0026-895X/91/0250-08$03.00/0

MOLECULAR PHARMACOLOGY, 39:250-257

Induction of Deoxycytidine Kinase by 5-Azacytidine in an HL-60 Cell Line Resistant to Arabinosylcytosine

XIANG-BIN KONG, WILLIAM P. TONG, and TING-CHAO CHOU

Laboratory of Biochemical Pharmacology, Memorial Sloan-Kettering Cancer Center, Cornell University Graduate School of Medical Sciences, New York, New York 10021

Received August 15, 1990; Accepted November 7, 1990

SUMMARY

Induction of 2′-deoxycytidine kinase (dCK) by 5-azacytidine (5-Aza-C) in a dCK-deficient HL-60 cell line resistant to 1-β-D-arabinofuranosylcytosine (Ara-C) (HL-60/Ara-C) was examined by measurement of the incorporation of [^{3}H]deoxycytidine ([^{3}H]dCyd) into cellular DNA, the kinetic properties of purified dCK, the cytotoxic potency (IC_{50} values), and the DNA methylation patterns of 5-Aza-C-treated and untreated cells. Following a 72-hr exposure to 1 μM 5-Aza-C, the incorporation of [^{3}H]dCyd into DNA was increased 6-fold and the total dCK activity was increased 12-fold, with a peak for both on day 6. The onset of dCK induction was dependent on the length of exposure time. The IC_{50} values for cell growth inhibition by Ara-C on day 3 were 0.08 μM for HL-60 cells, 12.5 μM for HL-60/Ara-C cells, and 0.55 μM for HL-60/Ara-C cells pretreated with 5-Aza-C for 40 hr. The K_m and V_{max} of dCyd for HL-60 dCK were similar to those for 5-Aza-C-induced HL-60/Ara-C dCK. The restriction enzymes *Hpa*II, which cleaves CCGG sequences but cannot cleave at sites methylated at the internal cytosines (5′-C^{Me}CGG), and *Msp*I, which cleaves sequences with internal methylated cytosine but cannot cleave at sites methylated at external cytosines (5′-^{Me}CCGG), were used for DNA methylation pattern determination. The newly synthesized DNA of HL-60 wild-type cells was cleaved by *Msp*I to a greater extent than that of HL-60/Ara-C cells. After exposure to 1 μM 5-Aza-C for 40 hr, methylation patterns of newly synthesized DNA reverted in HL-60/Ara-C cells to a cleavage pattern similar to that in HL-60 wild-type cells. When compared with untreated control, DNAs from 5-Aza-C-treated resistant cells were cleaved to a greater extent by *Msp*I than by *Hpa*II, suggesting that internal cytosine-residue methylation was relatively uninhibited. The data given above suggest that 1) dCK in HL-60/Ara-C cells was induced by 5-Aza-C and the resistance to Ara-C in the cells was partially reversed by 5-Aza-C, 2) the induced enzyme was similar in terms of its kinetic properties to the wild-type enzyme, 3) DNA from HL-60/Ara-C cells may have more methylation at the external cytosine residues of 5′-CCGG sequences than DNA from wild-type HL-60 cells, and 4) the DNA methylation at external cytosine residues was inhibited (i.e., hypomethylation state) when dCK expression was induced by 5-Aza-C treatment.

Mammalian DNA is modified shortly after replication by the enzymatic conversion of 3% of cytosines to 5-methylcytosine (1, 2). Although the biological function of DNA methylation at the molecular level is unknown, considerable evidence has accumulated demonstrating that DNA methylation is involved in cell differentiation (3, 4) and regulation of gene expression (5–9). 5-Aza-C has a broad spectrum of biological activities; one of its unusual properties is its ability to inhibit DNA methylation (10). The inhibition of DNA methylation by 5-Aza-C results in the activation of certain genes, presumably by reversal of the usual methyl-mediated state of repression (11). The correlation between hypomethylation of the promoter or control regions of genes and their high level expression has been documented in a variety of experimental systems (5).

This study was supported by Grants CA 27569 and CA 18856 from the National Cancer Institute and by the Elsa U. Pardee Foundation.

It has been established that the resistance to Ara-C in an HL-60 cell line is mainly due to a deficiency in dCK activity (12). This cell line shares numerous biological and biochemical features with the parent cell line and displays a stable resistant phenotype in the absence of continued selection, i.e, the cells have been maintained in Ara-C-free medium for 6 months without spontaneous reversion to the parent phenotype (12). Recently, the sequences of cDNA clones for human dCK and analysis of expression in cells with and without enzyme activity have been reported by Huang *et al* (13). They found that there were no differences in the amount or size of dCK protein and mRNA between CCRF/CEM (or L1210) leukemia cells that express and those that do not express dCK activity. Genomic restriction fragments were similar between the active and inactive CCRF/CEM cell lines. These data suggest that gene amplification is not involved in the mechanism of Ara-C resistant. The precise mechanisms of the genetic defect(s) in the

ABBREVIATIONS: 5-Aza-C, 5-azacytidine; dCK, 2′-deoxycytidine kinase; Ara-C, 1-β-D-arabinofuranosylcytosine; dCyd, deoxycytidine; dThd, deoxythymidine; BSA, bovine serum albumin; FPLC, fast protein liquid chromatography.

TABLE 1

Incorporation of [^{3}H]dCyd into DNA in HL-60/Ara-C cells after treatment with 1 μM 5-Aza-C

The incorporation of [^{3}H]dCyd into DNA in HL-60 wild-type cells was measured under the same conditions, with a value of 13,860 ± 1,370 cpm/10^6 cells from day 1 to day 19. Values are mean of two experiments. The untreated control value was 1134 ± 254 cpm/10^6 cell.

Treatment	Exposure time	Incorporation of [^{3}H]dCyd into DNA							
		1[a]	3	6	9	10	12	16	19
	hr	*% of untreated control*							
5-Aza-C	24	ND[b]	80	561	190	199	220	ND	ND
5-Aza-C	72	86	87	630	202	380	230	153	290

[a] Days after exposure to 5-Aza-C.
[b] ND, not determined.

structural gene responsible for the deficiency of dCK activity in Ara-C-resistant cells remain unclear. Studies have shown that 5-Aza-C treatment is capable of inducing the expression of dCK in CCRF/CEM/dCK$^-$ cells *in vitro* and reestablishing sensitivity toward Ara-C in pediatric patients who were resistant to Ara-C (14, 15).

The purpose of this study was to explore the possible differences in DNA methylation patterns between the wild-type HL-60 and HL-60/Ara-C cells and to correlate DNA methylation patterns and induction of dCK activity after treatment with 5-Aza-C in HL-60/Ara-C cells. In this study, the time course and the ability of 5-Aza-C to induce dCK in HL-60/Ara-C cells were examined by measurement of the incorporation of [^{3}H] dCyd into cellular DNA, the activity of FPLC-purified dCK, the reversibility of resistance to Ara-C, the similarity of K_m values for the dCK purified from wild-type HL-60 cells and from HL-60/Ara-C cells induced by 5-Aza-C, and the changes in DNA methylation patterns before, during, and after the 5-Aza-C treatment. Comparisons have been also made for the DNA methylation patterns between the wild-type HL-60 cell line and the HL-60/Ara-C cell line.

