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5-Azacytidine induction of mouse endogenous type C virus and suppression of DNA methylation

(cytidine analogue/hypomethylation/gene expression)

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ABSTRACT **5-Azacytidine was found to induce the expression of BALB:virus-1 and BALB:virus-2 from K-BALB cells and ecotropic endogenous virus from AKR2B cells. Efficiency of the induction was high and comparable to that by 5-bromodeoxyuridine. The level of methylcytosine in newly synthesized DNA was drastically decreased when K-BALB cells were treated with 5-azacytidine. There was an inverse relationship between the level of DNA modification and the frequency of virus expression.**

Mammalian DNA contains about 2–7% of its cytosine methylated in the 5 position (1). Methylation of DNA was proposed to play a regulatory role in gene expression of eukaryotic cells (2), although the precise mechanism involved is unknown. 5-Azacytidine (5azaCyd), a cytidine analogue with nitrogen in the 5 position, has recently been shown to induce myotube formation in mouse embryonic fibroblast culture (3) and at the same time inhibit methylation of DNA (4).

All strains of laboratory mice carry DNA sequences of endogeneous type C viruses in an unexpressed form (5). Expression of the endogenous viruses can be induced by treatment of cells with various agents. These agents include halogenated pyrimidines such as 5-bromodeoxyuridine (BrdUrd) and 5-iododeoxyuridine (IdUrd) (6, 7), inhibitors of protein synthesis such as cycloheximide and puromycin (8), an amino acid analogue (9), and DNA-damaging agents (10–12). In this communication, we report that 5-azaCyd induces expression of endogenous virus as efficiently as does BrdUrd, one of the most potent inducers so far studied. Our data also indicate that DNA modification might be involved in the repression of endogenous virus genome in mammalian cells.

MATERIALS AND METHODS

Cell Culture. All cultures were grown in Eagle's minimal essential medium supplemented with 10% heat-inactivated calf serum. K-BALB cells (13) were obtained from S. A. Aaronson, National Cancer Institute, Bethesda, MD. AKR2B cells (14) were kindly given to us by W. P. Rowe, National Institute of Allergy and Infectious Diseases, Bethesda, MD. NIH 2e cells were a contact-inhibited cell line established in our laboratory from NIH Swiss mouse embryo fibroblasts, which were the gift of A. Ishimoto, Virus Research Institute, Kyoto University, Kyoto, Japan. Mink lung and rat NRK and XC cell lines were originally supplied by A. Declève, Department of Radiology, Stanford University, Stanford, CA.

Virus Induction and Assay. Procedures of endogenous virus induction and virus assay have been described (12). Briefly, 2×10^5 K-BALB cells or AKR2B cells were seeded onto plastic dishes (60 mm). On the following day, the cells were treated with various inducers for 24 hr. Virus production from K-BALB cells was monitored by the procedure developed by Aaronson (7). Ecotropic virus production was detected on NIH 2e cells and xenotropic virus on NRK cells. Virus production from AKR2B cells was tested by the reverse XC plaque assay (15).

Synchronization. For the synchronization of K-BALB cells a procedure described by other investigators (16) was used with a slight modification. Briefly, 5×10^5 cells were seeded onto plastic dishes (30 mm). After 3 days of incubation, the cultures were fed with 2 ml of fresh medium. At every 5 hr thereafter, 20 μl of medium containing various chemicals was added to the dishes to yield a final concentrations of 0.1 μCi/ml (1 Ci = 3.7×10^{10} becquerels) for [^{14}C]thymidine, 2 μg/ml for 5-azaCyd, 30 μg/ml for BrdUrd, or 20 μg/ml for cycloheximide. The dish receiving [^{14}C]thymidine was incubated for 10 min and the cells were treated with trypsin. Appropriate numbers of the cells were filtered onto glass-fiber filters. The filters were washed with 5% trichloroacetic acid and with ethanol. Radioactivity incorporated was used as a measure of DNA synthesis. The dishes were incubated for 5 hr with various inducers and assayed for virus production by the procedure described above.

