatographed with 1MeAde, 3MeAde, 7MeGua, and O^6MeGua standards; picomoles of methyl group per 10^6 cells chromatographing as each of these species were 0.069, 0.028, 0.069, and 0.013, respectively. No products chromatographed with 3MeGua, 1MeGua, 7MeAde, N^2MeGua, or N^2Me_2Gua standards (not shown). Our ability to resolve the elution products from the anion exchange column into peaks that cochromatograph with authentic methylated purines, and the fact that these products are those predicted to occur in significant yield, confirm our ability to isolate the deoxyribonucleotide pools.

A major site of reaction of MNU with DNA precursors should be the phosphate groups because of their electronegativity. The amount of phosphate methylation by MNU is given in Table 3. The unidentified category of methylated products contains methylated pyrimidines and total phosphate modification. Since methylation of pyrimidine bases represents a small fraction of total modification, we assume that phosphate modification is 0.644 of total modified. This represents 75% of total pool modification. Such a large amount was expected because the level of phosphate modification of DNA *in vitro*, in which there is only one phosphate moiety per residue, is ≈20% (14–17).

DISCUSSION

Our experiments show that MNU significantly modifies the DNA precursor pools of S-phase 10T½ cells in culture. Comparison of amounts of specific methyl adducts in the pool with those in the DNA shows 1.2–7.6 times more modification of the pools. This result gains added significance from measurements of amounts of deoxyribonucleotides per S-phase cell in the form of precursors and in the form of DNA residues. These measurements show (on average) 1,100-fold more residues in the DNA than in the pools. Thus, a residue in the precursor pool

Table 1. Distribution of radioactivity during isolation of dNTP from [^{3}H]MNU-treated 10T½ cells

	Radioactivity, cpm × $10^{-3}/10^6$ cells remaining after step						
				Precursor pool QAE-Sephadex elution			
Exp.	Enucleated cell lysate (step 3)	Phenol extraction (step 4)	Affi-Gel (step 5)	0.1 M NH_4OAc (step 6)	1.0 M NH_4OAc (step 7)	DNA	Pool/DNA
1	386	149	40	25	7.6 (4.7)	1.0 (0.63)	7.6
2	298	78	28	20	6.7 (4.2)	1.4 (0.88)	4.8
3	580	86	29	25	1.5 (0.91)	1.2 (0.75)	1.2

All data were normalized for differences in cell number between experiments. Results in parentheses represent pmol of methyl group per 10^6 cells and were calculated by dividing cpm × 10^{-3} by specific activity of the [^{3}H]MNU.

Table 2. Deoxyribonucleotide in pools and in DNA

	pmol per 10^6 cells
dATP	12.3 ± 4.9
dCTP	36.0 ± 0
dGTP	12.0 ± 1.2
dTTP and dUTP	15.8 ± 1.6
Total dNTP	76
DNA	85,900

is, on average, 1,300–8,400 times more susceptible to methylation than a residue in the DNA of these cells.

Since we could not be sure that the isolated pools were free of contaminating methylated cellular molecules, we made the more stringent comparison by reverse-phase HPLC of methylation of pool and DNA nucleotides at specific sites. Amounts of the four major methyl purines formed during reaction of MNU with the precursor pool are compared with the amounts formed in the DNA in Table 3. Also shown are unidentified products, which include methylated pyrimidines and all phosphate methylations. The results indicate that susceptibility to MNU methylation of a residue in the DNA precursor pool compared with that in the DNA varies with the site of modification. This is to be expected from DNA structural interactions. For example, the N-1 position of adenine is involved in hydrogen bonding and is located, out of reach of solvent, in the interstices of the helical duplex. On the other hand, this same position in the nucleotide precursor is readily available for modification. This difference is manifested as a 13,000-fold greater susceptibility of the N-1 position of dATP to methylation by MNU compared with the susceptibility of this same position in the adenosine residues in DNA. The N-7 position of guanine, meanwhile, is more available in the DNA than is the N-1 position of adenine because of its location in the major groove of the helix. This is reflected in a more moderate difference in susceptibility between precursor and DNA guanosine residues at this position. All of the sites show greater susceptibility in the triphosphate precursor compared with the DNA: greater amounts of methyl phosphate, 3MeAde, and O^6MeGua were also found per residue in the precursor pool than in the DNA. The larger differences are manifested by those sites least accessible in the DNA helix.