Materials and Methods

Chemicals and radioactive chemicals. Ara-C and 5-Aza-C were obtained from Sigma Chemical Co., (St. Louis, MO). ED-81 filter paper discs were from Whatman (Hillsboro, OR). Agarose, protein standards, and DNA size standards were purchased from Bio-Rad (Richmond, CA). BSA and the restriction enzymes *Hpa*II and *Msp*I were from Pharmacia (Piscataway, NJ). [*methyl*-^{3}H]dThd (68 Ci/mmol) and [5-^{3}H]dCyd (26 Ci/mmol) were products of ICN Biomedicals Inc. (Irvine, CA). [2-^{14}C]dThd (50 mCi/mmol) was from New England Nuclear (Boston, MA). All other chemicals, unless otherwise indicated, were from Sigma Chemical Co.

Cell lines and cell culture. HL-60 cells and HL-60 cells that were resistant to Ara-C (HL-60/Ara-C) (from Dr. Steven Grant of Medical College of Virginia, Richmond, VA) were propagated twice weekly in RPMI 1640 medium supplemented with 10% fetal calf serum, 100 U/ml penicillin, 100 μg/ml streptomycin, and 1% sodium pyruvate. The cells were kept in a 37°, 5% CO_2 atmosphere and routinely examined to ensure that they were free from *Mycoplasma* contamination. The HL-60/Ara-C cells were maintained in 1 μM Ara-C, which was added to medium twice weekly. Logarithmically growing cells were utilized for all experiments.

Drug treatment and measurement of IC_{50} values, kinetic parameters, and DNA synthesis. HL-60/Ara-C cells were treated with 1 μM 5-Aza-C for 24, 40, and 72 hr. 5-Aza-C was removed from the culture and the cells were resuspended in fresh drug-free medium. The cells were harvested at desired intervals after drug treatment. In another experiment, HL-60/Ara-C cells were continuously treated with 1 μM 5-Aza-C with replenishment of the drug. The viability of cells was determined by the trypan blue exclusion method, using a hemocytometer. The IC_{50} values of Ara-C for HL-60 cells, HL-60/Ara-C cells, and 5-Aza-C HL-60/Ara-C cells treated were calculated by the median-effect method (16), using computer software (17). The incorporation of [^{3}H]dCyd into DNA and the K_m and V_{max} values of dCK were measured by a previously described method (17, 18), except that FPLC-purified dCK was used.

Determination of dCK activity. Cells (1–5 × 10^7/ml) were washed once with phosphate-buffered saline and resuspended in buffer A (25 mM Tris·HCl, pH 8.0, 1 mM glutathione, 10% glycerol, 5 mM $MgCl_2$, 0.1 mM ATP). The cells were disrupted in a Tri-R tissue homogenizer with a Teflon pestle (scale position 4, 1 min), followed by centrifugation at 105,000 × *g* for 1 hr at 4°. The supernatants were further purified by 20–40% saturated ammonium sulfate fractionation. The 20–40% ammonium sulfate precipitates, dissolved in buffer A, were applied to a Sephacryl S-200 gel filtration column for FPLC (Pharmacia). Elution was carried out with buffer A at a flow rate of 0.5 ml/min. One milliliter/fraction was collected. The reaction mixture [6 mM ATP, 6 mM $MgCl_2$, 0.2 mM 3,4,5,6-tetrahyrouridine, 0.2% (w/v) BSA, 1 unit of creatine kinase, 6 mM creatine phosphate, 7 mM NaF, 2 mM dithiothreitol, 0.5 μCi of [^{3}H]dCyd (26 Ci/mmol), 0.1 M Tris·HCl, pH 7.5, and enzyme] was incubated at 37° for 60 min. The dCyd monophosphates formed were detected by a DE-81 disc method (18). The dCK activity was calculated and expressed as fmol of dCMP/min/μg of protein. The amount of protein was quantitated by the Bio-Rad method.

Assay of DNA methylation pattern by restriction enzymes *Hpa*II and *Msp*I. The HL-60/Ara-C cells were prelabeled with [^{14}C]

TABLE 2

dCK activity after exposure to 5-Aza-C in HL-60/Ara-C cells

The dCK activity in wild-type HL-60 cells was 7.2 fmol/min/μg of protein (mean of two experiments). All the values are mean of two experiments, except the values for untreated cells. For untreated cells, the values are mean of three to five experiments; standard deviations are 0.0037, 0.025, 0.0076, 0.008, and 0.007 fmol/min/μg of protein for days, 3, 6, 9, 12, and 19, respectively. Values in parentheses represent fold increase of dCK activity, compared with the untreated control.

Treatment	Exposure time	dCK activity Days after removal of 5-Aza-C				
		3	6	9	12	19
	hr	*fmol/min/μg of protein*				
None	0	0.086	0.094	0.080	0.083	0.088
5-Aza-C (1 μM)	24	0.1 (1.2)	0.46 (5)	0.41 (5.2)	0.58 (7.0)	0.24 (2.7)
5-Aza-C	40	0.86 (9.9)	0.70 (7.5)	0.63 (7.9)	0.21 (2.5)	ND[a]
5-Aza-C	72	0.22 (2.6)	1.19 (12.7)	ND	0.391 (4.7)	0.62 (7.1)
5-Aza-C	Continuous up to 19 days	0.082 (0.95)	0.13 (1.4)	0.36 (4.5)	0.73 (8.8)	0.25 (2.8)

[a] ND, Not determined.

TABLE 3

Comparison of cytotoxicity of Ara-C in HL-60, HL-60/Ara-C, and 5-Aza-C (1 μM, 40 hr)-treated HL-60/Ara-C cells

Values are the mean of two experiments. The median inhibitory concentration (IC_{50}) was determined from five different Ara-C concentrations, using median-effect plots and computer software (17).

Cells	Treatment	Exposure time	Ara-C IC_{50}
		hr	*μM*
HL-60	None		0.078
HL-60/Ara-C	None		12.5
HL-60/Ara-C	5-Aza-C	24	0.5
HL-60/Ara-C	5-Aza-C	72	0.6

TABLE 4

Comparison of V_{max} and K_m values for dCK purified from wild-type HL-60 cells and HL-60/Ara-C cells treated with 5-Aza-C

The variations (mean ± standard deviation) in V_{max} and K_m values in two separate experiments were 2.2 ± 0.14 and 0.52 ± 0.03 for wild-type HL-60 cells and 1.29 ± 0.03 and 0.43 ± 0.01 for 5-Aza-C-treated HL-60/Ara-C cells, respectively. There are no significant differences for the V_{max} ($p = 0.24$) and the K_m ($p = 0.25$) values between the wild-type HL-60 cells and 5-Aza-C-treated HL-60/Ara-C cells, as calculated by Mann-Whitney confidence interval test. Cells were exposed to 1 μM 5-Aza-C for 40 hr and dCK was purified as described in Materials and Methods.