Base Analysis. Methylcytosine content in the newly synthesized DNA strand was assayed as described by others (4) with minor modifications. K-BALB cells were seeded onto 60-mm dishes at 5×10^5 cells per dish. After overnight incubation, cultures were treated with 5azaCyd in medium containing [*methyl*-^{3}H]methionine at 4 μCi/ml and [2-^{14}C]thymidine at 0.005 μCi/ml. DNA was collected and hydrolyzed with 60% perchloric acid as described (4). After neutralization with 6 M KOH, 100 μl of the hydrolysate was spotted onto cellulose thin-layer glass plates together with 5 μg each of methylcytosine and thymine. The plates were then developed with H_2O in dimension 1 and with 1-butanol/methanol/H_2O/NH_4OH (60:20:20:1, vol/vol) in dimension 2 (17). The spots for thymine and 5-methylcytosine were marked under ultraviolet light, scraped off the plate, and extracted overnight with 200 μl of 0.1 M HCl at 37°C. The radioactivity of each spot was determined in 10 ml of Biofluor (New England Nuclear) with an Aloka liquid scintillation counter, using a program to calculate dpm for ^{3}H and ^{14}C isotopes. Ratio of ^{3}H and ^{14}C counts was used as a measure of the relative amount of methylcytosine in newly replicated DNA. More than 95% of the ^{3}H was found in the spot corresponding to methylcytosine.

Incorporation of [^{14}C]cytosine into DNA bases was monitored essentially by the same procedure described above except the DNA hydrolysate was analyzed with the addition of carrier thymine and cytosine.

Chemicals and Radiochemicals. Cycloheximide, 5-methylcytosine, and thymine were purchased from Nakarai Chemicals,

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Abbreviations: 5azaCyd, 5-azacytidine; BrdUrd; 5-bromodeoxyuridine; IdUrd, 5-iododeoxyuridine.

Kyoto, Japan. Cytidine was the product of Kohjin, Tokyo. BrdUrd and 5azaCyd were purchased from Sigma. Two different batches of 2′-deoxycytidine were obtained from Nakarai and Sigma. [2-^{14}C]Thymidine (58 mCi/mmol), [U-^{14}C]cytidine (500 mCi/mmol), and [*methyl*-^{3}H]methionine (78 Ci/mmol) were purchased from the Radiochemical Centre, Amersham, England.

RESULTS

Dose Response of 5azaCyd-Induced Endogenous Virus Expression. K-BALB cells are a BALB 3T3 cell line nonproductively transformed with the Kirsten strain of murine sarcoma virus and carry two biologically distinct endogenous viruses, ecotropic BALB:virus-1 and xenotropic BALB:virus-2 (18). Treatment of K-BALB cells with 5azaCyd induced viruses which formed foci on NRK cells and on NIH 2e cells (Fig. 1). Focus formation on NRK cells could be explained solely by the induction of BALB:virus-1, which infect rat cells as well with low efficiency. However, virus induction frequency assayed on NRK cells was always higher than that on NIH 2e cells. This demonstrates that infectious center formation on NRK cells was due to BALB:virus-2 induction. In addition, 5azaCyd-induced K-BALB cells formed infectious centers on mink lung cells. Thus, 5-azaCyd induced both BALB:virus-1 and BALB:virus-2 in K-BALB cells. Significant increases in the expression of both viruses were observed after treatment of the cells for 24 hr with 5azaCyd at a concentration as low as 0.2 μg/ml. The maximum induction frequencies were 2.73×10^{-2} for BALB:virus-1 and 1.53×10^{-1} for BALB:virus-2 with 5azaCyd at 1–2 μg/ml (4.1–8.2 μM). A decrease in the induction frequency was noted at higher doses of 5azaCyd, and in such cases the drug exerted strong cytotoxic effects. When treated with BrdUrd, K-BALB cells expressed both BALB:virus-1 and BALB:virus-2 (Fig. 1). With BrdUrd at 20 μg/ml (65 μM), induction frequencies for BALB:virus-1 and BALB:virus-2 were 2.4×10^{-2} and 1.1×10^{-1}, respectively, such being comparable to the values obtained for 5azaCyd-induced virus expression. The ratio of the maximum induction frequency of BALB:virus-1 to that of BALB:virus-2 was approximately 2×10^{-1} for both BrdUrd-induced and 5azaCyd-induced cultures. As is clear from the results of Fig. 1, the effective molar dose range for 5azaCyd was about 1/10th that for BrdUrd.

