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DNA Methylation in 5-Aza-2′-Deoxycytidine-Resistant Variants of C3H 10T1/2 C18 Cells

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A cell line (T17) was derived from C3H 10T1/2 C18 cells after 17 treatments with increasing concentrations of 5-aza-2′-deoxycytidine. The T17 cell line was very resistant to the cytotoxic effects of 5-aza-2′-deoxycytidine, and the 50% lethal dose for 5-aza-2′-deoxycytidine was ca. 3 μM, which was 30-fold greater than that of the parental C3H 10T1/2 C18 cells. Increased drug resistance was not due to a failure of the T17 cell line to incorporate 5-aza-2′-deoxycytidine into DNA. The cells were also slightly cross-resistant to 5-azacytidine. The percentage of cytosines modified to 5-methylcytosine in T17 cells was 0.7%, a 78% decrease from the level of 3.22% in C3H 10T1/2 C18 cells. The DNA cytosine methylation levels in several clones isolated from the treated lines were on the order of 0.7%, and clones with methylation levels lower than 0.45% were not obtained even after further drug treatments. These highly decreased methylation levels appeared to be unstable, and DNA modification increased as the cells divided in the absence of further drug treatment. The results suggest that it may not be possible to derive mouse cells with vanishingly low levels of 5-methylcytosine and that considerable de novo methylation can occur in cultured lines.

The pyrimidine analogs 5-azacytidine (5-aza-CR) and 5-aza-2′-deoxycytidine (5-aza-CdR) were originally developed for use as cancer chemotherapeutic agents (15). They have shown some activity against human acute myelogenous leukemia (19) and have also been effective in markedly increasing the lifespan of mice bearing L1210 leukemia (9). The development of resistance to 5-aza-CR has been associated with decreased levels of uridine-cytidine kinase (16), which is responsible for the primary activation of the analog. On the other hand, resistance to 5-aza-CdR has been attributed to lowered levels of deoxycytidine kinase (16–18), which is a distinct enzyme.

The aza nucleosides have also found recent application as activators of a wide variety of cellular and viral genes (11; P. A. Jones, *in* A. Razin, H. Cedar, and A. Riggs, ed., *DNA Methylation*, in press). This activity has been attributed to their ability to inhibit the methylation of cytosine residues in newly synthesized DNA (8, 14). Thus, cells of the C3H 10T1/2 C18 (10T1/2) line undergo marked phenotypic changes several days or weeks after analog exposure (2, 3, 13).

In the present study, we determined whether multiple treatments of 10T1/2 cells with 5-aza-CdR would result in the emergence of drug-resistant lines. A cell line was derived which was 30-fold more resistant to the aza nucleoside than were 10T1/2 cells and which was slightly more resistant to 5-aza-CR. This cell line efficiently incorporated the fraudulent base into DNA and had a DNA cytosine methylation level which was only 22% of the level in 10T1/2 cells. Interestingly, the depressed cytosine methylation level was not permanent and increased with cell division in the absence of further drug treatment.

MATERIALS AND METHODS

Derivation of T17 cells. 10T1/2 cells (10) were grown in Eagle basal medium (GIBCO Laboratories, Grand Island, N.Y.) supplemented with 10% heat-inactivated fetal bovine serum (Biological Associates, Los Angeles, Calif.), penicillin (100 U/ml), and streptomycin (100 μg/ml). The cells were passaged with trypsin and seeded into T-75 tissue culture flasks (Falcon Plastics, Oxnard, Calif.) at 10^5 cells per flask for 5-aza-CdR treatment. Drug treatment was always started 24 h after seeding and lasted for 24 h before the medium was changed and the cells were allowed to recover. Treated cells were passaged every 7 to 10 days, and the treatment cycle with increasing 5-aza-CdR (obtained from J. Vesely, Institute of Organic Chemistry and Biochemistry, Prague, Czechoslovakia) concentrations was repeated. The number of treatments was recorded, and the T17 cells were exposed to 5-aza-CdR 17 times. The cells were exposed 10 times to 1 μM 5-aza-CdR, 1 time to 3 μM 5-aza-CdR, 1 time to 10 μM 5-aza-CdR, 2 times to 20 μM 5-aza-CdR, 2 times to 50 μM 5-aza-CdR, and 4 times to 100 μM 5-aza-CdR.

