Variable effects of DNA-synthesis inhibitors upon DNA methylation in mammalian cells

Jonathan Nyce, Leonard Liu and Peter A. Jones

USC Comprehensive Cancer Center, 2025 Zonal Avenue, Los Angeles, CA 90033, USA

Received 15 January 1986; Revised and Accepted 18 April 1986

ABSTRACT

Post-synthetic enzymatic hypermethylation of DNA was induced in hamster fibro-sarcoma cells by the DNA synthesis inhibitors cytosine arabinoside, hydroxyurea and aphidicolin. This effect required direct inhibition of DNA polymerase α or reduction in deoxynucleotide pools and was not specific to a single cell type. At equivalently reduced levels of DNA synthesis, neither cycloheximide, actinomycin D nor serum deprivation affected DNA methylation in this way. The topoisomerase inhibitors nalidixic acid and novobiocin caused significant hypomethylation indicating that increased 5-mCyt content was not a necessary consequence of DNA synthesis inhibition. The induced hypermethylation (1) occurred predominantly in that fraction of the DNA synthesized in the presence of inhibitor; (2) was stable in the absence of drug; (3) was most prominent in low molecular weight DNA representing sites of initiated but incomplete DNA synthesis; and (4) occurred primarily within CpG dinucleotides, although other dinucleotides were overmethylated as well. Drug-induced CpG hypermethylation may be capable of silencing genes, an effect which may be relevant to the aberrantly expressed genes characteristic of neoplastic cells.

INTRODUCTION

The concept that methylation of eukaryotic genes may modulate their expression has received considerable experimental support (1-3). In general, methylation of specific CpG sites adjacent to or within actively transcribed genes appears to inactivate them, while undermethylation of such sites permits transcription (4,5). However, such correlations are not universal, and there is evidence for the expression of several heavily methylated genes (6,7). In most cases, however, undermethylation of 5' flanking sequences appears to be a necessary prerequisite for gene activation, although there are clearly also other requirements which are necessary to ensure that active transcription occurs (8).

Studies of the relationship between DNA methylation and gene expression have been aided by use of drugs capable of inducing DNA hypomethylation. Thus, 5-azacytidine (5-azaCyt) and other analogs of cytidine modified in the 5 position of the pyrimidine ring, induce profound changes in gene expression in a large number of experimental systems (9,10). 5-AzaCyt appears to have general activity in inducing gene expression in a manner related to its ability to cause DNA hypomethylation. These effects can also be induced by

a variety of other agents (11), but not to the extent observed with 5-azaCyt or its deoxy analogue.

Drug-mediated hypermethylation of DNA has also been reported. Following the initial findings of Burdon and Adams (12) and Kappler (13) that DNA methylation continues during inhibition of DNA synthesis, we (9) and others (14) have shown that cytosine arabinoside (araCyt), a chemotherapeutic agent which is a potent inhibitor of DNA synthesis, induces significant hypermethylation of the genome. It has also been reported that low dose araCyt induces differentiation of malignant myeloid cells (15).

Drug-mediated hypermethylation might induce the switching off of genes. Besides being of theoretical interest, this concept could have important implications regarding the aberrant gene expression characteristic of tumor cells (16-19). We therefore extended our previous work to include 2 further "direct" inhibitors of DNA synthesis, hydroxyurea (HU) and aphidicolin. HU is an inhibitor of ribonucleotide reductase and depletes deoxyribonucleotide pools (20); aphidicolin is a specific inhibitor of DNA- α polymerase and blocks the dCTP binding site of this enzyme (21). Both HU and aphidicolin have been shown to suppress the polymerization of newly synthesized DNA, resulting in the accumulation of low molecular weight DNA fragments (22-24). Over extended periods of synthesis inhibition (e.g., 24 h), a small percentage of these low molecular weight fragments undergo successful ligation to high molecular weight DNA characteristic of the mature polymer (24). We analyzed the relative degrees of methylation within these low molecular weight fragments compared to bulk DNA by isolating nascent DNA from mature high molecular weight polymer. This was accomplished by a procedure originally designed for the selective extraction of polyoma DNA from host cells (25) and later modified to enable the size separation of newly replicated versus mature eukaryotic DNA (26). Pre-chromosomal fragments and bulk DNA isolated in this way were compared for total 5-mCyt content and dinucleotide specificity of the hypermethylation phenomenon. The effects upon DNA methylation of cycloheximide-mediated inhibition of peptide synthesis, of actinomycin-D-mediated inhibition of mRNA synthesis, of depletion of required growth factors by serum deprivation, and of nalidixic acid and novobiocin-mediated inhibition of topoisomerase activity were also investigated.

We report here that DNA hypermethylation (1) required the direct inhibition of α -polymerase or of deoxyribonucleotide synthesis; (2) occurred to the greatest extent within low molecular weight DNA fragments synthesized during the presence of inhibitor; (3) occurred primarily within the heritable dinucleotide CpG, although other dinucleotides were overmethylated as well; and (4) could be induced in a number of cell types of both murine and human origin. The implications of drug-induced CpG hypermethylation for modulating gene expression are discussed.