K-BALB cells are unique among mouse cell lines used for induction studies in that they respond to inhibitors of protein synthesis to express endogenous xenotropic virus (8). To determine whether the virus-inducing activity of 5azaCyd is restricted to the K-BALB cell system, another cell line was tested for its response to this drug. AKR2B cells, a nonproducer cell line established from AKR mouse embryo fibroblasts (14), is widely used for the study of the endogenous virus induction. Upon treatment with BrdUrd, IdUrd, or hydroxyurea, this cell line produces endogenous ecotropic virus (6, 11). Treatment of AKR2B cells with 5azaCyd resulted in the expression of ecotropic AKR virus (Fig. 2). The optimal concentrations of 5azaCyd and BrdUrd for the induction of the virus were 2 μg/ml (8.1 μM) and 20 μg/ml (65 μM), respectively. The levels of endogenous virus expression were similar for 5azaCyd and BrdUrd-induced cultures. Higher concentrations of 5azaCyd lowered the induction frequency.

5azaCyd Activation of Endogenous Virus From Synchronized K-BALB Cells. It has been reported that BrdUrd and

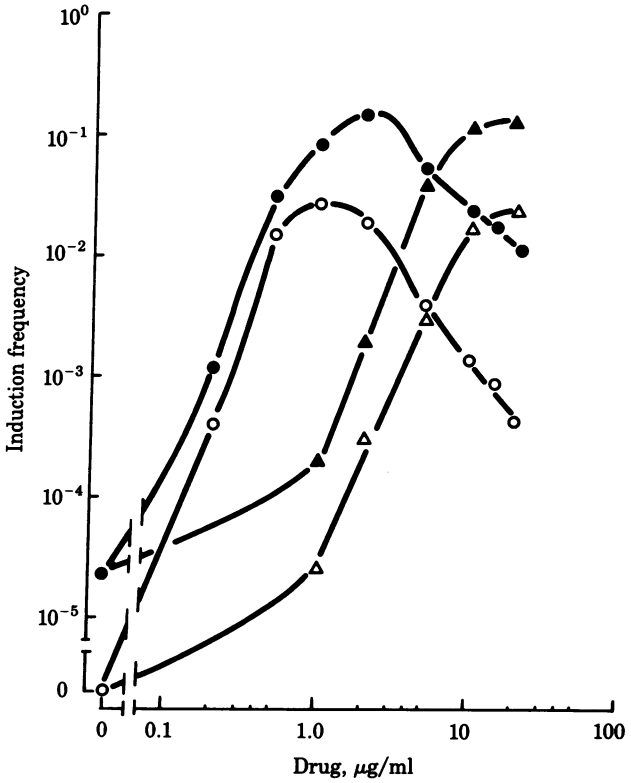

FIG. 1. Dose response of virus induction from K-BALB cells by 5azaCyd. K-BALB cells (2×10^5) seeded a day earlier were treated either with 5azaCyd or BrdUrd for 24 hr. The cells expressing ecotropic virus or xenotropic virus were assayed on NIH 2e cells or NRK cells, respectively. ●, 5azaCyd-induced xenotropic virus expression; ○, 5azaCyd-induced ecotropic virus expression; ▲, BrdUrd-induced xenotropic virus expression; △, BrdUrd-induced ecotropic virus expression.