Clones were isolated from the mass cultures by seeding 200 cells per 60-mm-diameter dish and allowing cells to grow as individual colonies. Well-separated discrete colonies were ring isolated, trypsinized, and grown for further studies.

Growth curves. Cells obtained from logarithmically growing stock cultures were seeded into 60-mm-diameter dishes at 20,000 cells per dish and treated 24 h later with 5-aza-CR (Sigma Chemical Co., St. Louis, Mo.) or 5-aza-CdR. Medium was changed after 24-h exposure periods, and control dishes were not treated with any drug. The growth rate was measured by counting the number of cells after trypsinization of duplicate dishes in each treatment regime every 24 h. The mean population doublings were calculated according to the equation: mean population doublings (ln of number of cells harvested/number of cells seeded)/ln 2. Each experiment was repeated three times.

Cytotoxicity assays. Cells (200 or 1,000) were seeded onto 60-mm dishes (four to six dishes per treatment) and treated 24 h later with different drug concentrations. The medium was changed twice weekly, the dishes were fixed 14 days

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after seeding and stained with Giemsa stain, and the number of colonies was counted. Each experiment was repeated three to five times.

Incorporation of nucleosides into DNA. [*methyl*-^{3}H] thymidine (60 Ci/mmol) was obtained from ICN, Irvine, Calif., and 5-[6-^{3}H]aza-CdR (9 Ci/mmol) was obtained from Moravek Biochemicals, Brea, Calif. Cells were seeded onto 60-mm-diameter dishes at 2×10^5 cells per dish and treated 24 h later with the indicated concentrations of radioactive precursors. The medium was aspirated after 24 h and the cells were washed twice with phosphate-buffered saline, harvested by trypsinization, and divided into three aliquots. One of the aliquots was used for determination of total radioactivity in DNA, another was used for determination of radioactivity in DNA, which was alkali labile and represented the radioactvity associated with the 6 position of incorporated 5-azacytosine, and the third sample was used for the fluorimetric estimation of DNA content (7). For the incorporation studies, each cell pellet was lysed by incubation with 1% sodium dodecyl sulfate–0.1 M NaCl–0.01 M EDTA (pH 8.0) at 37°C for 1 h. Carrier salmon sperm DNA (60 μg) was added, and the DNA was precipitated overnight by the addition of 3 volumes of 96% ethanol. The precipitate was resuspended in 1× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) and treated with RNase A (10 μg/ml) at 37°C for 1 h. For the measurement of base labile incorporation, an equal volume of 0.6 N NaOH was subsequently added to the RNase digest and incubated at 37°C for 20 h. Trichloroacetic acid (TCA) was added to a final concentration of 10%, and the pelleted DNA was washed twice with cold 5% TCA. DNA was hydrolyzed in 5% TCA at 100°C for 1 h, and the solubilized radioactivity was determined by counting in Biofluor (New England Nuclear Corp., Boston, Mass.).

DNA methylation levels. The percentage of cytosine residues modified to 5-methylcytosine (5-mCyt) was measured as previously described (5). Nonconfluent cells were grown in 2 ml of BME and exposed for 24 h to 5 μCi of [6-^{3}H]uridine per ml (20.7 Ci/mmol; Research Products International, Mount Prospect, Ill.). The cells were lysed in situ with 0.5% sodium dodecyl sulfate in the presence of 0.3 M NaOH and incubated at 65°C for 1 h to hydrolyze RNA labeled by the precursor. The NaOH was then neutralized with HCl, proteins were digested with 0.1 mg of proteinase K per ml (E. Merck AG, Darmstadt, Federal Republic of Germany) for 1 h at 65°C, and the pH was adjusted to 7.6 with 0.25 ml of Tris-hydrochloride buffer (0.5 M). Carrier salmon sperm DNA (20 μg) was added, the DNA was precipitated with 10% TCA, and the pellet was washed twice with cold 5% TCA and once with 70% ethanol. The DNA was hydrolyzed to bases with 88% formic acid at 180°C for 30 min in sealed vials, and the formic acid was blown off with nitrogen gas. Marker bases (cytosine and 5-mCyt; Sigma Chemical Co.) were added, and the bases were separated by high-pressure liquid chromatography on a Waters Z module fitted with an SCX column (12). The bases were eluted with 0.08 M potassium phosphate buffer, pH 2.5, at a flow rate of 4 ml/min and a pressure of 400 lb/in^2. The bases were identified relative to the elution times of standard marker bases and detected by absorbance at 280 nm. The fractions containing cytosine and 5-mCyt were collected and assayed for radioactivity in a scintillation counter after the addition of Aquasol II (New England Nuclear Corp.). The percentage of cytosines modified to 5-mCyt was calculated according to the equation: % 5-mCyt = [5-mCyt/(5-mCyt + cytosine)] × 100.