The site exhibiting the largest amount of methylation (75% of total methylation), and one of the largest differences in susceptibility, was found to be methylation of the phosphate moiety. This is not surprising since MNU is believed to act through a strongly electrophilic intermediate (for review, see ref. 18) that should be influenced by the highly electronegative phosphate group (19). The large difference in susceptibility of the phosphates of precursor and DNA (Table 3) is probably due to protection of DNA by binding of positively charged proteins such as histones. The effect of dNTP precursors with methylated phosphates on DNA replication is unknown.

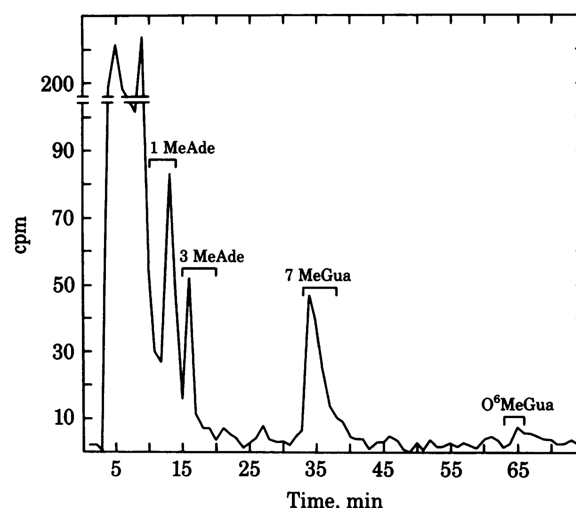

FIG. 2. HPLC separation of methylated purines resulting from the reaction of MNU with the DNA precursor pool of 10T½ cells. Acid-hydrolyzed precursors were chromatographed on a C_{18} reverse-phase column at a flow rate of 0.7 ml/min.

The great susceptibility of the dNTP precursors to methylation suggests that, during treatment of an S-phase cell with a methylating agent such as MNU, there will be a significant concentration of methylated precursors competing with normal precursors for incorporation into the DNA daughter strand. Evidence that such precursors are indeed incorporated comes from our results that show the ability of products of the reaction of MNU with dATP to be incorporated by phage T4 DNA polymerase during replication of ϕX174 DNA *in vitro* (1). This suggests that the large quantitative differences between precursor and DNA methylation found may actually be underestimates.

Table 3. Site susceptibility of nucleotide residues in precursor pool and DNA to methylation by MNU

Modification	Methyl groups, pmol per 10^6 cells		Pool/DNA	Relative pool susceptibility†
	Pools	DNA*		
1MeAde	0.069	0.011 (0.005–0.017)	6.3	13,000
3MeAde	0.028	0.056 (0.039–0.071)	0.50	1,000
7MeGua	0.069	0.502 (0.457–0.585)	0.13	190
O^6MeGua	0.013	0.047 (0.026–0.058)	0.28	400
Unidentified‡	0.644	0.146 (0.112–0.184)	4.4	5,000

* Data for extent of methylation of DNA at these sites were obtained from refs. 14–17 and are mean (range). The mean values were used to determine the pool/DNA ratio and the relative pool susceptibility. The reported values agree well with each other and with our preliminary measurements.

† Determined from the pool/DNA methylation ratio (this table), for a particular site, normalized for the large difference (Table 2) in nucleotide content between pool and DNA. Normalization was achieved by multiplying the pool/DNA methylation ratio by the appropriate ratio of DNA nucleotide content/pool nucleotide content per cell. The latter ratios were determined to be 1,432 for guanine, 2,095 for adenine, and 1,130 for total nucleotide assuming a DNA G/C content of 40% for 10T½ cells and using the measured nucleotide content per cell values of Table 2.

‡ Determined to be mainly phosphate methylation by treatment of the deoxynucleotides with acid to hydrolyze purines and with alkaline phosphatase to remove unmethylated phosphates. With both treatments, the majority of radioactivity cochromatographed on reverse-phase HPLC with a propyl phosphate standard provided by Richard Wolfendon but not with standards indicating base methylation.

