Proc. Natl. Acad. Sci. USA
Vol. 91, pp. 11797–11801, December 1994
Medical Sciences

Toxicity of 5-aza-2′-deoxycytidine to mammalian cells is mediated primarily by covalent trapping of DNA methyltransferase rather than DNA demethylation

(DNA methylation/drug resistance/cancer treatment)

RUTH JÜTTERMANN, EN LI*, AND RUDOLF JAENISCH

Whitehead Institute for Biomedical Research and Department of Biology, Massachusetts Institute of Technology, 9 Cambridge Center, Cambridge, MA 02142

Communicated by Robert A. Weinberg, August 23, 1994

ABSTRACT **The deoxycytidine analog 5-aza-2′-deoxycytidine (5-azadCyd) has been widely used as a DNA methylation inhibitor to experimentally induce gene expression and cellular differentiation. Prior to the availability of mutant mice with altered DNA methyltransferase levels, treatment of cells with drugs has been the only means to experimentally manipulate the level of genomic DNA methylation in mammalian cells. Substitution of DNA with 5-azadCyd leads to covalent trapping of the enzyme, thereby depleting the cells of enzyme activity and resulting in DNA demethylation. 5-AzadCyd or 5-azacytidine treatment causes multiple changes in treated cells, including activation of silent genes, decondensation of chromatin, and induction of cellular differentiation, all of which are believed to be consequences of drug-induced demethylation. 5-AzadCyd is highly toxic in cultured cells and animals and is utilized as a potent antitumor agent for treatment of certain human cancers. It has been postulated that the toxicity of the drug in mammalian cells is also due to its inhibition of DNA methylation. The chemistry of the methylation reaction is consistent, however, with an alternative mechanism: the cytotoxic effect of 5-azadCyd may be directly mediated through the covalent binding of DNA methyltransferase to 5-azadCyd-substituted DNA. We have tested this possibility by using embryonic stem cells and mice with reduced levels of DNA methyltransferase due to a targeted mutation of the gene. When exposed to 5-azadCyd mutant embryonic stem cells or embryos were significantly more resistant to the toxic effects of the drug than wild-type cells and embryos, respectively. These results strongly suggest that the cellular DNA methyltransferase itself, rather than the secondary demethylation of genomic DNA, is the primary mediator of 5-azadCyd cytotoxicity. In light of our results, some conclusions from previous studies using 5-azadCyd in order to experimentally manipulate cellular methylation levels may have to be reassessed. Also, our data make clear predictions for cancer treatment: tumor cells with elevated DNA methyltransferase levels would be expected to be susceptible to treatment with 5-azadCyd, whereas tumors with reduced levels of the enzyme would be resistant.**

5-Aza-2′-deoxycytidine (5-azadCyd) is widely used as a DNA methylation inhibitor to induce gene expression and cellular differentiation. To exert its biological effects, the drug must be incorporated into the DNA (1). 5-AzadCyd-substituted DNA or DNA substituted with the ribose analog 5-azaCyd forms covalent adducts with cellular DNA methyltransferase (MTase), thereby depleting the cells of enzyme activity and causing demethylation of genomic DNA as a secondary consequence (1, 2). Treatment with 5-azadCyd or 5-azaCyd also causes various other changes in cells, including activation of silent genes (3, 4), decondensation of chromatin (5), and alteration of DNA replication timing (6), all of which are believed to be consequences of drug-induced demethylation.

In addition to causing multiple changes in cell physiology, 5-azaCyd and 5-azadCyd are highly toxic in cultured cells (7, 8) and animals (9, 10). For this reason, 5-azadCyd is utilized as a potent anticancer agent for treatment of certain leukemias, such as acute myeloid leukemia, and myelodysplastic syndromes (11). Phase I and II trials have been conducted with different types of cancer, including colorectal cancer, head and neck cancers, renal carcinomas, and malignant melanomas (12, 13). The toxicity of the drug in mammalian cells has been attributed to its inhibitory effect on DNA methylation (14). It has been postulated that the antitumor effect of the drug might be due to the transcriptional activation of differentiation genes leading to terminal differentiation of undifferentiated cancer cells (15–17).