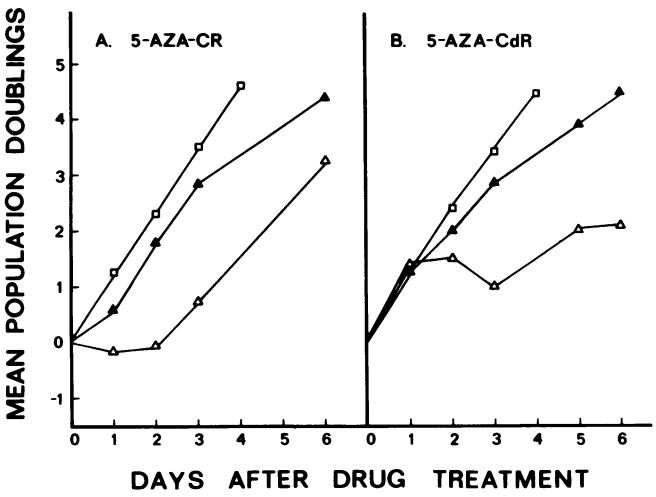

FIG. 1. Effects of 5-aza-CR and 5-aza-CdR on the growth of 10T1/2 cells. Cells (20,000) were seeded into 60-mm-diameter dishes and treated 24 h later with 5-aza-CR (A) or 5-aza-CdR (B) for a further 24 h. The medium was then changed, and the growth rate was determined by trypsinizing duplicate dishes at the indicated times. Data represent one of two experiments which gave essentially the same results. Symbols: □, untreated 10T1/2 cells; ▲, 1 μM analog; △, 10 μM analog.

RESULTS

Derivation of T17 cells. The effects of 24-h treatments of 5-aza-CdR and 5-aza-CR on the growth of 10T1/2 cells were determined to select concentrations of the analogs most suitable for the derivation of lines with depressed DNA methylation levels (Fig. 1). 5-Aza-CR had an immediate effect on the growth of 10T1/2 cells, and decreased cell numbers were seen 24 h after exposure to 1 or 10 μM analog (Fig. 1A). The deoxy compound, on the other hand, had a more delayed effect, and changes in cell numbers were not observed until 48 h after treatment had started (Fig. 1B). These differences in response to the analogs were possibly due to the fact that 5-aza-CR interferes with both RNA and DNA metabolism, whereas the deoxy analog only affects DNA metabolism (15). We therefore used 5-aza-CdR in further experiments because of its more direct interference with DNA cytosine methylation.

The treatment of 10T1/2 cells with 1 μM 5-aza-CdR for 24 h resulted in a ca. 85% decrease in the extent of modification of those cytosine residues incorporated into DNA during drug exposure (data not shown). However, in agreement with reports for 10T1/2 (8) and other cells (6), the decrease in methylation was not permanent, and the overall methylation level in the mass culture increased to ca. 2.5% 7 days after drug treatment (Fig. 2). To obtain cells with permanently decreased 5-mCyt levels, 10T1/2 cells were treated with sequentially increasing concentrations of 5-aza-CdR. Cultures were treated for 24 h each time they were passaged, and the extent of cytosine modification was measured ca. 7 days after exposure to 5-aza-CdR (Fig. 2). Many treatments with very high drug concentrations (up to 100 μM) were therefore required to obtain cells with depressed modification levels, and after 20 treatments, the lowest level reached was 0.45%.

Methylation levels in clones isolated from the mass cultures were also measured to determine whether they were populated by cells with different 5-mCyt contents (Fig. 2).