MATERIALS AND METHODS

Cells and Media

Syrian hamster A(T1)Cl-3 fibrosarcoma cells (27) were grown in McCoy's 5A medium, supplemented with 10% heat inactivated fetal calf serum (Tissue Culture Biochemicals, Tulare, CA), 100 units/ml penicillin and 100 mg/ml streptomycin (Gibco Laboratories, Grand Island, NY). T1 human fibroblasts (28) and HT-1080 human fibrosarcoma cells (29) were grown in minimal essential medium from the same supplier and similarly supplemented. Cells were passaged prior to confluency with 0.05% trypsin (Difco Laboratories, Detroit, MI) in isotonic phosphate buffered saline (PBS) at pH 7.4.

Chemicals

All chemicals and drugs were obtained from Sigma Chemical Co., St. Louis, MO, unless otherwise indicated and were dissolved in sterile isotonic PBS before addition to culture media, with the following exceptions: Aphidicolin was dissolved in absolute ethanol and added to a final concentration in the medium of 0.5%; Nalidixic acid was dissolved in .01 M KOH and sterilized by filtration.

5-mCyt Determinations

A(T1)Cl-3 hamster fibrosarcoma cells (2.5×10^5) were seeded into 60 mm culture dishes (Falcon Plastics, Oxnard, CA) and treated with drugs 24 h later. Concurrently with addition of drug, [^3H]-6-uridine (20 Ci/mmol, Research Products International, IL) or [^3H]-6-deoxycytidine (6 Ci/mmol, Moravsek Biochemicals, Brea, CA) were added to the cultures to a final concentration of 10-20 uCi/ml, depending on the drug utilized or the requirements of the experiment. DNA was prepared for analysis according to the procedure of Flatau et al (29) after 24 h in the presence of both labelled precursor and appropriate drug. The percentage of total DNA cytosines that became methylated during the treatment period was then determined by high pressure liquid chromatography (HPLC) of the formic acid hydrolyzed samples (29).

Estimations of DNA Synthesis

The ratio of newly incorporated cytosine (cpm co-eluting with cytosine peak) per nmol total cytosine within the sample provided an estimate of new DNA synthesis. This method could not be used where drugs affecting changes in nucleotide pool sizes were considered, e.g., HU. In such cases, DNA synthesis was estimated by relative rates of incorporation of inorganic [^{32}P]-phosphate (ICN, Irvine, CA) into deoxynucleotide monophosphates of control versus treated samples.

Inorganic Phosphate Labelling of Cellular DNA and Enzymatic Hydrolysis to Mononucleotides

A(T1)Cl-3 cells (2.5×10^6) were seeded into each of 7 T-75 tissue culture flasks (Falcon Plastics, Thousand Oaks, CA) containing 10 ml of McCoy's 5A medium. Twenty-four h later the medium was changed and 0.25 mCi of inorganic [^{32}P]-phosphate was

added directly to each flask. Additionally, HU (10^{-3} M) was added to five of these flasks. Cells were harvested by trypsinization after 24 h, and DNA extracted by a modified Marmur procedure (30). Enzymatic hydrolysis to deoxyribonucleotide monophosphates was effected essentially according to the procedure of Jensen (31). A portion of the hydrolysate (50 μ l) was injected onto a uBondapak reversed-phase C-18 column (Millipore/Waters, Milford, CT) and eluted with 0.2 M dibasic potassium phosphate, pH 5.5. 5'-Deoxymononucleotides, monitored by absorption at 280 nm, eluted in positions identical to those of authenticated standards (Sigma, St. Louis, MO).

Isolation of Nascent, Low Molecular Weight DNA

DNA was divided into 2 fractions consisting of newly replicated small molecular weight DNA and mature high molecular weight DNA by an adaptation of the procedures of Hirt (25) and Coyle and Strauss (26). Hamster fibrosarcoma cells (10^6) were plated into 60 mm tissue culture dishes, and allowed to grow overnight. Cultures were then treated with 2.5×10^{-3} M HU or PBS alone, in the presence or absence of 5 μ Ci/ml [3 H]-6-deoxycytidine. Twenty-four h later, cell cultures were washed twice with 1.0 ml PBS. Two ml of fresh PBS were then added followed by 2.0 ml of 1.2% SDS in 0.2 M EDTA, pH 7.5, to lyse the cells. The resulting viscous suspension was very carefully transferred without pipetting to 5 ml SW-50.1 polylallylmer centrifuge tubes (Beckman, Fullerton, CA), and 1.0 ml of 1 M NaCl was added. It was critical that mixing of the resulting layers was done gently; five gentle inversions of each tube were found to be sufficient. Samples were cooled at 4°C for 18 h, and centrifuged at 17,000 rpm in an SW-50-1 rotor at 4°C for 30 min. The supernatant containing low molecular weight DNA was carefully removed, and the pellets containing high molecular weight DNA allowed to air dry. These precipitates were taken up in a volume of PBS equivalent to the decanted supernatant. Following ethanol precipitation of both fractions at -80°C overnight, total incorporation of [3 H]-6-deoxycytidine into respective fractions was measured by retention of labelled TCA-precipitated DNA on Whatman GF/C filters. Alternatively, DNA was worked up for 5-mCyt determinations as described above or for nearest neighbor analysis by the method of Gruenbaum et al (32). For this latter procedure, enzymatic digestion of DNA was carried to completion and the released 3'-deoxymononucleotides were quantitated via HPLC under conditions identical to those reported above for 5'-deoxymononucleotides, except that the pH of the eluting buffer was lowered to 4.2.