Enzyme source	V_{max}		K_m	
	Expt. 1	Expt. 2	Expt. 1	Expt. 2
	fmol/min/μg of protein		*μM*	
Wild-type HL-60	2.10	2.30	0.50	0.54
5-Aza-C-treated HL-60/Ara-C	1.27	1.31	0.42	0.44

dThd for 48 hr, followed by incubation in [^{14}C]dThd-free medium for 24 hr, and then treated with 1 μM 5-Aza-C for 40 hr. The cells were then pulse-labeled during the last hour of treatment with [^{3}H]dThd and washed twice with phosphate-buffered saline. The cell DNAs were extracted by the method described by Davis *et al* (19). The DNA concentrations were determined spectrophotometrically, and aliquots of 5 μg of DNA were incubated with *Hpa*II or *Msp*I, at a final concentration of 3 units/μg of DNA, at 37° for 2 hr. The assay buffer for *Hpa*II was 10 mM Tris·HCl, pH 7.5, 50% glycerol, 50 mM KCl, 1 mM dithiothreitol, 0.1 mM EDTA, 300 μg/ml BSA; for *Msp*I, 10 mM Tris·HCl, pH 7.5, 10 mM $MgCl_2$, 10 mM KCl, 1 mM β-mercaptoethanol, 100 μg/ml BSA. The reaction mixture was stopped by addition of 5 μl of sodium dodecyl sulfate-EDTA and was subjected to 1% agarose gel electrophoresis in 40 mM Tris-acetate, 10 mM EDTA buffer, pH 7.2. The gel lanes were cleaved from the slabs and sliced into 2-mm fractions using stacked razor blades. Slices were digested with gel solubilizer (Solusol; National Diagnostics) and counted for radioactivity by addition of scintillation fluid (Soluscint A; National Diagnostics). The size of the DNA from 5-Aza-C-treated cells and untreated cells, after being cleaved with *Hpa*II or *Msp*I, was determined by comparison of the radiography patterns of the gel with the DNA size standards (Pharmacia, Piscataway, NJ). The completeness of *Hpa*II and *Msp*I digestion was confirmed by the observation of identical gel patterns when 2-fold higher enzyme concentrations were used.

Results

Incorporation of [^{3}H]dCyd into DNA. As a pilot experiment, the incorporation of [^{3}H]dCyd into DNA of HL-60/Ara-C cells, after treatment with 1 μM 5-Aza-C for the indicated time intervals, is given in Table 1. Compared with the untreated cells, after 24- or 72-hr exposure to 1 μM 5-Aza-C the incorporation of [^{3}H]dCyd into DNA was increased 5.6–6.3-fold on day 6 after removal of the drug from the cultures. An approximately 2-fold increase was persistantly maintained during the period of 12–19 days after the removal of the drug. The increases in incorporation of [^{3}H]dCyd into DNA (1–2-fold higher than that of control) were continuously observed until the experiments were terminated (after 30 days) (data not shown).

dCK activity. dCK activity in wild-type HL-60 and HL-60/Ara-C cells was purified and measured as described in Materials and Methods. The dCK activity in wild-type HL-60 cells was 7.2 fmol/min/μg of protein. The dCK activity in HL-60/Ara-C cells before, during, and after treatment with 1 μM 5-Aza-C, at different time intervals after removal of the drug, is summarized in Table 2. Compared with the untreated cells, after treatment with 1 μM 5-Aza-C for 24 hr the dCK activity was increased 1.2–7-fold during the period of days 3–12 after removal of the drug, with the maximal increase on day 12. After a 40-hr exposure to 1 μM 5-Aza-C the dCK activity was increased 8–10-fold, with a peak on day 3 after the removal of the drug. Following treatment with 1 μM 5-Aza-C for 72 hr the dCK activity reached its maximum, which was 12.7-fold higher than the activity of the untreated cells on day 6 after removal of the drug. Continuous exposure (without removal of the drug) to 1 μM 5-Aza-C did not result in an increase in dCK activity until day 9; the maximal increase was observed on day 12. By comparing the onset time of the maximum increase in dCK activity in different treatment regimens, we found that the onset of maximal increase in the dCK activity was delayed when exposure time was shortened (e.g., 24 hr) or when exposure time was continuous. When cells were exposed to 5-Aza-C for 40 hr, the dCK activity was induced at the maximum level (9.9-fold) as early as day 3 after removal of 5-Aza-C. Longer exposure to the drug did not lead to an early onset of dCK activity induction. From day 19 after removal of the drug to the termination of the experiment (30 days), the dCK activity was still 2–3-fold higher than that of untreated cells (data not shown). The difference in the maximum increases, after a 24-hr exposure to 5-Aza-C, for the incorporation of [^{3}H]dCyd into DNA and for dCK activity measurements may be due to the fact that the incorporation of [^{3}H]dCyd into DNA is affected by the extent of DNA synthesis at different cell phases, whereas dCK activity is not. Based on the dCK activity measurements, 40 hr was chosen as the optimal exposure time for the following experiments.

IC_{50} concentrations of Ara-C in cells with or without pretreatment with 1 μM 5-Aza-C. Comparisons of IC_{50} values for cell growth inhibition, after exposure to Ara-C for 3 days, in HL-60 cells, HL-60/Ara-C cells, and HL-60/Ara-C cells pretreated with 1 μM 5-Aza-C are presented in Table 3. The IC_{50} of Ara-C in HL-60/Ara-C cells was approximately 160-fold higher than that of Ara-C in wild-type HL-60 cells. When the HL-60/Ara-C cells were pretreated with 1 μM 5-Aza-C for 40 hr, the drug was removed, and cells were harvested for determination of cytotoxicity of Ara-C at 3 days after removal of the drug, the pretreated cells became much less resistant to Ara-C, with an IC_{50} 23-fold higher than that of wild-type HL-60 cells.

K_m and V_{max} values of dCK. As shown in Table 4, comparisons of K_m and V_{max} values for dCK purified from wild-type HL-60 cells and from HL-60/Ara-C cells pretreated with 5-Aza-C were made. The K_m values for dCK from wild-type HL-60 cells and from HL-60/Ara-C cells pretreated with 5-Aza-C were not significantly different.