An alternative mechanism of drug-induced cytotoxicity is suggested by the chemistry of the methylation reaction. The methylation of cytosine involves the covalent binding of the MTase to the cytosine residue through a nucleophilic attack of a cysteine thiolate at the C-6 position of the cytosine ring. The covalent binding of the enzyme at C-6 allows for a nucleophilic attack at C-5 by the methyl group of the *S*-adenosylmethionine methyl donor and the subsequent transfer of a methyl group to C-5. The resulting intermediate is resolved by β-elimination of the enzyme at C-6 with abstraction of the hydrogen from C-5 (18–20) (Fig. 1*A*). When dCyd is replaced by 5-azadCyd in the DNA, C-6 of the cytosine residue reacts with the cysteine thiolate of the enzyme to form a 5,6-dihydropyrimidine adduct as described above. In contrast to normal DNA, however, the enzyme remains covalently bound under physiological conditions and cannot be released from the 5-azadCyd-substituted DNA, thus decreasing the cellular concentration of MTase (1, 2, 21) (Fig. 1*B*). This leads subsequently to demethylation of genomic DNA.

In this study we sought to establish the mechanism of 5-azadCyd toxicity in cells and animals. If demethylation of genomic DNA is the primary cause of drug toxicity, as conventionally assumed (16, 17), cells expressing reduced levels of MTase should be more sensitive to 5-azadCyd than normal cells. In contrast, if covalent binding of the enzyme to 5-azadCyd-substituted DNA rather than genomic DNA demethylation was the primary mechanism of drug-induced toxicity, mutant cells expressing lower levels of MTase should be more resistant to 5-azadCyd. To distinguish between these two possibilities, we compared the drug sensitivity of mutant cells or mice expressing reduced levels of

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

Abbreviations: 5-azadCyd, 5-aza-2′-deoxycytidine; 5-azaCyd, 5-azacytidine; MTase, DNA methyltransferase; ES cells, embryonic stem cells.

*Present address: Massachusetts General Hospital-East, Cardiovascular Center, 149 13th Street, 4th Floor, Charlestown, MA 02129.

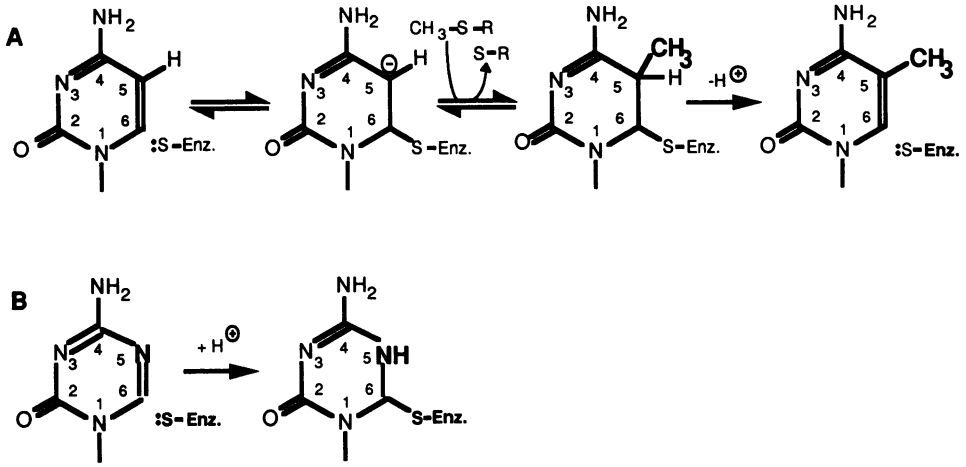

FIG. 1. (*A*) Reaction mechanism of cytosine MTase. It was shown for bacterial DNA cytosine-5-methyltransferases that a covalent intermediate is formed between cytosine within the DNA and the enzyme during the catalytic reaction (18, 19). This intermediate is generated by nucleophilic attack on the C-6 position of cytosine by a cysteine residue of the enzyme. The activated C-5 of cytosine then attacks the methyl group of *S*-adenosylmethionine which subsequently is added to C-5, followed by deprotonation of C-5 and release of the enzyme by β-elimination. (*B*) Proposed reaction mechanism when 2′-deoxycytidine is replaced by the analog 5-azadCyd. The MTase is trapped in an inactivating intermediate (21).