RESULTS

We had observed in previous work that DNA hypermethylation was induced during the period that DNA synthesis was inhibited by araCyt in a murine cell line (9). This suggested that DNA methylation continued even though movement of the replication fork had been severely retarded. To determine if such "supermethylation" of DNA was a

Figure 1. Comparative rates of DNA synthesis and methylation following HU or aphidicolin treatment. A(T1)Cl-3 hamster fibrosarcoma cells were administered HU or aphidicolin at the indicated dosages for a period of 24 h in the presence of labelled DNA precursor as described in text. Results are displayed as % of control and are the average of 6-10 determinations. Open bars represent % of cytosine methylation. Closed bars represent rate of DNA synthesis. All samples showed standard deviations ± 3.6 .

general result of inhibition of DNA synthesis, we tested a variety of direct and indirect inhibitors of replication for their abilities to elicit this effect in hamster fibrosarcoma cells. We first verified that araCyt ($4 \mu\text{M}$) induced a 28% increase in the methylation level of DNA that was synthesized during inhibition of replication in A(T1)Cl-3 cells (data not shown). Both qualitatively and quantitatively, this inverse relationship between DNA synthesis and DNA methylation resembled that which was previously reported (9). We then extended our investigation to determine if the ribonucleotide reductase inhibitor HU or the direct α polymerase inhibitor aphidicolin also produced hypermethylated DNA (Figure 1). Exposure of hamster fibrosarcoma cells to either HU or aphidicolin induced increased methylation within the DNA synthesized during the period of replication inhibition. This effect was substantial and dose dependent for both agents. Hypermethylation was therefore induced by 2 more inhibitors of DNA synthesis with pharmacological sites of action different from araCyt.

Inhibition of DNA synthesis to comparable levels by the topoisomerase antagonists nalidixic acid and novobiocin did not induce hypermethylation (Figure 2). Indeed, exposure of cells to these drugs caused significant hypomethylation of the DNA synthesized during the period of replication inhibition. This effect was most pronounced with nalidixic acid. These results indicate clearly that DNA synthesis inhibition *per se* was insufficient to induce hypermethylated DNA. Other factors related to the method of synthesis inhibition were therefore involved.

Figure 2. Comparative rates of DNA synthesis and methylation following nalidixic acid or novobiocin treatment. A(T1)Cl-3 hamster fibrosarcoma cells were administered nalidixic acid or novobiocin at the indicated doses for a period of 24 h in the presence of labelled DNA precursor as described in text. Results are displayed as % of control and are the average of 6-10 determinations. Open bars represent % of cytosine methylation. Closed bars represent rate of DNA synthesis. All samples showed standard deviations ± 2.9 .

Experiments were also performed in which DNA replication was indirectly inhibited by blocking RNA synthesis with actinomycin D, protein synthesis with cycloheximide, or by depletion of necessary growth factors by serum deprivation (Table 1). Under these conditions no hypermethylation was observed in the DNA synthesized during the period of treatment. In fact, each of these agents induced moderate levels of hypomethylation at low drug concentrations. Although not tested, the results within table 1 suggest that concentrations of cycloheximide and actinomycin D that induced more pronounced inhibition of DNA synthesis might then simultaneously induce DNA hypermethylation. The reported results suggested that the continuation of methylation during inhibition of DNA synthesis required direct interference with the process of replication. Agents with modes of action not associated directly with DNA polymerization, at least at concentrations that permitted some replication to occur, showed little or the opposite effect.

It was also of interest to test the possibility that the hypermethylation response to direct DNA synthesis inhibition might be confined to rodent cell lines. Treatment of human fibroblast and human fibrosarcoma cells with HU also induced hypermethylation within the fraction of DNA synthesized in the presence of inhibitors (Table 2). Hypermethylation resulting from the direct inhibition of DNA synthesis by HU was thus shown not to be a peculiarity of the metabolism or action of the drug in A(T1)Cl-3 cells only. Rather, it occurred in several cell types of different species origin.