DNA methylation patterns of HL-60 cells and HL-60/

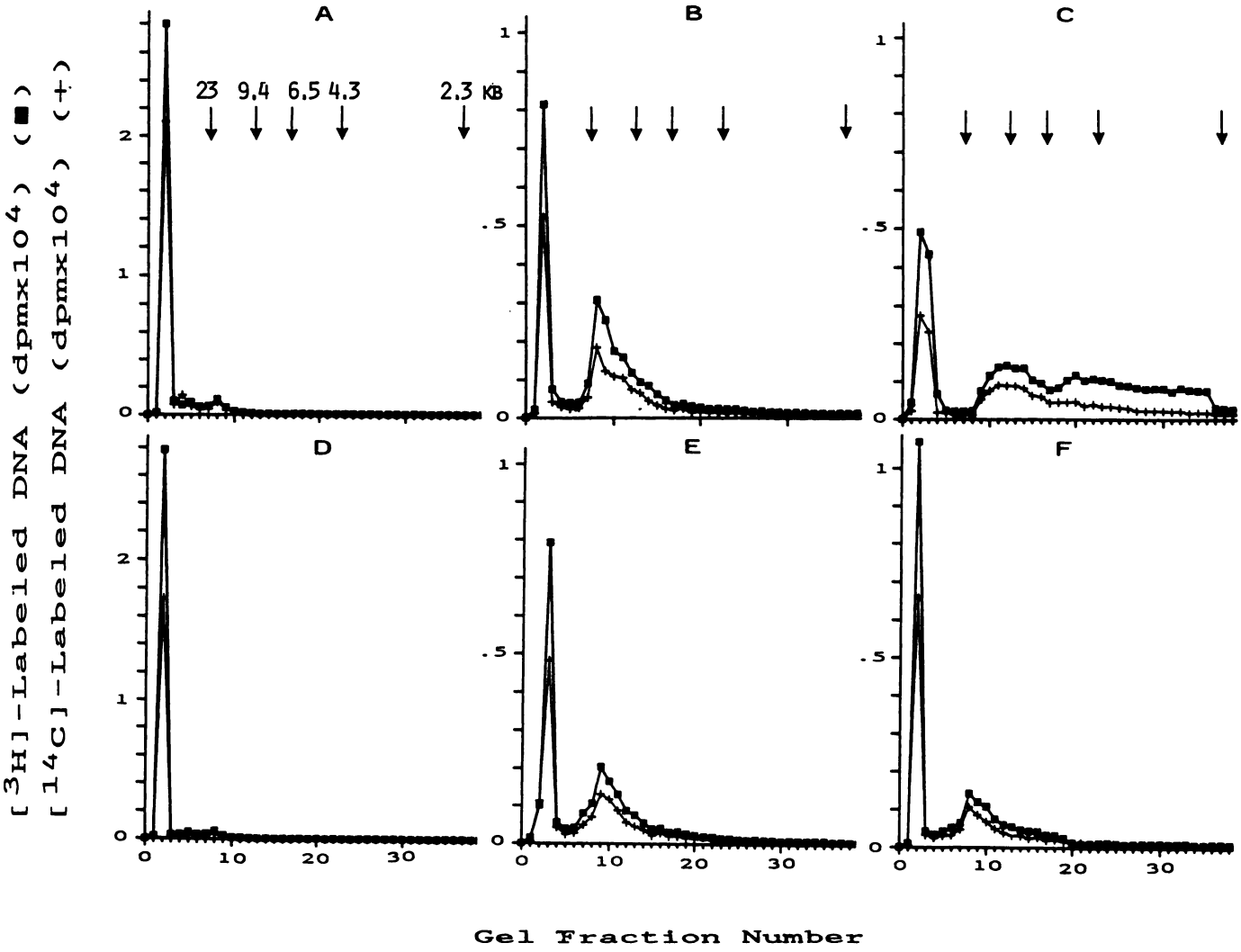

Fig. 1. Comparison of DNA methylation patterns between HL-60 cells (A–C) and HL-60/Ara-C cells (D–F), as analyzed by agarose gel electrophoresis. The parental DNA was labeled by [^{14}C]dThd (+) and the newly synthesized DNA was labeled by [^{3}H]dThd (■). Restriction enzymes *Msp*I and *Hpa*II were used for the determination of DNA methylation. For the catalytic DNA-cleavage properties of these enzymes, see Table 5. A–C, HL-60 cell DNA. A, Uncleaved; B, cleaved by *Hpa*II; C, cleaved by *Msp*I; D–F, HL-60/Ara-C cell DNA. D, Uncleaved; E, cleaved by *Hpa*II; F, cleaved by *Msp*I. The details of procedures are described in Materials and Methods. Similar observations were obtained in a separate experiment.

TABLE 5

Effect of methylation of DNA on specificity of *Hpa*II and *Msp*I cleavage, and the DNA methylation patterns in HL-60, HL-60/Ara-C, and 5-Aza-C-treated HL-60/Ara-C cells

For details see Ref. 20.

Restriction enzyme	Sequence cleaved	Sequences not cleaved	Cleavage		
			HL-60	HL-60/Ara-C	5-Aza-C-treated HL-60/Ara-C
*Hpa*II	5′-CCGG-3′	C^{Me}CGG	Limited cleavage	Limited cleavage	Limited cleavage
*Msp*I	CCGGC^{Me}CGG	^{Me}CCGG	Extensive cleavage	Limited cleavage	Extensive cleavage

Ara-C cells. The effect of 5-Aza-C on DNA methylation patterns was studied using the restriction enzymes *Hpa*II and *Msp*I. These enzymes are isoschizomers and both cleave the sequence 5′-CCGG, but *Hpa*II does not cleave if the internal cytosine residue is methylated and *Msp*I does not cleave if the external cytosine residue is methylated (20). Thus, by comparison of the electrophoretic patterns on agarose gel produced by digestion of a given DNA with each of the two sources of enzyme(s), the extent and pattern of the enzymatic DNA methylation at internal and external cytosines of 5′-CCGG-3′ sequences in this DNA can be estimated. The parental DNA was labeled with [^{14}C]dThd, and the DNA that was newly synthesized during various times after 5-Aza-C treatment was labeled with [^{3}H]dThd. As shown in Fig. 1, the uncleaved DNAs from HL-60 cells and HL-60/Ara-C cells all demonstrated similar mobilities (Fig. 1, A and D). The DNAs from HL-60 cells and HL-60/Ara-C cells were cleaved by *Hpa*II to the same extent (Fig. 1, B and E). However, the newly synthesized DNA from HL-60 cells was cleaved by *Msp*I to a much greater extent than the DNA from HL-60/Ara-C cells, as indicated by the increased proportion of low molecular weight DNA, which appeared in bands of approximately 5.0–2.3 kilobases (Fig. 1,

C and F); the parental DNA from both cell lines did not reveal appreciable differences (Fig. 1). The difference between untreated HL-60 wild-type and HL-60/Ara-C cells, in terms of their digestion patterns, was that DNA from HL-60/Ara-C cells was not cleaved by the restriction enzyme *Msp*I, which is known to cleave CCGG-C^{Me}CGG sequences but not to cleave 5′-^{Me}CCGG-3′ sequences. These data suggest that HL-60/Ara-C DNAs may be predominantly methylated at external rather than internal cytosine residues in 5′-CCGG sequences.