MTase due to a targeted mutation of the gene with that of their wild-type counterparts.

MATERIALS AND METHODS

Embryonic Stem (ES) Cell Culture and Drug Treatment. ES cells were cultured essentially as described (22). Murine wild type or MTase mutant ES cells were seeded into 12-well tissue culture dishes at 1500 cells per well. One day after seeding, the ES cells were treated with various concentrations of freshly prepared 5-azadCyd (Sigma) dissolved in Dulbecco's modified Eagle's medium.

Embryonic Fibroblasts. Embryos from crosses between MTase mutant and wild-type mice were removed from placenta and extraembryonic membranes at day 13.5 of gestation and washed twice with Hepes-buffered saline under sterile conditions. After the embryos were decapitated, the inner organs were removed and each torso was individually trypsinized [2.5% trypsin (GIBCO) and 1 mM EDTA in Hepes-buffered saline] and the fibroblasts derived from single embryos were plated into gelatinized T175 Falcon cell culture flasks containing Dulbecco's modified Eagle's medium with 10% calf serum (GIBCO).

Cocultivation of Wild-Type and MTase Mutant Cells. Equal numbers of wild-type and mutant cells were mixed and plated on T25 flasks previously seeded with feeder cells. The cocultures were treated every second day for 24 hr, starting at 12 hr after seeding with various concentrations of 5-azadCyd. Cultures were passaged once at day 5 after seeding and DNA was prepared (23) at day 9 after four rounds of treatment. Embryonic fibroblasts were seeded into T25 flasks and treated with 5-azadCyd. The 5-azadCyd-containing medium was changed every day for 4 days, cells were passaged when confluent, and DNA was prepared at day 5.

Southern Blot Analysis. Purified DNA was digested with restriction endonuclease, fractionated by electrophoresis through 0.8 or 1.0% agarose gels, denatured in 0.5 M NaOH/1.5 M NaCl for 30 min, neutralized with 0.5 M HCl/1.5 M NaCl/0.1 M Tris·HCl, pH 7.5, and blotted for 14 hr with 10× standard saline citrate (SSC) onto Zetabind filters (American Bioanalytical). Probes were radioactively labeled by oligonucleotide priming. After hybridization in CHURCH buffer (34) for 12 hr, blots were washed twice with 1% SDS/0.2× SSC at 65°C. When *Hpa* II digestions were performed, blots were stripped and rehybridized with mitochondrial DNA probes to control for partial digestion.

RESULTS

MTase Mutant Cells Are More Resistant to 5-AzadCyd Than Wild-Type Cells. Previously, we have derived mutant mice carrying a targeted mutation of the MTase gene (22). The recessive mutation causes a 50% reduction of enzyme levels in heterozygous (+/−) cells and a >90% reduction of enzyme levels in homozygous (−/−) mutant cells. Homozygous mutant ES cells proliferate normally with their genomic DNA content of 5-methylcytosine reduced to 30% of that seen in heterozygous or wild-type cells (22). Mice heterozygous for the mutation are normal, whereas homozygous mutant embryos die at midgestation. Mutant cells and mice were used to investigate whether demethylation was the primary cause of 5-azadCyd-mediated cytotoxicity.

To measure the sensitivity to the toxic effect of the drug, ES cells were exposed to various concentrations of 5-azadCyd 1 day after seeding. The drug was removed after 24 hr and the cells were grown in normal medium for 9 days before colonies were counted. The number of surviving wild-type ES cell colonies declined rapidly with increasing concentrations of 5-azadCyd, with 50% survival at 0.05 μM drug concentration (Fig. 2). Exposure of heterozygous MTase mutant ES cells to the drug resulted in a slightly increased survival. ES cells homozygous for the mutation were substantially more resistant to the drug than the normal control cells, with 50% survival at a 10-fold higher concentration (0.5 μM 5-azadCyd).