Table 1. Effect of Indirect Inhibitors of DNA Synthesis Upon DNA Methylation

Treatment	5-mCyt/5-mCyt + Cyt	DNA synthesis (% Control)
Control	2.50 $\pm$ 0.18 (100)	100
Actinomycin D		
0.08 nM	2.19 $\pm$ 0.17 (88)	50
0.80 nM	2.48 $\pm$ 0.21 (99)	29
Cycloheximide		
0.33 μ M	2.32 $\pm$ 0.09 (93)	71
1.0 μ M	2.54 $\pm$ 0.07 (102)	41
Serum deprivation		
0.5% replacing 5%	2.24 $\pm$ 0.11 (90)	15

A(T1)Cl-3 hamster fibrosarcoma cells (2.5×10^5 per 60 mm dish) were administered indicated drugs and [3 H]-uridine or [3 H]-dCyt (10 uCi) 24 h after plating. Drugs and label were washed from the cultures 24 h later, the cells lysed, and DNA extracted and analyzed as described in Methods. Numbers in parentheses represent % of control. DNA synthesis values are given as percent of controls. Depending upon the experimental conditions, different methods of determining relative rates of DNA synthesis were employed, as discussed in Methods. The values reported are the average of 3 determinations $\pm$ standard deviation.

The Stability of Induced Hypermethylation

Methylation patterns must be heritable to be biologically significant. We therefore determined whether the hypermethylation induced in hamster fibrosarcoma cell DNA by HU was stable after the inhibitor had been removed. Following a 24 h treatment with HU in the presence of [3 H]-dCyt, drug was removed and the labelled nucleoside chased. Twenty-four h later, cells were harvested and analyzed for DNA 5-mCyt content. The

Table 2. Effect of HU on DNA Methylation in Different Cell Types.

Cell Type	HU Concentration	5-mCyt/5-mCyt + Cyt	% Control
Hamster fibrosarcoma cells A(T1)Cl-3	0	2.50 $\pm$ 0.18	100
	1 mM	3.35 $\pm$ 0.44	134
	10 mM	3.69 $\pm$ 0.45	148
Human fibroblasts (T ₁)	0	2.80 $\pm$ 0.14	100
	1 mM	3.65 $\pm$ 0.18	130
Human fibrosarcoma cells (HT-1080)	0	2.17 $\pm$ 0.10	100
	1 mM	3.14 $\pm$ 0.31	145

Cells were treated for 24 h with 1 mM HU in the presence of 10 uCi/ml [3 H]-6-dCyt. The level of 5-mCyt was then determined by DNA hydrolysis and HPLC. Results of at least 3 separate determinations are presented, $\pm$ standard deviation.

Table 3. Stability of Hypermethylated DNA After Removal of Synthesis Inhibition

Treatment	24 hr pulse chase	5-mCyt/5-mCyt + Cyt
Control	No	2.38 $\pm$ 0.10 (100)
	Yes	2.44 $\pm$ 0.01 (103)
HU	No	2.83 $\pm$ 0.07 (119)
	Yes	3.01 $\pm$ 0.11 (126)
Aphidicolin	No	3.03 $\pm$ 0.11 (127)
	Yes	2.73 $\pm$ 0.08 (115)

Replicate cultures of hamster fibrosarcoma cells (2.5×10^5) were exposed to HU (10^{-5} M) or aphidicolin (5×10^{-5} M) and simultaneous labelling with 10 μ Ci/ml of [3 H]-6-dCyt. Twenty-four h later, cells of these and control cultures were either harvested or chased with cold dCyt and allowed to grow in the absence of either drug or label for a further 24 h. Values are given as the mean of at least three determinations $\pm$ standard deviations. Values in parentheses represent % of control.

ratio of 5-mCyt to Cyt increased slightly following removal of HU (Table 3) suggesting that not only was DNA hypermethylation maintained in the absence of inhibitor, but that cytosines incorporated in the presence of drug were further methylated during the post-labelling period. This effect was not as evident in the DNA from aphidicolin-treated cells (Table 3). Since exposure to aphidicolin leads to the accumulation of a large pool of intracellularly trapped [3 H]-dCTP (21), this result may have been due to incorporation from this pool immediately after aphidicolin was removed. Thus the methylation level determined after a 24 h chase would represent the average value of the pre-existing hypermethylated sequences and the newly incorporated normally methylated [3 H] - dCTP.

The Sequence Composition of Hypermethylated DNA

The drug-induced hypermethylation observed might have been due to preferential replication of sequences enriched in cytosine and guanine, since primarily CpG sequences act as methyl-acceptor sites in vertebrate DNA (1). The base composition of DNA from cells treated with HU in the presence of radiolabelled inorganic phosphate was therefore determined. DNA from control or treated cells was extracted, hydrolyzed to 5'-deoxymononucleotides, and the radiolabeled base composition determined by HPLC (Table 4). Ratios of A+T/G+C obtained from limit enzymatic digests of DNA to 5'-deoxymononucleotides showed no bias toward the synthesis of sequences enriched in cytosine and guanine in treated vs control cultures. The hypermethylation response during inhibition of DNA synthesis was therefore not due to the selective synthesis of DNA of inherently greater methyl-accepting capacity.