DNA methylation patterns during the induction of dCK by 5-Aza-C. To determine whether DNA methylation patterns were different during the induction of dCK by 5-Aza-C and to study the correlation between DNA methylation patterns and induction of dCK, the HL-60/Ara-C cells were treated with 1 μM 5-Aza-C for 40 hr, as described above. The DNA methylation patterns were studied, by means of the restriction enzymes *Hpa*II and *Msp*I, during the time course from 0 to 96 hr after removal of 5-Aza-C. As shown in Fig. 2, the DNA synthesis (total [^{3}H]dThd incorporation) was inhibited to different degrees during and after the treatment with 5-Aza-C. The newly synthesized DNA at 24–96 hr after 40-hr treatment with 5-Aza-C was cleaved by *Hpa*II to a similar extent as untreated control (Fig. 2, F–I). In contrast, *Msp*I cleaved 5-Aza-C-treated DNAs to a greater extent than the untreated control, as indicated by the increased proportion of small sizes of DNA, which have molecular weights ranging from 5.0 to 2.3 kilobases (Fig. 2, L–O). The DNAs synthesized during the 5-Aza-C treatment (time 0, after 5-Aza-C removal; Fig. 2, E and K) were poorly cleaved by both *Hpa*II and *Msp*I. The inhibitory effects of 5-Aza-C on DNA methylation were also observed in separate experiments in which the 5-methyl-deoxycytosine levels of newly synthesized DNA were measured by high performance liquid chromatography (data not shown). A comparison of DNA methylation patterns in HL-60/O cells, HL-60/Ara-C cells, and 5-Aza-C-treated HL-60/Ara-C cells is summarized in Table 5.

Discussion

In this study, we demonstrated that, when HL-60/Ara-C cells were treated with 1 μM 5-Aza-C for 40 hr, different DNA methylation patterns, as shown by DNA cleavage with *Msp*I, were observed 24–96 hr after removal of 5-Aza-C, during which time the dCK activity was induced and was accompanied by the reacquisition of sensitivity to Ara-C. Five days after the removal of 5-Aza-C, the DNA methylation patterns, as shown by the gels, returned to patterns comparable to those of the untreated control. Our finding is in agreement with what Antonsson *et al.* (14) reported concerning the induction of dCK in CCRF/CEM/dCK$^-$ cells. Although the DNA methylation changes were not observed directly from dCK genes, these results suggest that this transient genome-wide hypomethylation is in a unique pattern in 5-Aza-C-treated HL-60/Ara-C cells and is associated with the reexpression of previously inactive genes coding for dCK, which are silent probably because of hypermethylation.

We also found that, although the DNA methylation patterns in 5-Aza-C-treated HL-60/Ara-C cells were restored to the untreated control patterns, an increase in dCK activity was detected until day 19 after the termination of 5-Aza-C treatment, or until day 15 after the DNA methylation patterns were back to the untreated control patterns. This observation supports the view that the activation of gene expression by 5-Aza-C is inheritable for many generations in the absence of further drug treatment. However, this observation also raises the question of whether the DNA hypomethylation is really necessary to keep the gene in active status. The answer is yet unknown,

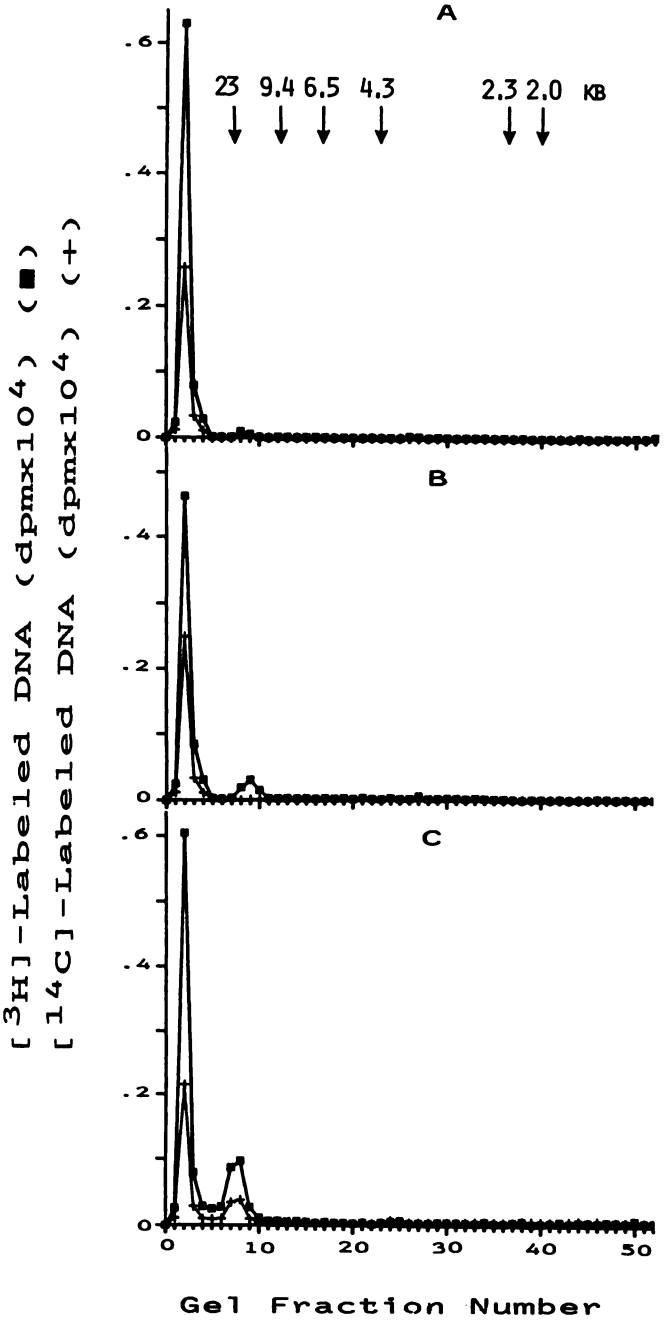

Fig. 2. Time course of DNA methylation patterns following 5-Aza-C treatment (1 μM, 40 hr) in HL-60/Ara-C cells, as analyzed by agarose gel electrophoresis. Parental DNA was labeled with [^{14}C]dThd (+) and newly synthesized DNA was labeled with [^{3}H]dThd (■). A, 5-Aza-C-untreated control; B, 5-Aza-C-treated, at 0 hr after removal of drug; C, 5-Aza-C-treated, at 24 hr after removal of drug. D–I, Labeled DNAs that were cleaved with *Hpa*II. D, 5-Aza-C-untreated control. E–I, 5-Aza-C-treated, at 0–96 hr after removal of 5-Aza-C. E, 0 hr; F, 24 hr; G, 48 hr; H, 72 hr; I, 96 hr after removal of the drug. J–O, Labeled DNAs that were cleaved with *Msp*I. J, 5-Aza-C untreated control. K–O, 5-Aza-C-treated, at 0–96 hr after removal of 5-Aza-C. K, 0 hr; L, 24 hr; M, 48 hr; N, 72 hr; O, 96 hr after removal of the drug. Similar observations were obtained in a separate experiment.