To assess whether repeated exposure to low concentrations of the drug would lead to a growth advantage of mutant cells with reduced levels of MTase over normal cells, an equal number of wild-type and heterozygous cells or of wild-type and homozygous ES cells were seeded into the same dish. The mixed cultures were treated every 2 days with 5-azadCyd, DNA was prepared after a total of 9 days (four treatments), and Southern blot analysis was used to quantify the ratio of mutant to wild-type cells. In this analysis, a 5.3-kb band corresponding to the wild-type MTase allele is found in wild-type ES cells, two bands of 5.3 kb and 6.1 kb are found in heterozygous cells, and a single band at 6.1 kb corresponding to the mutant allele (22) is seen in homozygous cells. Mutant ES cells outgrew wild-type cells when continuously

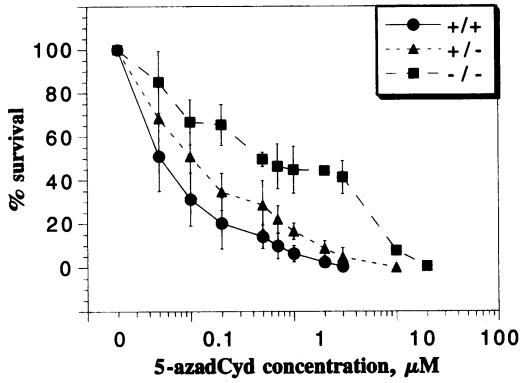

FIG. 2. Colony assay of wild-type or mouse MTase mutant ES cells treated with 5-azadCyd. One day after seeding, ES cells were treated with 0 (as a control), 0.05, 0.1, 0.2, 0.5, 0.7, 1.0, 2.0, 3.0, 10.0, or 20.0 μM 5-azadCyd. The percentage of surviving cells (as compared with untreated cells) is plotted against the concentration of 5-azadCyd. In each experiment, cells were seeded and counted in triplicates; the graph represents the mean values of two independent experiments. Bars indicate the standard deviation. +/+, Wild-type ES cells; +/−, heterozygous MTase mutant ES cells; −/−, homozygous MTase mutant ES cells.

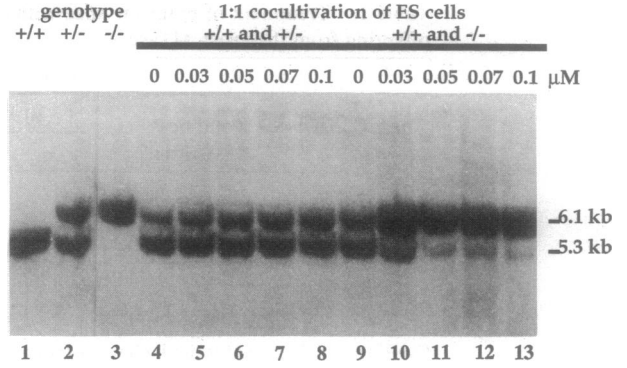

FIG. 3. Growth competition between wild-type and MTase mutant cells by cocultivation in 5-azadCyd-containing medium. Wild-type (+/+) ES cells and heterozygous MTase mutant (+/−) ES cells or wild-type (+/+) and homozygous MTase mutant (−/−) ES cells were mixed in a 1:1 ratio (1.2×10^6 cells of each cell line) and seeded into T25 cell culture flasks. The cocultures were treated every second day for 24 hr, starting at 12 hr after seeding, with 0 (as a control), 0.03, 0.05, 0.07, or 0.1 μM 5-azadCyd. Cultures were passaged once at day 5 after seeding, and DNA was prepared at day 9 after four rounds of treatment. Southern blot analysis was performed by cutting the DNA with *Xba* I and probing with pBB (22) (an external MTase probe, which distinguishes between wild-type and MTase mutant cells). Wild-type (+/+) ES cells (lane 1) show two copies of the 5.3-kb wild-type band, homozygous mutant (−/−) ES cells (lane 3) show two copies of the 6.1-kb mutant band, and heterozygous (+/−) mutant ES cells (lane 2) show one copy of each band. Untreated 1:1 cocultivated +/+ and +/− cells (lane 4) are expected to have three copies of the wild-type band and one copy of the mutant band. Even after treatment with 0.03 μM 5-azadCyd this 1:3 ratio of band intensities is shifted toward a 1:1 ratio, which is expected if heterozygous mutant ES cells have a growth advantage. Cocultivated +/+ and −/− cells (lane 9) (untreated) show a 1:1 ratio of the two bands, corresponding to two copies of each band. 5-AzadCyd treatment (lanes 10–13) leads to a decrease in the wild-type band. Quantitative phosphorimaging analyses of band intensities confirmed that both homozygous and heterozygous MTase mutant ES cells overgrew wild-type ES cells with increasing 5-azadCyd concentrations.