Treatment of cells with HU or aphidicolin leads to the accumulation of low MW

Table 4. Base Composition of Hypermethylated DNA

Treatment	$\frac{A+T}{G+C}$
Control	0.96 $\pm$ 0.01 (100%)
HU	1.01 $\pm$ 0.04 (105%)

A(T1)Cl-3 cells were labelled with [^{32}P]-inorganic phosphate (25uCi/ml) for 24 h in the presence or absence of 10^{-3}M HU. Extracted DNA was enzymatically digested to 5'-deoxynucleotide monophosphates, and the reaction products analyzed by HPLC and liquid scintillation counting. Differences between control and HU-treated groups in A+T/G+C ratios are not significant as determined by Student's T test. A + T = adenine + thymine. G + C = guanine + cytosine. Results are presented as the average of at least 3 determinations $\pm$ standard deviation.

DNA fragments (21-24). Such fragments presumably represent small regions of DNA in which synthesis was initiated but not elongated to the high MW stage characteristic of the mature polymer. If the period of synthesis inhibition is sufficiently brief, the low MW fragments do become ligated into high MW DNA (24). To determine whether hypermethylated sequences were confined to low MW fragments resulting from drug treatment, nascent and mature DNA were separated by a modification of the procedures of Hirt (25) and Coyle and Strauss (26). These techniques take advantage of the fact that DNA molecules of varying size can be separated according to their solubilities in 1M NaCl in the presence of SDS and 0.01M EDTA. Fragments smaller than approximately 20 S are soluble in this mixture, while larger fragments are not and can be pelleted upon centrifugation.

DNAs were fractionated in this way from control or HU-treated hamster fibrosarcoma cells labelled with [^3H]-dCyt over a 24 h period (Table 5). Almost all of the incorporated label in control cells was found in the fraction representing mature, high molecular weight DNA. Since DNA is replicated in small fragments which are then ligated together (24), this result indicated that maturation proceeded completely under the conditions of our experiment in the absence of inhibitor. On the other hand, most of the incorporated label in HU-treated cells was found in the fraction representing nascent, low molecular weight DNA fragments. Thus, HU prevented maturation into high molecular weight DNA. The small amount of incorporated label occurring in the high molecular weight fraction of HU-treated cellular DNA presumably represented the few initiated DNA fragments that underwent successful maturation.

This ability to separate nascent DNA made in the presence of inhibitor from bulk DNA allowed us to undertake a more detailed analysis of the small fraction of DNA which was synthesized during HU exposure. The degree of methylation within low and high molecular weight DNAs separated by the Hirt procedure were therefore determined by

Table 5. Analysis of Nascent and Mature DNA from Control and HU-treated Hamster Fibrosarcoma Cells

Treatment	Cpm in mature DNA fraction	Cpm in nascent DNA fraction	% of total label in nascent fraction
Control	22808 $\pm$ 1438	1721 $\pm$ 255	7.0 $\pm$ 0.8
2.5 $\times 10^{-3}$ M HU	4066 $\pm$ 725	7440 $\pm$ 674	65.0 $\pm$ 2.5

A(T1)Cl-3 cells (2.0×10^6 cells per 100 mm tissue culture dish) were labelled with [^3H]-6-dCyt (10 $\mu\text{Ci/ml}$) and simultaneously treated with 2.5×10^{-3} M HU for 24 h. At the end of this period DNA was isolated and fragments of nascent (low molecular weight) and mature polymer (high molecular weight) were separated as described in Methods. Equal aliquots (50 μl) of both nascent and mature fractions were precipitated by the addition of 25 μg of salmon sperm DNA, followed by TCA to a final concentration of 10-15%. Precipitates were collected on Whatman GF/C filters and scintillation counted. Results are given as CPM (counts per minute) $\pm$ standard deviation, and were computed based upon 6 separate experimental determinations for each value.

[^3H]-dCyt labelling and HPLC (Table 6). Hypermethylation of DNA in HU-treated cells occurred to the greatest degree within the low molecular weight fraction, representing DNA strands in which replication fork movement and subsequent maturation had been retarded. Hypermethylation of the high molecular weight fraction in HU-treated cellular DNA occurred to a lesser extent. The extent of methylation in nascent vs. mature DNA was clearly different in both control and HU-treated cells. Since nascent DNA from control cells had the lowest levels of methylation and nascent DNA from HU-treated cells had the highest, the relative differences were most pronounced between these 2 groups.