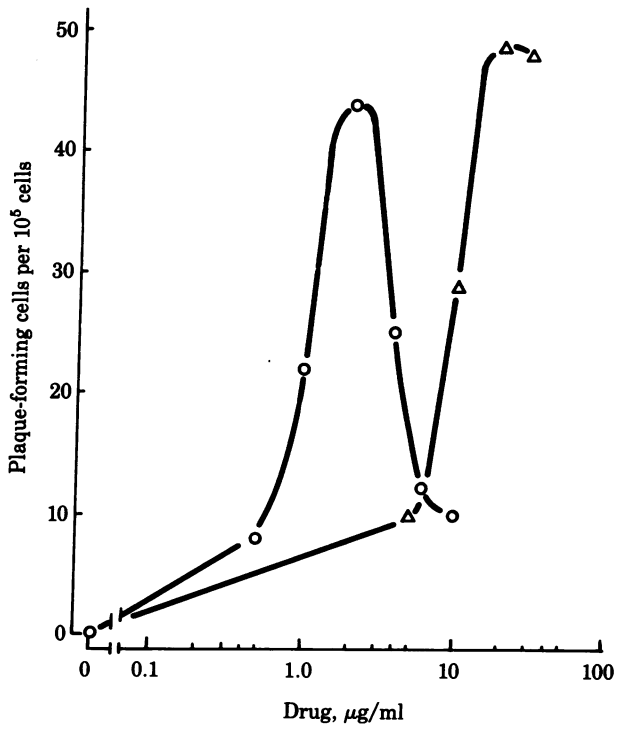

FIG. 2. Dose response of virus induction from AKR2B cells by 5azaCyd. AKR2B cells (2×10^5) seeded a day earlier were treated either with 5azaCyd or BrdUrd for 24 hr. After the treatment, the cells were further incubated for 24 hr. The cultures were then treated with trypsin, cells were counted, and appropriate numbers of the cells were transferred into dishes together with 5×10^5 XC cells. XC plaques were scored 3 days later. ○, 5azaCyd-induced virus expression; △, BrdUrd-induced virus expression.

cycloheximide activate endogenous virus only from cells in S phase (16). In order to see whether this is also the case for 5azaCyd induction of endogenous virus, synchronized K-BALB cells were tested for their response to various inducers. K-BALB cells were cultured for 3 days without changing medium. Under these conditions all the cells were in G1 phase of the cell cycle and the incorporation of [^{14}C]thymidine into the acid-insoluble fraction of the cells was negligible. After the addition of fresh medium, the cells started synthesizing DNA in a synchronous fashion. The peak of DNA synthesis was at around 15 hr (Fig. 3A). This peak was followed by the division of the cells, and the cell number per dish doubled during 35-hr incubation after the exposure of the cells to fresh medium. Fig. 3B is the virus induction frequency from K-BALB cells exposed to 5azaCyd, BrdUrd, or cycloheximide under these conditions. Endogenous virus expression was observed when the cells were induced in S phase of the cell cycle. Frequency of the virus-producing cells peaked when DNA synthesis was at its maximum. The frequency of BALB:virus-1- and BALB:virus-2-producing cells decreased thereafter, in accord with the decline of DNA synthesis of the cells. Within the resolution of our experiment, we did not observe much difference among three inducers used here—5azaCyd, BrdUrd, and cycloheximide—in the pattern for virus inducibility throughout the cell cycle.