treated with low concentrations of 5-azadCyd (Fig. 3). The growth advantage of mutant over wild-type cells was already seen at 0.03 μM 5-azadCyd for homozygous mutant ES cells and at a slightly higher concentration for heterozygous mutant cells. A growth advantage of heterozygous mutant cells over wild-type cells was also found for embryonic fibroblasts, albeit at drug concentrations 10 times higher than those used for ES cells (data not shown).

The data in Figs. 2 and 3 indicate that cells with reduced levels of MTase were significantly more resistant to 5-azadCyd-induced growth inhibition than cells with normal enzyme levels. Inhibition was proportional to MTase levels, suggesting that the enzyme itself, rather than genomic DNA demethylation, mediates cellular drug toxicity.

MTase Mutant Embryos Are More Resistant to 5-AzadCyd Treatment than Wild-Type Siblings. A single intraperitoneal injection of the ribose analog of 5-azadCyd into pregnant mice causes substantial embryo lethality with frequent runting of surviving pups (9). It was of interest to investigate whether embryos heterozygous for the MTase mutation would be more resistant to the toxic effects of 5-azadCyd than wild-type embryos as would be expected from the observations described above. Wild-type females were mated with heterozygous MTase mutant males and the pregnant females were injected with a single dose of 5-azadCyd at day 14 of gestation and allowed to deliver their pups. Offspring were genotyped by PCR analysis. As expected, ≈50% of the offspring from females injected with saline were wild type, the rest being heterozygous for the mutation (Table 1). Injection of 5-azadCyd resulted in a lower fraction of wild-type offspring at birth, which further decreased in a dose-dependent manner when the mice were genotyped at weaning. The frequency of wild-type weanlings from females injected with 200 and 300 μM 5-azadCyd was 30% and 22%, respectively. Significantly, of a total of 24 wild-type mice derived from drug-injected females, 18 were severely sick, 14 being runts and 4 displaying severe hair loss and skin ulceration. In contrast, only 3 of a total of 64 heterozygous litter mates were visibly affected, whereas the remainder were apparently healthy and of normal weight. Fig. 4 shows a typical example of offspring from a drug-injected mother, the wild-type animal being runted and one-third the size of the normal-weight heterozygous littermate. We conclude that MTase mutant embryos, like mutant ES cells, are significantly more resistant to the deleterious effects of 5-azadCyd.

DISCUSSION

The role of DNA methylation in gene expression has been the subject of intense investigations over the last two decades. Important tools for these studies have been the cytosine analogs 5-azaCyd and 5-azadCyd, which allow experimental reduction of the level of DNA methylation; only recently have genetic approaches to the study of DNA methylation become feasible, with the generation of MTase mutations in plants (25), *Neurospora* (26), and mice (22). In fact, prior to the generation of mice with a targeted mutation of the MTase gene, treatment of cells with these drugs has been the only available means to experimentally cause demethylation of genomic DNA in mammalian cells, and a large body of work is based on experiments using drug-induced DNA hypomethylation in cultured cells. The principal conclusions from these studies have been that demethylation of DNA results in activation of silent genes, including reactivation of the inactive X chromosome (3, 4), in the change of DNA replication timing (6), and in decondensation of centromeric chromatin (5). The second major consequence of treating cells with 5-azadCyd is cytotoxicity. However, the relation between cytotoxicity and drug-induced DNA demethylation has not been resolved in previous experiments.