One explanation for the hypermethylation observed may have been that sequences

Table 6. 5-mCyt Content of Nascent and Mature DNA from Control and HU-treated Hamster Fibrosarcoma Cells

Treatment	Nascent 5-mCyt/5-mCyt+Cyt (% of control)	Mature 5-mCyt/5-mCyt+Cyt (% of control)
Control	1.86 $\pm$ 0.04	2.13 $\pm$ 0.06
2.5 $\times 10^{-3}$ M HU	3.54 $\pm$ 0.10 (190)	3.27 $\pm$ 0.36 (155)

A(T1)Cl-3 cells (2.0×10^6 cells per 100 mm tissue culture dish) were labelled with [^3H]-6-dCyt (10 $\mu\text{Ci/ml}$) and simultaneously treated with 2.5×10^{-3} M HU for 24 h. Isolated DNA was separated into nascent (low molecular weight) and mature (high molecular weight) fractions and worked up for analysis of 5-mCyt content as described in Methods. Results are given $\pm$ standard deviation, and were computed based upon 6 separate experimental determinations for each value. Values for control vs. HU-treated samples were determined to be statistically different for both fractions using Student's t test (p $\leq$.1)

Table 7. Extent of Cytosine Methylation Occurring Within Various Dinucleotide Sequences in Nascent and Mature DNA from HU-treated and Control Hamster Fibrosarcoma Cells

	Percentage of dinucleotides containing 5-mCyt			
	CpA	CpG	CpC	CpT
Nascent DNA from control cells	1.11	24.4	0.59	N.D.
Nascent DNA from HU-treated cells	1.57 (141)	36.2 (148)	0.85 (144)	L.D.
Mature DNA from control cells	1.66	26.6	0.49	1.3
Mature DNA from HU-treated cells	3.68 (222)	28.1 (106)	1.42 (290)	2.8 (215)

A(T1)Cl-3 cells were grown to near confluence in ten T-75 tissue culture flasks. Eight flasks were treated with 2.5×10^{-3} M HU for 24 h. Nascent and mature fractions of DNA were isolated from these and the 2 control flasks and subjected to nearest neighbor analysis as described in Methods. Results are given as % of each dinucleotide containing 5-mCyt on the 5' side. Values reported are the average of two separate experiments. Numbers in parenthesis represents % of control cultures not treated with HU. N.D. = not detected. L.D. = unmeasurable, observed only at the limits of detection.

other than CpG became methylated when DNA synthesis was inhibited. Nearest neighbor analysis of the DNA in bulk and nascent DNA fractions was therefore conducted (Table 7). Such analysis permitted the determination of the frequency with which each base was joined by a phosphodiester bond to an upstream Cyt or 5-mCyt. Values are reported as percentage of total methylation occurring 5' to any of the four major bases, A,G,C or T. Hypermethylation was observed in all possible 5-mCyt-containing dinucleotides. However, the vast majority of hypermethylated cytosines was found within the sequence CpG. Of these, the greatest increase was observed in the low molecular weight fraction corresponding to immature DNA. These results strongly suggest that drug-induced hypermethylation cannot be explained by a reduction in methylase sequence specificity, and that the supermethylation observed remains primarily confined to potentially heritable CpG sequences.

DISCUSSION

Our results demonstrate that inhibition of DNA synthesis had variable effects upon DNA methylation. When highly tumorigenic A(T1)Cl-3 hamster fibrosarcoma cells were

treated with concentrations of ara-Cyt, HU or aphidicolin that inhibited DNA synthesis, that small fraction of total DNA that underwent replication during the treatment period became hypermethylated. This hypermethylation response to DNA synthesis inhibition was dose-dependent for each of the 3 inhibitors. It was observed in 2 human cell lines as well, indicating that it was not specific for a single cell type.

Szyf et al (33) have recently suggested that prokaryotic DNA methylation patterns are determined by the intracellular levels of methylase. These authors have defined a "methylation quotient" which represents the ratio of methylation capacity to newly replicated methylation sites. If the methylation capacity of a cell was balanced with the rate of generation of new sites of potential modification, then slowing the rate of replication fork movement might increase the effective methylation capacity allowing de novo modification to occur. Inhibition of DNA synthesis might therefore increase the time available for DNA methylase(s) to act, resulting in the stimulation of DNA modification we have observed.

Hypermethylation required the direct inhibition of replicative synthesis. Indirect inhibition by cycloheximide-mediated inhibition of protein synthesis, by actinomycin D mediated inhibition of RNA synthesis, or by depletion of necessary growth factors by serum deprivation did not induce hypermethylation. To the contrary, each of these agents induced modest decreases in 5-mCyt content of DNA synthesized during the period of inhibition. This result might be explained by the parallel diminution of proteins involved in methylation along with those of replication. This would occur in the case of cycloheximide by a direct inhibition of synthesis of methylation-required proteins, and in the case of actinomycin D by inhibition of the transcription of mRNA coding for these same proteins. Since DNA methylase has been shown recently to undergo cell-cycle-dependent regulation (34), cycloheximide and actinomycin D treatment may result in hypomethylated DNA by inhibiting synthesis of this important enzyme. Serum deprivation may interfere with DNA methylation by a combination of generalized depression of macromolecular syntheses and depletion of required growth factors. In this regard, DNA methylation has been shown to be modulated by unidentified cytoplasmic components (35), and to be inducible by cell growth factors in a cycle-dependent manner (34). It is reasonable to expect, therefore, that depletion of such factors by serum deprivation might lead to hypomethylation of DNA as reported here.