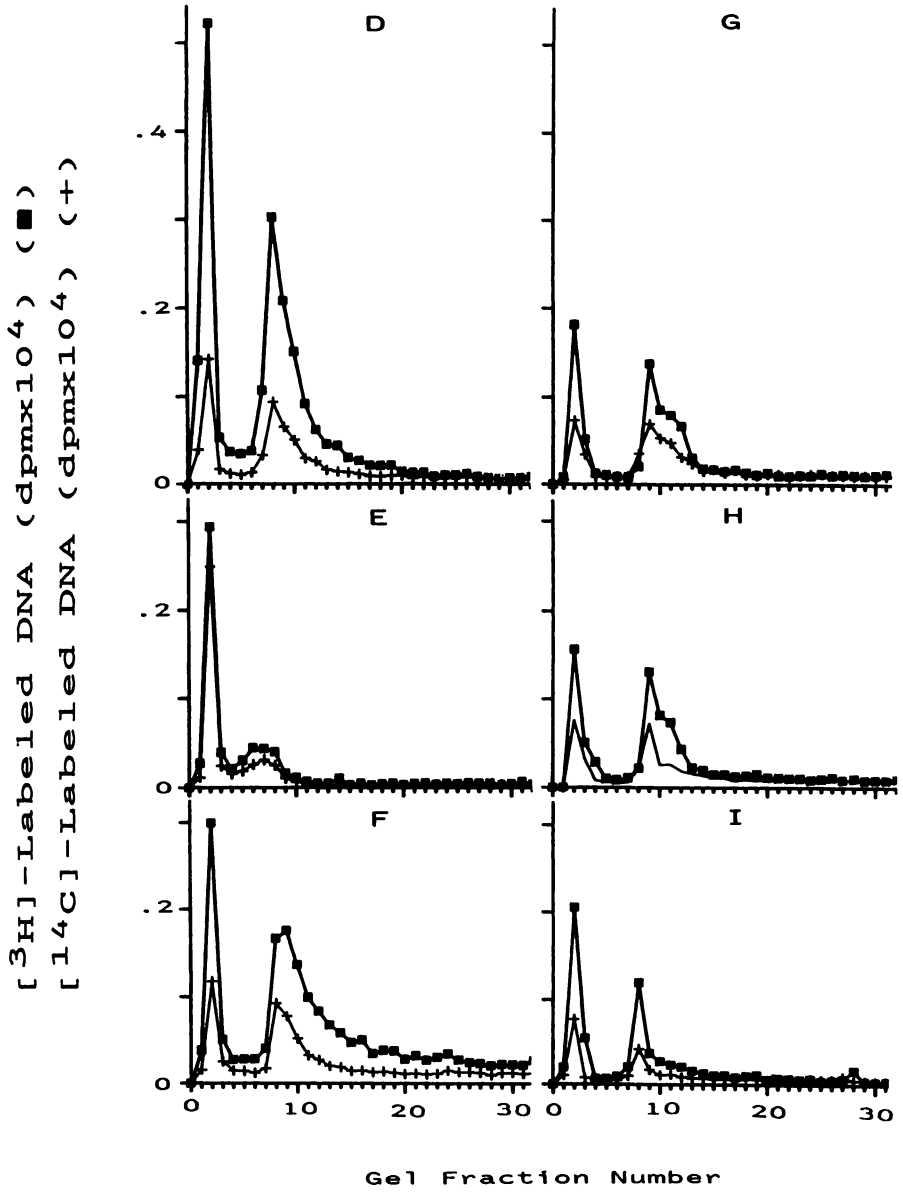

Fig. 2.

because how well DNA methylation patterns are copied in cell culture is poorly understood at the present time. Previous studies suggest that DNA methylation patterns in 5-Aza-C-treated cells are unstable, with remethylation and reversion to the pretreated phenotype occurring shortly after drug removal (21–25). It seems very important that a more precise and more quantitative method for the determination of transient DNA demethylation and remethylation be developed.

In this study, our data showed that the DNA of HL-60 cells was cleaved by *Msp*I to a greater extent than that of HL-60 cells deficient in dCK. This result suggests that the DNA from HL-60/Ara-C cells has more methylation in the external cytosine residues of 5′-CCGG sequences than DNA from wild-type HL-60 cells, and this is consistent with the previous reports that *in vitro* Ara-C treatment was able to induce a genome-wide elevated level of enzymatic DNA methylation (26, 27). As described in this study, 5-Aza-C is able to inhibit DNA methylation, induce reexpression of dCK activity, and reestablish sensitivity to Ara-C in HL-60/Ara-C cells. It has been reported that the ability of 5-Aza-C to activate genes is selective, rather than involving a generalized increase in gene expression (7). Taken together, these data support the notion that methylation in individual gene loci is one of the factors governing gene expression in eukaryotic cells.

A very intriguing observation was that the DNAs synthesized 24–96 hr after termination of 5-Aza-C treatment were cleaved by *Msp*I but not by *Hpa*II, although both of these enzymes cleave DNA at 5′-CCGG-3′ sequences. Singer *et al* (28) have demonstrated that, in mouse liver DNA, the 5′-CCGG sites present in the genome are cleaved by *Msp*I much more frequently than by *Hpa*II and 77% of the total 5′-CCGG sites are modified for some reason to protect them from being cleaved by *Hpa*II. One possible explanation for these results is that the methylation of internal cytosine residues in 5′-CCGG sites that is affected by 5-Aza-C treatment is not significant enough to be detected by *Hpa*II restriction assay, probably because 5-Aza-C is incorporated into only one DNA strand during the 40-hr treatment. The parental strand and the progeny strands that