Some methyl adducts we detected in the DNA may have come from incorporation of modified precursors during the 30 minutes of replication taking place while the cells were treated with MNU. Indeed, detailed characterization of the DNA isolated from these cells indicates greater than five-fold preferential methylation (compared with that expected from random methylation) of the DNA replicated during the time of treatment of the cells with MNU (unpublished results). Moreover, we expect such incorporation to contribute to mutation and neoplastic transformation. Modified residues, now in the template strand, may misdirect incorporation of precursors during the next round of DNA replication in the cell. For example, deoxy-1-methyladenosine triphosphate, which we found to be one of the major modified purine products produced during incubation of 10T½ cells with MNU, has been shown to cause incorporation errors during "transcription" of synthetic ribopolymers containing methylated bases by DNA-dependent RNA polymerase (20). Methylated template residues may also be subject to high rates of depurination, which has recently been suggested, under certain conditions, to be mutagenic (21). Moreover, some DNA modifications may induce error-prone repair systems in the cell (22). Finally, it is interesting to note that deoxy-N^6-methyladenosine triphosphate is expected as a minor product of pool methylation. Incorporation of this modified precursor into DNA could have significant biological consequences since deoxy-N^6-methyladenosine in helical DNA is involved in the restriction-modification system in bacteria (for review, see ref. 23) and possibly in control of gene expression and differentiation in eukaryotic cells (24, 25).

We thank M. Cordeiro-Stone for the measurements of methylation and nucleotide content of cell DNA and David Toorchen for synthesizing O^6-MeGua. This work was supported in part by Grants GM 24798 and CA 28632 from the U.S. Public Health Service and Predoctoral Training Grant ES07017 to M.S.B. from the National Institute of Environmental Health Sciences.

1. Topal, M. D., Hutchison, C. A., III, Baker, M. S. & Harris, C. (1980) in *Mechanistic Studies of DNA Replication and Genetic Recombinations*, ICN–UCLA Symposium on Molecular and Cellular Biology, ed. Alberts, B. (Academic, New York), Vol. 19, pp. 725–734.
2. Hauschka, P. V. (1973) in *Methods in Cell Biology*, ed. Prescott, D. (Academic, New York), Vol. 8, pp. 361–462.
3. Tong, C., Fazio, M. & Williams, G. M. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 7377–7379.
4. Marquardt, H. (1974) *Cancer Res.* **34**, 1612–1615.
5. Grisham, J. W., Greenberg, D. S., Kaufman, D. G. & Smith, G. J. (1980) *Proc. Natl. Acad. Sci. USA* **77**, 4813–4817.
6. Cerda-Olmeda, E., Hanawalt, P. C. & Guerola, N. (1968) *J. Mol. Biol.* **33**, 705–719.
7. Hince, T. A. & Neale, S. (1974) *Mutat. Res.* **24**, 383–387.
8. Reznikoff, C. A., Brankow, D. W. & Heidelberger, C. (1973) *Cancer Res.* **33**, 3231–3238.
9. Brookes, P. & Lawley, P. D. (1960) *J. Chem. Soc.*, 539–545.
10. Lawley, P. D. & Thatcher, C. J. (1970) *Biochem. J.* **116**, 693–707.
11. Balsiger, R. W. & Montgomery, J. A. (1960) *J. Org. Chem.* **25**, 1573–1575.
12. Weith, H. L., Wiebers, J. L. & Gilham, P. T. (1970) *Biochemistry* **9**, 4396–4401.
13. Skoog, L. (1970) *Eur. J. Biochem.* **17**, 202–208.
14. Newbold, R. J., Warren, W., Medcalf, A. S. C. & Ames, J. (1980) *Nature (London)* **283**, 596–599.
15. Lawley, P. D., Orr, D. J., Shah, S. A., Farmer, P. B. & Jarman, M. (1973) *Biochem. J.* **135**, 193–201.
16. Walker, I. G. & Ewart, D. F. (1973) *Mutat. Res.* **19**, 331–341.
17. Kirtikar, D. M. & Goldthwart, D. A. (1974) *Proc. Natl. Acad. Sci. USA* **71**, 2022–2226.
18. Lawley, P. D. (1976) in *Chemical Carcinogens*, ed. Searle, C. (Am. Chem. Soc., Washington, DC), pp. 83–244.
19. Pullman, B., Perahia, D. & Cauchy, D. (1979) *Nucleic Acids Res.* **6**, 3821–3829.
20. Kroger, M. & Singer, B. (1979) *Biochemistry* **18**, 3493–3500.
21. Schaaper, R. M. & Loeb, L. A. (1981) *Proc. Natl. Acad. Sci. USA* **78**, 1773–1777.
22. DasGupta, U. B. & Summers, W. C. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 2378–2381.
23. Meselson, M., Yuan, R. & Heywood, J. (1972) *Annu. Rev. Biochem.* **41**, 447–466.
24. Holliday, R. & Pugh, J. R. (1975) *Science* **187**, 226–232.
25. Sager, R. & Kitchin, R. (1975) *Science* **189**, 426–433.