Exposure of cells to concentrations of the topoisomerase inhibitors valdixic acid or novobiocin that inhibited DNA synthesis to levels comparable to those observed with ara-Cyt, HU or aphidicolin induced significant DNA hypomethylation. Since topoisomerase enzymes regulate the topological conformation of DNA (36,37), this result suggests that DNA methylation is a supercoil-dependent phenomenon. This is particularly interesting in view of the finding that DNA methylation itself does not seem to alter the degree of

supercoil formation in cellular DNA (38). It therefore appears from our limited data that a certain topological conformation may provide the best substrate for methylase action. Vardimon and Rich (39) found that poly (dG_pdC) plasmid inserts capable of undergoing transition to Z-form DNA were very poor substrates for prokaryotic Hha I methylase. Our results suggest that eukaryotic methylases prefer DNA substrates that have been exposed to nalidixate- or novobiocin-sensitive topoisomerase action.

The stimulation of DNA methylation induced by HU may have particular significance in our ability to silence eukaryotic genes because such hypermethylation was found to be stable in the absence of drug. Although only a small fraction (+6%) of the total genome became methylated during inhibitor treatment, the predominant sequence modified was found to be the normal methyl-acceptor CpG. Assuming sufficient methylase activity within the cell to maintain hypermethylated CpGs, these sequences may prove to be heritable. However, most of the hypermethylation was observed in the Hirt supernatant; since a substantial portion of this low molecular weight DNA made in the presence of HU does not join bulk chromosomal DNA (24), some of these sequences may be permanently lost from the genome.

The biological implications of DNA hypermethylation may not be limited to gene silencing. HU is known to cause chromosome fragmentation and gene amplification (40), so drug-induced hypermethylation and such phenomena may in some way be linked. Mariani and Schimke (40), for example, recently demonstrated that hydroxyurea induced a rapid increase in dihydrofolate reductase gene amplification in CHO cells and a similar finding was reported for aphidicolin by Huang and colleagues in Chinese hamster V-79 cells (41). Hypermethylation has been reported in amplified DNA of transformants of *Neurospora crassa* (47), and within amplified ribosomal RNA genes in a rat hepatoma cell line (45). If hypermethylation does play a role in the process of gene amplification, it will be important to investigate the respective levels of gene amplification and gene silencing caused by drug-induced hypermethylation.

Whether or not the DNA hypermethylation we have observed is biologically relevant remains an open question. Nevertheless, the potential heritability of the response is an exciting finding since it suggests the use of HU, aphidicolin and similar drugs in silencing genes. We have achieved some initial success demonstrating the feasibility of this approach utilizing a selectable thymidine kinase system in Chinese hamster ovary cells (Nyce and Jones, unpublished). The ability to switch off genes might allow us to reach a greater understanding of the exact relationship between DNA methylation and gene expression. It might also have clinical significance since agents capable of eliciting this response are currently used in cancer chemotherapy.

ACKNOWLEDGEMENTS

This work was supported by grants CA 39913 from the National Institute of Health to Peter A. Jones and CA 07644-01A from the National Cancer Institute to Jonathan Nyce.