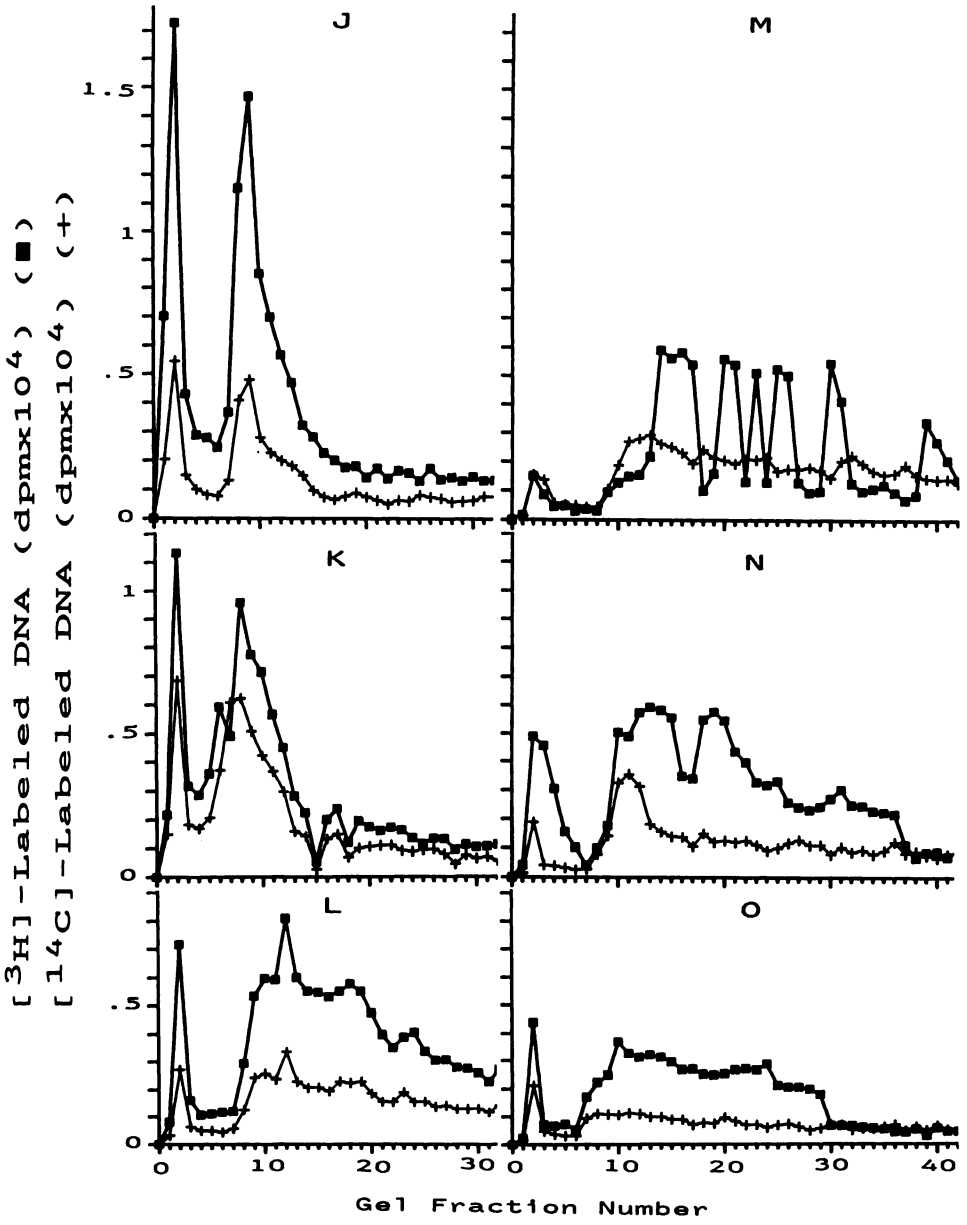

Fig. 2.

are subsequently synthesized using this 5-Aza-C-incorporated sequence as template would then be fully methylated and, therefore, not sensitive to cleavage by *Hpa*II. In fact, these results are consistent with our finding that the DNA of wild-type HL-60 cells is cleaved only by *Msp*I to a greater extent than that of HL-60/Ara-C. Whether the hypermethylation in DNA of HL-60/Ara-C cells occurs predominantly at the external cytosines of 5′-CCGG sequences or whether 5-Aza-C specifically inhibits DNA methylation in such sites of 5′-CCGG sequences remains to be determined.

The K_m values should be similar if the enzymes are from the same source, regardless of the enzyme concentration used and the range of substrate concentrations involved in the assay. In this study, we have shown that the K_m value of dCK from HL-60/Ara-C cells induced by 5-Aza-C is close to that of dCK from wild-type HL-60 cells, although the enzyme activities are somewhat different. These results indicate that the induced enzyme may not be different from that of wild-type cells. They could be the products of the same dCK gene. Recently, more studies have shown that epigenetic rather than genetic mechanisms are involved in the control of eukaryotic gene expression (5–8, 29, 30). Furthermore, 5-Aza-C does not show mutagenic activity (31). Our data indirectly support the concept that epigenetic controls of gene expression in eukaryotic cells play an important role in the development of clonal populations of cells that are resistant to some therapeutic agents.

The DNA methyltransferase activity is inhibited by 5-Aza-C because of the formation of a tight noncovalent complex or a covalent linkage between the enzyme and 5-Aza-C residues in DNA (10). In this study, we demonstrated that a 40-hr exposure to 5-Aza-C was the most efficient in terms of changes in DNA methylation patterns and in induction of dCK. This period of treatment time approximately corresponds to the doubling time of HL-60/Ara-C cells, during which enough S phase cells of the

total cell population are exposed to the 5-Aza-C and therefore more 5-Aza-C will be incorporated into DNA. However, continuous exposure does not induce the enzyme activity as efficiently as 40-hr exposure but delays the onset of enzyme induction, possibly because of the cytotoxicity of 5-Aza-C.

The dCK activity induced by 5-Aza-C can be a result of increased dCK gene transcription with increased mRNA or of the expression of an altered dCK with different kinetic properties. Because our kinetic analysis revealed no significant differences in K_m or V_{max} values for dCKs isolated from wild-type HL-60 and 5-Aza-C-treated HL-60/Ara-C cells, it is reasonable to suggest that an increased gene expression at the transcriptional level (or reexpression of the gene) is a more likely mechanism. However, unexpected results have been observed by Avramis *et al.* (32), showing that there were no differences in mRNA levels between CEM or L1210 leukemic cell lines that express and do not express dCK activity, using a cDNA coding for dCK. It was suggested that the cDNA clone used did not represent the major dCK but another enzyme or activator with low Ara-C kinase activity (32). It will be of interest to test the 5-Aza-C-induced transient DNA methylation inhibition at the dCK gene once a specific cDNA coding for the major dCK becomes available.

Acknowledgments

We express our gratitude to Dr. Steven Grant of the Medical College of Virginia (Richmond, VA) for providing the HL-60/Ara-C cell line and to Ms. Kathryn J. Brown and Ms. Marilyn Moran for their assistance in the preparation of the manuscript.