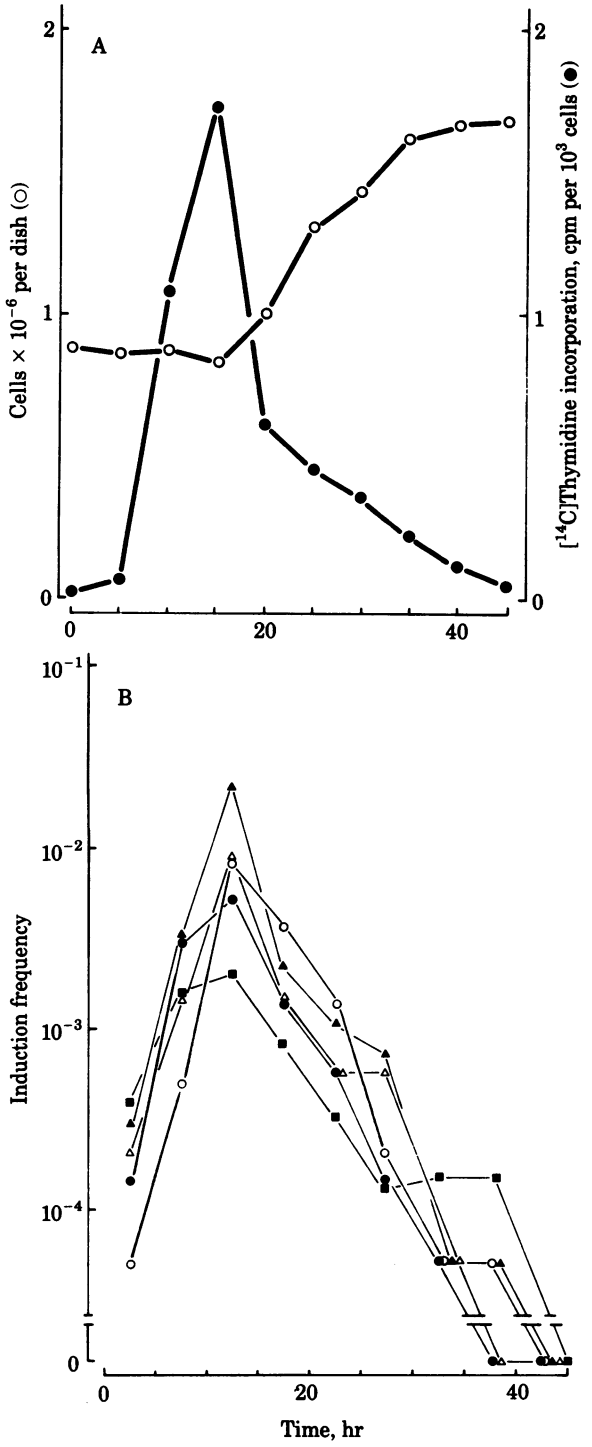

FIG. 3. Virus induction from synchronized K-BALB cells. K-BALB cells were synchronized and treated with various inducers. (A) [^{3}H]Thymidine incorporation (●) and cell number per dish (○). (B) Virus induction frequency by BrdUrd (○, ●), by 5azaCyd (△, ▲), or by cycloheximide (□, ■). Open symbols are for ecotropic virus and closed symbols are for xenotropic virus.

Competition of the Effect of 5AzaCyd with Cytidine. 5azaCyd can be incorporated into DNA as well as into RNA in place of cytosine. To test whether or not this analogue exerts the virus-inducing effect through incorporation into nucleic acids, K-BALB cells were treated with 5azaCyd at 2 μg/ml together with various concentrations of cytidine or deoxycytidine. Cytidine at the concentrations higher than 7.2 μg/ml completely blocked ecotropic and xenotropic virus expression induced by 5azaCyd, whereas deoxycytidine at a concentration as high as 20 μg/ml did not have any effect on 5azaCyd-induced virus expression (Fig. 4). This experiment has been repeated several times, using deoxycytidine purchased from two different companies, all with the same results.