Importantly, 5-azadCyd is clinically used as an anticancer agent which is particularly effective for treatment of leukemias (11, 17). In contrast to our present understanding of the mechanisms of 5-azadCyd-mediated gene activation, little is known about the mechanism of drug-induced cytotoxicity or development of drug resistance by tumor cells. While all the

Table 1. Treatment of pregnant mice with 5-azadCyd: Preferential survival of heterozygous offspring from the cross MTase +/+ × MTase +/−

5-azadCyd, μg	Total no. of offspring	Genotype distribution of offspring, %				No. of sick survivors/total	
		At birth		At weaning			
		+/−	+/+	+/−	+/+	+/−	+/+
0	82	43	57	43	57	0/35	0/47
200	103	65	35	70	30	3/43	13/18
300	44	59	41	78	22	1/21	5/6

Males heterozygous for the MTase mutant S allele (24) were mated with wild-type FVB females and offspring were genotyped by PCR. At day 14 of gestation, females were injected with a 0.85% solution of NaCl as a control or with 200, 300, or 400 μg of 5-azadCyd per 25 g of body weight. In five out of five litters all littermates died when mothers were injected with 400 μg of 5-azadCyd. Day 14 of gestation was chosen as a time of rapid cell growth after organogenesis is completed. Pups were genotyped after death or at weaning. Sick animals were runts with one-third to one-half the size of normal littermates or had severe skin ulceration. The mean weight of healthy +/− offspring at weaning was 15.7 ± 1.15 g and that of runts 7.84 ± 1.24 g.

drug-induced physiological alterations may contribute to the anticancer activity of the drug, DNA demethylation has been thought to be the most important parameter of the cytotoxic effect of the drug (14, 17). It was hypothesized that demethylation results in inappropriate gene expression, which then induces terminal differentiation of undifferentiated tumor cells (8, 16). The well-established chemistry of the methylation reaction, however, suggested to us an alternative mechanism: covalent and irreversible binding of the enzyme to drug-substituted DNA rather than secondary DNA demethylation due to enzyme depletion may be the principal cause for cytotoxicity.

We used cells and mice carrying a targeted mutation of the MTase gene, which permitted us to experimentally alter enzyme concentration as well as genomic DNA methylation levels by genetic means rather than by drug treatment. Our results directly correlated the level of MTase with cytotoxicity. Mutant cells or mice expressing about 50% of MTase levels, as compared with wild-type controls, and maintaining normal levels of overall genomic DNA methylation were more resistant to the cytotoxic effects of drug treatment than the respective normal controls. Homozygous mutant cells, which were already partially demethylated due to greatly decreased MTase activity, were substantially more resistant to the toxic effect of the demethylating drug 5-azadCyd than the heterozygous or wild-type cells with normal DNA methylation levels. The most reasonable explanation which is consistent with the chemistry of the methylation reaction is that covalent binding of the enzyme rather than secondary DNA demethylation is the key mediator of drug-induced cytotoxicity.

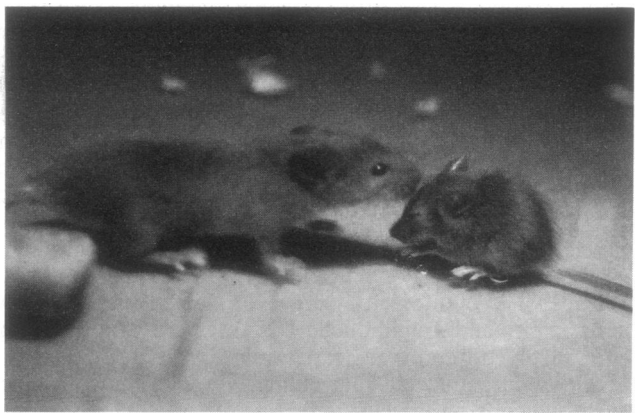

FIG. 4. Four-week-old littermates from a mother who had been injected with 300 μg of 5-azadCyd per 25 g of body weight at day 14 of gestation. The wild-type animal is runted, dehydrated, and one-third the size of the normal-weight heterozygous littermate.