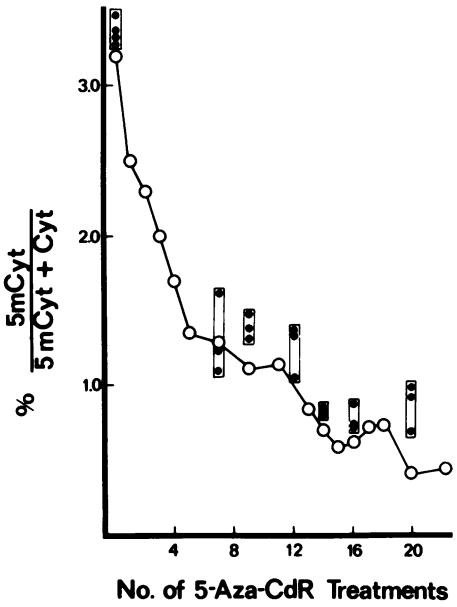

FIG. 2. Effects of multiple 5-aza-CdR treatments on the overall DNA methylation level (○) in 10T1/2 cells. Mass cell cultures were treated every 7 to 10 days with increasing 5-aza-CdR concentrations as detailed in the text. Methylation levels were measured after [^{3}H]uridine incorporation ca. 7 days after analog exposure. Subclones derived from mass cultures after various numbers of drug treatments were also isolated, and their methylation levels were determined (●).

Methylation levels in four clones isolated from untreated 10T1/2 cells were similar to the value of 3.2% found for the mass culture. The 10T1/2 cell line was therefore homogeneous with regard to overall 5-mCyt content. Clones isolated from mass cultures exposed to 5-aza-CdR for different numbers of times showed methylation levels similar to those of the respective mass cultures (Fig. 2). Clones with methylation levels below 0.5% were never isolated, and 5-mCyt contents were, in most cases, higher than those of the parent cultures. This suggests that some de novo methylation might occur in the clones because of the large number of cell divisions which occurred during their isolation. A mass culture derived after 17 treatments (T-17) was selected for studies on de novo methylation and for further characterization.

The T-17 cells had a methylation level of 0.7% when assayed soon after their last treatment with 5-aza-CdR (Fig. 2 and 3). This decreased DNA modification level was not stable, and the genomic content of 5-mCyt increased when the cells were passaged in the absence of further drug treatment (Fig. 3). The tendency for increasing overall methylation levels with cell division was also seen in two other independently isolated lines (data not shown) and suggested that the higher levels seen in clones when compared to mass cultures (Fig. 2) might be due to de novo methylation. It was also similar to what we had observed earlier during the establishment of mouse cell lines from senescing populations (20).

Drug resistance of T-17 cells. The T-17 line was considerably more resistant to the toxic effects of 5-aza-CdR than the parental 10T1/2 cells (Fig. 4). The 50% lethal dose for the T17 cells was ca. 3 μM, which was 30-fold greater than that for 10T1/2 cells.

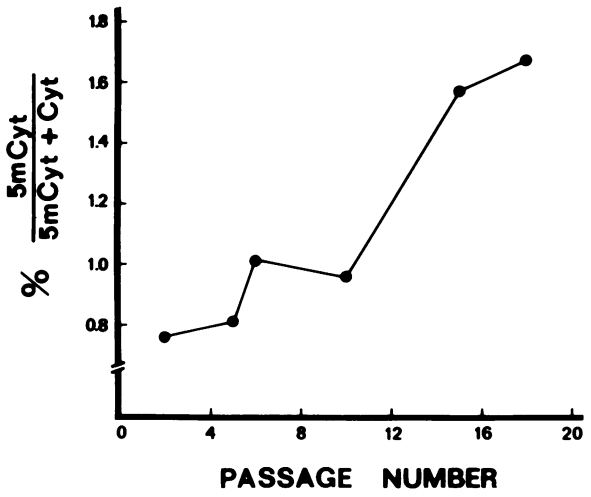

FIG. 3. Kinetics of remethylation of T-17 cells cultured in the absence of further drug treatment. T-17 cells were grown for several passages after their last treatment with 5-aza-CdR, and the levels of 5-mCyt were measured periodically after [^{3}H]uridine labeling. Each point was done in duplicate and similar results were obtained with two other independently isolated lines with depressed 5-mCyt levels.