Inhibition of DNA Modification by 5AzaCyd. It has been

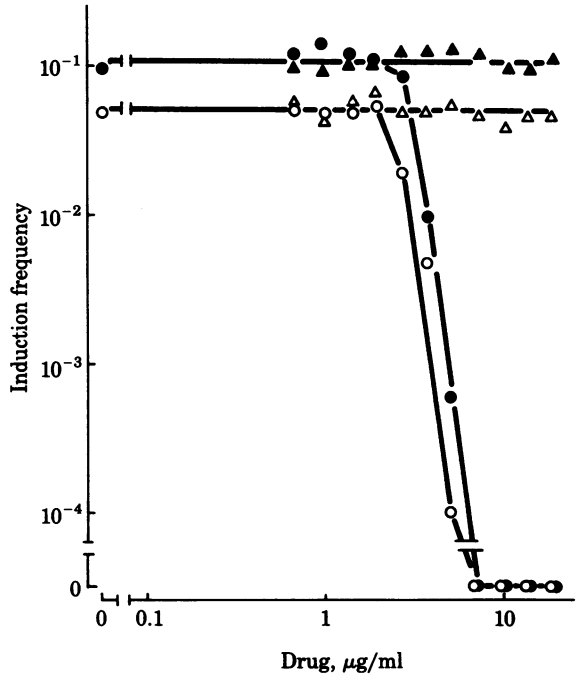

FIG. 4. Competition of the virus-inducing effect of 5azaCyd with cytidine and with deoxycytidine. K-BALB cells (2 × 10^5) seeded a day earlier were treated for 24 hr with of 5azaCyd at 2 μg/ml together with various concentrations of either cytidine or deoxycytidine. The cells were then assayed for virus production. ●, Xenotropic virus induction from K-BALB cells treated with 5azaCyd plus cytidine; ○, ecotropic virus induction from K-BALB cells treated with 5azaCyd plus cytidine; ▲, xenotropic virus induction from K-BALB cells treated with 5azaCyd plus deoxycytidine; △, ecotropic virus induction from K-BALB cells treated with 5azaCyd plus deoxycytidine.

Table 1. Inhibition of 5azaCyd-induced hypomethylation by cytidine or deoxycytidine

Treatment	Methylcytosine content, %
None	100
5azaCyd	3.0
5azaCyd + cytidine	110.6
5azaCyd + deoxycytidine	6.6

5azaCyd was added at 2 μg/ml; cytidine or deoxycytidine, at 20 μg/ml.

reported that the DNA replicated in the presence of 5azaCyd is undermethylated (4). Experiments were designed to see if the hypomethylation of DNA has any relationship to the activation of endogenous virus genomes. K-BALB cells were incubated in the presence of 5azaCyd at 2 μg/ml together with cytidine or deoxycytidine at 20 μg/ml, and the level of methylcytosine in the DNA replicated during the incubation period was measured. Table 1 summarizes the results of these experiments. 5azaCyd inhibited methylation of cytosine to 3% of the control level. The addition of cytosine abolished this inhibition, and the level of methylcytosine recovered up to 110%. On the other hand, deoxycytidine had only a minor effect, and the extent of the cytosine methylation recovered only to 6% of the control level. Thus, cytidine competed with 5azaCyd both for endogenous virus induction and for the inhibition of DNA methylation, whereas deoxycytidine did not. These results may be taken as an indication that 5azaCyd exerts its biological and biochemical effects without being incorporated into DNA. However, this cannot be concluded. An experiment was performed to find out whether the addition of deoxycytidine really inhibits the incorporation of 5azaCyd into DNA. 5azaCyd is assumed to follow the same metabolic pathway as does cytidine. After incubation of K-BALB cells with [^{14}C]cytidine together with cytidine or deoxycytidine DNA was hydrolyzed and analyzed by thin-layer chromatography. As shown in Table 2, radioactivity was found in equal quantities in cytosine and in thymine. About 5% of the radioactivity was found in methylcytosine. Addition of cytidine at 20 μg/ml decreased the radioactivity in cytosine as well as in thymine. ^{14}C in methylcytosine was also lowered by cytidine. On the other hand, the incorporation of [^{14}C]cytidine into thymine instead of cytosine was inhibited in the presence of deoxycytidine at 20 μg/ml. To our surprise, the level of the radioactivity in cytosine was absolutely unaffected by deoxycytidine. Therefore, deoxycytidine must be converted into deoxyuridine by deamination and subsequently incorporated into DNA in the position of thymine.