REFERENCES

1. Cedar, H. (1984) in DNA Methylation: Biochemistry and Biological Significance. A. Razin, H. Cedar and A.D. Riggs, eds. Springer-Verlag, NY. pp. 146-164.
2. Naveh-Man, T. and Cedar, H. (1981) Proc. Natl. Acad. Sci. (USA) **78**, 4246-4250.
3. Mohandas, T., Sparkes, R.S., Shapiro, L.J. (1981) Science **211**, 393-396.
4. McGhee, J.D. and Ginder, G.D. (1979) Nature **280**, 419-420.
5. van der Ploeg, L.H.T. and Flavell, R.A. (1980) Cell **19**, 947-958.
6. McKeon, C., Ohkubo, H., Paston, I. and de Crombrughe, B. (1982) Cell **29**, 203-210.
7. Gerber-Huber, S., May F.E.B., Westley, B.R., Felber, E.K., Hosback, H.A., Andres, A.C., and Ryffel, G.U. (1983) Cell **33**, 43-51.
8. Conklin, K.F. and Groudine, M. (1984) in DNA Methylation: Biochemistry and Biological Significance. A. Razin, H. Cedar, and A.D. Riggs, eds. Springer-Verlag, NY. pp. 293-351.
9. Jones, P.A. and Taylor, S.M. (1980) Cell **20**, 85-93.
10. Groudine, M., Eisenman, R., and Weintraub, H. (1981) Nature **292**, 311-312.
11. Woodcock, D.M., Adams, J.K., Allan, R.G., and Cooper, L.A. (1983) Nucl. Acids Res. **11**, 489-499.
12. Burdon, R.H. and Adams, R.L.P. (1969) Biochem. Biophys. Acta **174**, 322-329.
13. Kappler, J.W. (1970) J. Cell. Physiol. **75**, 21-32.
14. Boehm, T.L.J. and Drahovsky, D. (1982) Cancer Res. **42**, 1537-1540.
15. Griffin, J.D., Major, P.P., Munroe, D., Kufe, D., (1982) Exp. Hematol. **10**, 774-781.
16. Rees, L.H. (1975) J. Endocrin. **67**, 143-157.
17. Imura, H. (1980) Adv. Cancer Res. **33**, 39-64.
18. Weinhouse, S. (1972) Gann Monograph on Cancer Res. **13**, 1-12.
19. Weinhouse, S. (1980) Cancer **45**, 2975-2980.
20. Timson, J. (1975) Mutation Res. **32**, 115-132.
21. Spadariet, S., Sala, F. and Pedrali-Noy, G. (1984) Adv. Exp. Med. Biol. **179**, 169-181.
22. Martin, R.F., Radford, I. and Pardee, M. (1977) Biochem. Biophys. Res. Commun. **74**, 9-15.
23. Radford, I.R., Martin, R.F., and Finch, L.R. (1982) Biochim. Biophys. Acta **696**, 145-153.
24. D'Anna, J.A., Crissman, H.A., Jackson, P.J. and Tober, R. (1985) Biochemistry **24**, 5020-5026.
25. Hirt, B. (1967) J. Mol. Biol. **26**, 365-369.
26. Coyle, M.B. and Strauss, B. (1970) Cancer Res. **30**, 2314-2319.
27. Benedict, W.F., Banerjee, A., Gardner, A., Jones, P.A. (1977) Cancer Res. **37**, 2202-2208.
28. Wilson, V.L. and Jones, P.A. (1983) Science **220**, 1055-1057.
29. Flatau, E., Bogenmann, E., and Jones, P.A. (1983) Cancer Res. **43**, 4901-4905.
30. Marmur, J. (1961) J. Mol. Biol. **3**, 208-218.
31. Jensen, D.E. (1978) Biochemistry **17**, 5108-5113.
32. Gruenbaum, Y., Szyf, M., Cedar, H., and Razin, A. (1983) Proc. Natl. Acad. Sci. (USA) **80**, 4919-4921.
33. Szyf, M., Avraham-Haetyni, K., Reifman, A., Shlomai, J., Kaplan, F., Oppenheim, A., and Razin, A. (1984) Proc. Natl. Acad. Sci. (USA) **81**, 3278-3282.
34. Szyf, M., Kaplan, F., Mann, V., Giloh, H., Kedar, E., and Razin, A. (1985) J. Biol. Chem. **260**, 8653-8656.
35. Kautiainen, T.L. and Jones, P.A. (1985) Biochemistry **24**, 5575-5581.
36. Wang, J.C. (1985) Ann. Rev. Biochem. **54**, 665-697.
37. Mattern, M.R. and Scudiero, D.A. (1981) Biochim. Biophys. Acta **653**, 248-258.
38. Rich, A., Nordheim, A., Wang, A.H. (1984) Ann. Rev. Biochem. **53**, 791-846.

39. Vardimon, L. and Rich, A. (1984) *Proc. Natl. Acad. Sci. (USA)* 81, 3268-3272.
40. Mariani, B.D. and Schimke, R.T. (1984) *J. Biol. Chem.* 259, 1901-1910.
41. Huang, Y., Chang, C., and Trosko, J.E. (1983) *Cancer Res.* 43, 1361-1364.
42. Taylor, J.H. (1978) in DNA Synthesis: Present and Future, Molineux, I. and Kohiyama, M. eds. Plenum Press, NY. pp. 143-159.
43. Meuth, M. and Green, H. (1974) *Cell* 3, 367-374.
44. Lewis, W.H. and Wright, J.A. (1978) *J. Cell. Physiol.* 97, 73-86.
45. Sugino, A., Nakayama, K. (1980) *Proc. Natl. Acad. Sci. (USA)* 77, 7049-7053.
46. Sabourin, C.L.K., Bates, P.F., Glatyes, L., Chans, C.-C., Trosko, J.E., Bolyi, J.A. (1981) *Som. Cell Genet.* 7, 255-268.
47. Bull, J.H. and Wootton, J.C. (1984) *Nature* 310, n701-704.
48. Tantravahi, U., Guntaka, R.V., Erlanger, B.F., and Miller, O.J. (1981) *Proc. Natl. Acad. Sci. (USA)* 78, 489-493.