It could be argued that DNA demethylation, leading to alterations in gene expression, could affect drug uptake and, as a consequence, render mutant cells more resistant to drug-induced cytotoxicity than wild-type controls. The following considerations strongly argue against this possibility. (*i*) Neither differences in methylation levels nor differences in gene expression have been detected between wild-type and heterozygous mutant MTase cells or mutant mice (22, 24). In fact, heterozygous mice, living up to 2 years of age (unpublished observation), are indistinguishable from normal littermates, suggesting that a 2-fold decrease in MTase is of little biological consequence. Yet both heterozygous ES cells and fibroblasts as well as heterozygous embryos were more resistant to the toxic effects of the drug than wild-type controls. This argues that the level of MTase rather than some other undefined cellular alteration is the main determinant of cytotoxicity. (*ii*) Drug treatment induces DNA demethylation in mutant as well as wild-type cells (preliminary results), which is not consistent with the MTase mutation interfering with drug uptake.

In light of our results it appears important to reevaluate previous drug studies to assess whether the observed cellular alterations were caused by DNA demethylation as widely assumed rather than by other effects due to the binding of MTase to 5-azadCyd-substituted DNA. We note that experiments using 5-azadCyd as a means to induce demethylation may lead to different conclusions regarding the role of methylation in gene expression than the genetic approach. For example, it has been reported that 5-azadCyd activates the imprinted (inactive) *Igf2* allele in cultured cells (35) as well as embryos (27), suggesting that methylation suppresses *Igf2* transcription. In contrast, we have shown (24) that embryos homozygous for the MTase mutation inactivate the active rather than activate the inactive *Igf2* allele, leading to the opposite conclusion.

The role of DNA methylation patterns in the genesis of cancer is controversial (36). Decreased 5-methylcytosine content was found in several tumors (28, 30) and demethylation of specific genes has been associated with colon and lung cancer (29), whereas in similar tumors hypermethylation of genes and an increase of MTase activity were reported by other investigators (31, 32). More recently, overexpression of MTase in 3T3 cells has been shown to result in cellular transformation (33). Our results predict that a tumor with increased MTase activity should be more sensitive to the anticancer activity of 5-azadCyd and that the treatment may, in effect, select for tumor variants with decreased enzyme expression which would, as a consequence, be resistant to the drug. The availability of a defined animal system which allows genetic manipulation of MTase levels should help to

define the mechanisms of the drug's antitumor activity as well as the molecular parameters of tumor resistance.

We thank C. Beard for her technical assistance and advice, H. Wu and X. Liu for helpful discussions, and P. Soloway, H. Leonhardt, K. Tucker, L. Jackson-Grusby, P. Laird, and B. Panning for constructive comments on the manuscript. R. Jüttermann was supported by a postdoctoral fellowship from the Deutsche Forschungsgemeinschaft and R. Jaenisch by National Institutes of Health Grant 5-R35-CA44339.