References

1. Kalousek, F., and R. Morris. The purification and properties of deoxyribonucleic acid methylase from rat spleen. *J. Biol. Chem.* **224**:1157–1163 (1969).
2. Doskocil, J., and F. Sorm. Distribution of 5-methylcytosine in pyrimidine sequences of deoxyribonucleic acids. *Biochim. Biophys. Acta* **55**:953–959 (1962).
3. Jones, P. A., and S. M. Taylor. Cellular differentiation, cytidine analogs and DNA methylation. *Cell* **20**:85–93 (1980).
4. Creusot, F., G. Acs, and J. K. Christman. Inhibition of DNA methyltransferase and induction of Friend erythroleukemia cell differentiation by 5-azacytidine and 5-aza-2′-deoxycytidine. *J. Biol. Chem.* **257**:2041–2048 (1982).
5. Riggs, A., and P. A. Jones. 5-Methylcytosine, gene regulation, and cancer. *Adv. Cancer Res.* **40**:1–25 (1983).
6. Jones, P. A. DNA methylation and cancer. *Cancer Res.* **46**:461–466 (1986).
7. Jones, P. A. Altering gene expression with 5-azacytidine. *Cell* **40**:485–486 (1985).
8. Cedar, H. DNA methylation and gene activity. *Cell* **53**:3–4 (1988).
9. Busslinger, M., J. Hurst, and R. A. Flavell. DNA methylation and the regulation of global gene expression. *Cell* **34**:197–206 (1983).
10. Santi, D. V., A. Norment, and C. E. Garrett. Covalent bond formation between a DNA-cytosine methyltransferase and DNA containing 5-azacytosine. *Proc. Natl. Acad. Sci. USA* **81**:6993–6997 (1984).
11. Harris, M. Induction of thymidine kinase in enzyme-deficient Chinese hamster cells. *Cell* **29**:483–492 (1982).
12. Bhall, K., R. Nayak, and S. Grant. Isolation and characterization of a deoxycytidine kinase-deficient human promyelocytic leukemic cell line highly resistant to 1-β-D-arabinofuranosylcytosine. *Cancer Res.* **44**:5029–5037 (1984).
13. Huang, S.-H., J. M. Tomich, H. Wu, A. Jong, and J. Holcenberg. Human deoxycytidine kinase: sequence of cDNA clones and analysis of expression in cell lines with and without enzyme activity. *J. Biol. Chem.* **264**:14762–14768 (1989).
14. Antonsson, B., V. I. Avramis, J. Nyce, and J. S. Holcenberg. Effect of 5-azacytidine and congeners on DNA methylation and expression of deoxycytidine kinase in the human lymphoid cell lines CCRF/CEM/O and CCRF/CEM/dCK$^-$. *Cancer Res.* **47**:3672–3678 (1987).
15. Avramis, V. I., R. Biener, B. Antonsson, and J. S. Holcenberg. Reversal of Ara-C resistance by treatment with dihydro-5-azacytidine (DHAC). *J. Cell. Biochem.* **10A(suppl)**:52 (1986).
16. Chou, T.-C., and P. Talalay. Quantitative analysis of dose-effect relationships: the combined effects of multiple drugs or enzyme inhibitors. *Adv. Enzyme Regul.* **22**:27–55 (1984).
17. Chou, J., and T.-C. Chou. *Dose-Effect Analysis with Microcomputers: Quantitation of ED_{50}, LD_{50}, Synergism, Antagonism, Low-Dose Risk, Receptor-Ligand Binding and Enzyme Kinetics*, Software and manual. Elsevier-Biosoft, Cambridge, UK (1987).
18. Cheng, Y. C., B. Domin, and L. S. Lee. Human deoxycytidine kinase: purification and characterization of the cytoplasmic and mitochondria isoenzymes derived from blast cells of acute myelocytic leukemia patients. *Biochim. Biophys. Acta* **481**:481–492 (1977).
19. Davis, L. G., M. D. Dibner, and J. F. Battery. *Basic Methods in Molecular Biology*. Elsevier Science Publishing Co., New York, 44–46 (1986).
20. Maniatis, T., E. F. Fritsch, and J. Sambrook. *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY, 103 (1982).
21. Olsson, L., and J. Forchhammer. Induction of the metastatic phenotype in a mouse tumor model by 5-azacytidine and characterization of an antigen associated with metastatic activity. *Proc. Natl. Acad. Sci. USA* **81**:3389–3393 (1984).
22. Christman, J. K., N. Mendelsohn, D. Herzog, and N. Schneiderman. Effect of 5-azacytidine on differentiation and DNA methylation in human promyelocytic leukemia cells (HL-60). *Cancer Res.* **43**:763–769 (1983).
23. Lu, L., and K. Randerath. Long term instability and molecular mechanism of 5-azacytidine-induced DNA hypomethylation in normal and neoplastic tissues *in vivo*. *Mol. Pharmacol.* **26**:594–603 (1984).
24. Ley, T. J., Y. L. Chian, D. Haidaris, N. P. Anagnou, V. L. Wilson, and W. F. Anderson. DNA methylation and regulation of the human β-globin-like genes in mouse erythroleukemia cells containing human chromosome 11. *Proc. Natl. Acad. Sci. USA* **81**:6618–6622 (1984).
25. Bouck, N., D. Kokkinaskis, and J. Ostrowski. Induction of a step in carcinogenesis that is normally associated with mutagenesis by nonmutagenic concentrations of 5-azacytidine. *Mol. Cell. Biol.* **4**:1231–1237 (1984).
26. Boehm, T. L. J., and D. Drahovsky. Elevated level of enzymatic DNA methylation in cells treated with 1-β-D-arabinofuranosylcytosine. *Cancer Res.* **42**:1537–1540 (1982).
27. Nyce, J., L. Lin, and P. A. Jones. Variable effects of DNA synthesis inhibitors upon DNA methylation in mammalian cells. *Nucleic Acids Res.* **14**:4353–4367 (1986).
28. Singer, J., E. J. Roberts, and A. D. Riggs. Methylation of mouse liver DNA studied by means of the restriction enzymes *Msp*I and *Hpa*II. *Science (Washington D. C.)* **203**:1019–1020 (1979).
29. Harris, M., and K. Collier. Phenotypic evolution of cells resistant to bromodeoxyuridine. *Proc. Natl. Acad. Sci. USA* **77**:4206–4210 (1980).
30. Kerbel, R. S., P. Frost, R. G. Literplo, D. A. Carlow, and B. E. Elliott. Possible epigenetic mechanisms of tumor progression: induction of high frequency heritable but phenotypically unstable changes in the tumorigenic and metastatic properties of tumor cell populations by 5-azacytidine treatment. *J. Cell. Physiol.* **3(suppl.)**:87–97 (1984).
31. Landolph, J. R., and P. A. Jones. Mutagenecity of 5-azacytidine and related nucleosides in C3H/10T1/2 clone 8 and V79 cells. *Cancer Res.* **42**:817–823 (1982).
32. Avramis, V. I., S.-H. Huang, and J. S. Holcenberg. Drug synergism, antagonism, and collateral sensitivity involving genetic changes, in *Synergism and Antagonism in Chemotherapy* (T.-C. Chou and D. Rideout, eds.). Academic Press, New York, 585–619 (1991).

Send reprint requests to: Ting-Chao Chou, Ph.D., Laboratory of Biochemical Pharmacology, Memorial Sloan-Kettering Cancer Center, 1275 York Avenue, New York, NY 10021