The T-17 cells were, however, only slightly less sensitive to 5-aza-CR than was the 10T1/2 line; thus, marked cross-resistance to the ribo analog was not induced by the 5-aza-CdR treatments (Fig. 5). We also observed, in another series

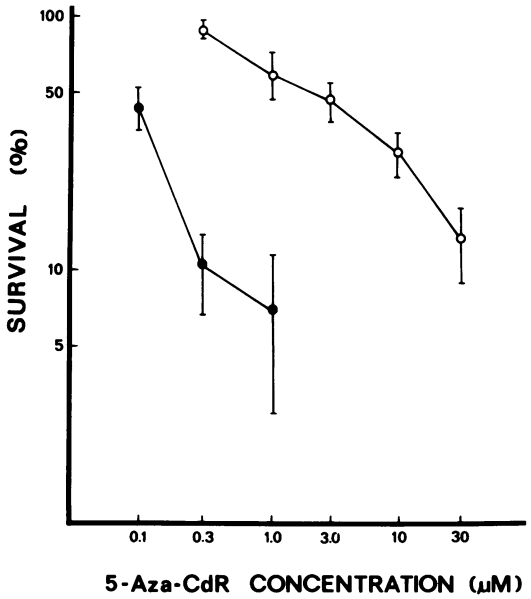

FIG. 4. Colony assay of 10T1/2 or T-17 cells treated with 5-aza-CdR. Cells (200 or 1,000) were seeded into 60-mm-diameter plastic dishes and treated 24 h later with analog. The cells were exposed to drugs for 24 h, cultures were fixed 12 days after treatment and stained with Giemsa stain, and the number of colonies was counted. The percentage of survival of the treated cells was compared to that of untreated cells. Each experiment was done in triplicate, and the results represent the mean ± the standard deviation (bars) of three independent experiments. Symbols: ●, 10T1/2 cells; ○, T-17 cells.

TABLE 1. Incorporation of labeled precursors into DNA of 10T1/2 and T-17 cells

Precursor	Concn (μM)	Incorporation into DNA[a] (pmol/μg of DNA)		% Substitution of 5-azacytosine for cytosine[b]	
		10T1/2	T-17	10T1/2	T-17
[^{3}H]5-aza-CdR	0.05	0.96	0.70	0.17	0.12
	1.0	15.50	8.60	2.71	1.50
	10.0	52.10	47.10	9.09	8.20
[^{3}H]5-aza-CdR (hydrolyzed)[c]	1.0	0	0		
[^{3}H]thymidine[d]	0.0017	0.46	0.41		

[a] 10T1/2 or T-17 cells were seeded at 2×10^5 cells per 60-mm-diameter dish and treated 24 h later with the indicated precursors for 24 h. The incorporation of precursors into DNA was determined as detailed in the text. Data are given as the mean values for triplicate determinations, and similar results were obtained in a separate experiment.

[b] Calculated as 5-azacytosine incorporated as a fraction of total cytosine.

[c] Radioactive 5-aza-CdR was hydrolyzed for 2.5 h at pH 11.0 before being added to the cells.

[d] Measured without consideration of endogenous thymidine.

of experiments which are not shown here, that it was not possible to induce more than a threefold change in the 50% lethal dose for 10T1/2 cells for 5-aza-CR even after 16 5-aza-CR treatments (E. Flatau and P. A. Jones, unpublished data). Resistance to the deoxy analog was therefore more readily induced.

Resistance to fraudulent nucleosides is often due to a failure of treated cells to incorporate the nucleosides into DNA. We therefore measured the incorporation of [^{3}H]5-aza-CdR into 10T1/2 and T-17 cells (Table 1). Determination of incorporation of labeled 5-aza-CdR into DNA is complicated by the fact that the tritiated 6 position of the aza pyrimidine ring is easily hydrolyzed (15), and the breakdown products can enter nucleic acids. We therefore defined 5-aza-CdR incorporation into DNA as the radioactivity which was insensitive to RNase, precipitable by TCA, but sensitive to alkaline hydrolysis. This definition works because naturally occurring bases in DNA are insensitive to alkali, whereas incorporated 5-azacytosine, labeled in the 6 position, loses its radiolabel after base hydrolysis.

Both 10T1/2 cells and T-17 cells actively incorporated 5-aza-CdR into DNA in a concentration-dependent manner (Table 1). The T-17 cells were slightly less efficient in assimilating the fraudulent base than were 10T1/2 cells over a wide concentration range. Although T17 cells incorporated only half as much of the 5-aza-CdR as did 10T1/2 at 1 μM, it was unlikely that the 30-fold difference in the 50% lethal dose observed in Fig. 4 could be explained by these differences in the levels of drug incorporation. There was no 5-aza-CdR incorporation (defined as outlined above) when the drug was hydrolyzed before being added to the cells (Table 1). Similar levels of DNA synthesis occurred in the two cell types as shown by [^{3}H]thymidine incorporation (Table 1). At 10 μM 5-aza-CdR, the substitution of cytosine residues by 5-azacytosine was 9.09% in 10T1/2 DNA and 8.20% in T-17 DNA (Table 1). The T-17 cells could therefore tolerate large amounts of 5-azacytosine in their DNA without serious loss of viability (Fig. 4).