1. Creusot, F., Acs, G. & Christman, J. K. (1982) *J. Biol. Chem.* **257,** 2041–2048.
2. Michalowsky, L. A. & Jones, P. A. (1987) *Mol. Cell. Biol.* **7,** 3076–3083.
3. Jones, P. A., Taylor, S. M., Mohandas, T. & Shapiro, L. J. (1982) *Proc. Natl. Acad. Sci. USA* **79,** 1215–1219.
4. Jones, P. A. (1985) *Cell* **40,** 485–486.
5. Haaf, T. & Schmid, M. (1989) *Chromosoma* **98,** 93–98.
6. Jablonka, E., Goitein, R., Marcus, M. & Cedar, H. (1985) *Chromosoma* **93,** 152–156.
7. Flatau, E., Gonzales, F. A., Michalowsky, L. A. & Jones, P. A. (1984) *Mol. Cell. Biol.* **4,** 2098–2102.
8. Davidson, S., Crowther, P., Radley, J. & Woodcock, D. (1992) *Eur. J. Cancer* **28,** 362–368.
9. Jaenisch, R., Schnieke, A. & Harbers, K. (1985) *Proc. Natl. Acad. Sci. USA* **82,** 1451–1455.
10. Richel, D. J., Colly, L. P., Lurvink, E. & Willemze, R. (1988) *Br. J. Cancer* **58,** 730–733.
11. Pinto, A. & Zagonel, V. (1993) *Leukemia* **7,** 51–60.
12. van Groeningen, C. J., Leyva, A., O'Brien, A. M., Gall, H. E. & Pinedo, H. M. (1986) *Cancer Res.* **46,** 4831–4836.
13. Abele, R., Clavel, M., Dodion, P., Bruntsch, U., Gundersen, S., Smyth, J., Renard, J., van Glabbeke, M. & Pinedo, H. M. (1987) *Eur. J. Cancer Clin. Oncol.* **23,** 1921–1924.
14. Wilson, V. L., Jones, P. A. & Momparler, R. L. (1983) *Cancer Res.* **43,** 3493–3496.
15. Jones, P. A., Taylor, S. M. & Wilson, V. (1983) *J. Exp. Zool.* **228,** 287–295.
16. Momparler, R. L., Bouchard, J. & Samson, J. (1985) *Leuk. Res.* **9,** 1361–1366.
17. Willemze, R., Archimbaud, E. & Muus, P. (1993) *Leukemia* **7,** 49–50.
18. Wu, J. C. & Santi, D. V. (1987) *J. Biol. Chem.* **262,** 4778–4786.
19. Chen, L., MacMillan, A. M., Chang, W., Ezaz-Nikpay, K., Lane, W. S. & Verdine, G. L. (1991) *Biochemistry* **30,** 11018–11025.
20. Klimasauskas, S., Kumar, S., Roberts, R. J. & Cheng, X. (1994) *Cell* **76,** 357–369.
21. Santi, D. V., Norment, A. & Garrett, C. E. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 6993–6997.
22. Li, E., Bestor, T. H. & Jaenisch, R. (1992) *Cell* **69,** 915–926.
23. Laird, P. W., Zijderveld, A., Linders, K., Rudnicki, M. A., Jaenisch, R. & Berns, A. (1991) *Nucleic Acids Res.* **19,** 4293.
24. Li, E., Beard, C. & Jaenisch, R. (1993) *Nature (London)* **366,** 362–365.
25. Vongs, A., Kakutani, T., Martienssen, R. A. & Richards, E. J. (1993) *Science* **260,** 1926–1928.
26. Foss, H. M., Poberts, C. J., Claeys, K. M. & Selker, E. U. (1993) *Science* **262,** 1737–1741.
27. Efstratiadis, A. (1994) *Curr. Opin. Genet. Dev.* **4,** 265–280.
28. Gama-Sosa, M. A., Slagel, V. A., Trewyn, R. W., Oxenhandler, R., Kuo, K. C., Gehrke, C. W. & Ehrlich, M. (1983) *Nucleic Acids Res.* **11,** 6883–6894.
29. Feinberg, A. P., Gehrke, C. W., Kuo, K. C. & Ehrlich, M. (1988) *Cancer Res.* **48,** 1159–1161.
30. Feinberg, A. P. & Vogelstein, B. (1983) *Nature (London)* **301,** 89–92.
31. Makos, M., Nelkin, B. D., Lerman, M. I., Latif, F., Zbar, B. & Baylin, S. B. (1992) *Proc. Natl. Acad. Sci. USA* **89,** 1929–1933.
32. Issa, J. P., Vertino, P. M., Wu, J., Sazawal, S., Celano, P., Nelkin, B. D., Hamilton, S. R. & Baylin, S. B. (1993) *J. Natl. Cancer Inst.* **85,** 1235–1240.
33. Wu, J., Issa, J. P., Herman, J., Bassett, D. E., Jr., Nelkin, B. D. & Baylin, S. B. (1993) *Proc. Natl. Acad. Sci. USA* **90,** 8891–8895.
34. Church, G. M. & Gilbert, W. (1984) *Proc. Natl. Acad. Sci. USA* **81,** 1991–1995.
35. Eversole-Cire, P., Ferguson-Smith, A. C., Sasaki, H., Brown, K. D., Cattanach, B. M., Gonzales, F. A., Surani, M. A. & Jones, P. A. (1993) *Mol. Cell. Biol.* **13,** 4928–4938.
36. Laird, P. W. & Jaenisch, R. (1994) *Hum. Mol. Genet.* **3,** 1487–1495.