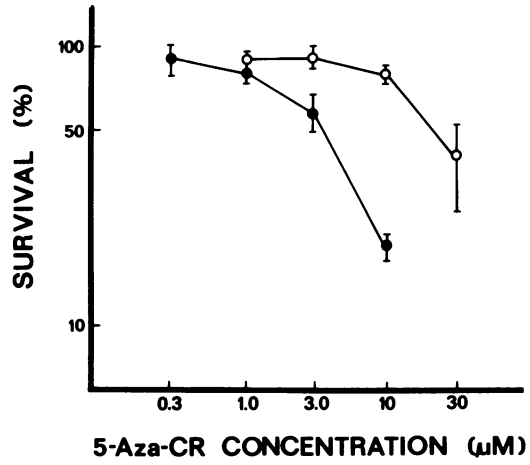

FIG. 5. Colony assay of 10T1/2 (●) or T-17 (○) cells treated with 5-aza-CR. Conditions for the experiment were the same as those reported in the legend to Fig. 4, except that 5-aza-CR was used instead of 5-aza-CdR.

DISCUSSION

Our results show that DNA cytosine methylation levels in cultured cells are very flexible. Thus, although 5-aza-CdR had marked and immediate effects on the 5-mCyt levels in DNA replicated during analog exposure, there was a tendency for the cells to remethylate their DNA after the drug had been removed. Rapid remethylation of DNA was also observed in mouse erythroleukemia cells after azanucleoside treatment (4). It was therefore only possible to obtain cells with substantially decreased methylation levels by sequential treatments with very high 5-aza-CdR concentrations.

The lowest methylation levels induced (15% of control) after many sequential 5-aza-CdR treatments were still compatible with the life of the cells. However, there was a clear tendency for increases in DNA methylation to occur in such cell lines upon further division. Remethylation has previously been observed with the passage of 5-aza-CR-treated T-lymphoid cells (6), and substantial increases in genomic methylation occur during the establishment of immortal cell lines from senescing mouse embryo fibroblasts (20). Our results do not address the question of whether the increased methylation is due to de novo methylation or to the selection of cells with high methylation levels. However, the studies of Gasson et al. (6) were done with clones, which supports the idea that considerable de novo methylation can occur in cell lines. On the other hand, the tendency to remethylate DNA may not be universal since Adams et al. (1) obtained variants of mouse L929 cells with apparently stably decreased 5-mCyt levels after 5-aza-CdR treatment. The final level of DNA modification reached may therefore be dependent on the cell line.

The present experiments do not show whether the markedly decreased overall methylation levels also occurred within specific genes in the mouse cells. We are therefore conducting a clonal analysis of several expressed and nonexpressed genes to determine whether substantially different patterns were generated by multiple drug treatments. It is also not clear whether our failure to derive cell lines with less than 0.45% 5-mCyt was due to the remethylation phenomenon discussed earlier, or whether methylation levels below this do not permit the division and growth of mouse cells. However, if methylation has an important role in controlling gene expression, then there may exist a lower methylation limit below which abnormal patterns of gene expression would result in cell death.

The derivation of these cell lines which are markedly resistant to 5-aza-CdR may also be important in furthering our understanding of the mechanisms of drug resistance and in the control of DNA methylation within cells. Resistance to 5-aza-CdR is often conferred by decreases in deoxycytidine kinase (15), the enzyme responsible for the primary activation of the analog. Alternatively, resistance can be due to increased deaminase levels which are capable of inactivating a fraudulent base. It was therefore highly significant that the cells derived in the present study actively incorporated 5-aza-CdR into their DNA. This implies that they could withstand a much higher concentration of azacytosine in their DNA than could 10T1/2 cells. We are therefore determining the biochemical mechanisms for this resistance, and preliminary karyotypes have shown the presence of double-minute chromosomes within several of the resistant clones generated (A. Banerjee, L. A. Michalowsky, and P. A. Jones, unpublished observations). The mechanisms of resistance may therefore be related to gene amplification.

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