Level of Methylcytosine and the Extent of Endogenous Virus Expression. The relative amount of methylcytosine was measured in newly replicated DNA of K-BALB cells treated with various concentrations of 5azaCyd to see whether or not the level of DNA methylation correlates with that of endogenous virus expression. The methylcytosine content in newly replicated DNA decreased exponentially with increasing doses of 5azaCyd and reached the plateau of about 2% of the control level at 1 μg/ml of 5azaCyd (Fig. 5). On the other hand, the frequency of xenotropic virus expression reached the maximal level at 5azaCyd concentrations higher than 1 μg/ml (Fig. 5). Thus, the level of DNA methylation had good correlation with the level of virus expression in K-BALB cells.

Table 2. Effect of cytidine and deoxycytidine on the incorporation of [^{14}C]cytidine into DNA bases

Treatment	Radioactivity, dpm		
	Cytosine	Methyl-cytosine	Thymine
[^{14}C]Cytidine	3361	311	3076
[^{14}C]Cytidine + cytidine	1152	196	1005
[^{14}C]Cytidine + deoxycytidine	2805	331	850

[^{14}C]Cytidine was added at 0.01 μCi/ml; unlabeled cytidine or deoxycytidine, at 20 μg/ml.

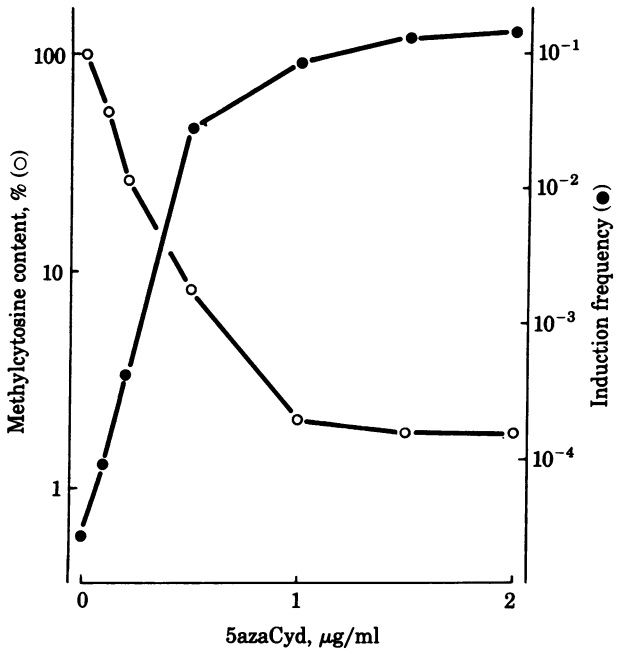

FIG. 5. Dose response of the inhibition of DNA methylation and virus induction by 5azaCyd. K-BALB cells (5×10^5) seeded a day earlier were treated with various concentrations of 5azaCyd in the medium containing [*methyl*-^{3}H]methionine at 4 μCi/ml and [2-^{14}C]thymidine at 0.005 μCi/ml. After 24 hr of incubation at 37°C the relative amount of methylcytosine in newly synthesized DNA was analyzed. The cells to be tested for virus production were treated with 5azaCyd in medium lacking the radiochemicals. ○, Percent methylcytosine in the newly replicated DNA; ●, xenotropic virus induction frequency.

DISCUSSION

All strains of mice so far studied carry endogenous type C virus genomes in a repressed state (5). Some of these endogenous viruses can be activated by a variety of treatments. Yet some of these endogenous viruses, such as ecotropic viruses, once induced, can infect mouse cells carrying the same virus in an endogenous form; in this case the cells become permanent producers of the virus. Thus, the repression system for the endogenous virus does not suppress the expression of the exogenously infecting virus. The virus expressed under these circumstances is likely the one infecting from outside of the cells and not the endogenous one carried by the cells from the begining. BrdUrd- or cycloheximide-induced K-BALB cells, upon transfer into fresh medium free of the drugs, gradually stop producing the viruses, suggesting that the repression system for endogenous viruses is reversibly derepressed. One possible explanation for the phenomenon described here is that the repression system is *cis*-acting. DNA modification, if it is involved in the control of eukaryotic gene expression, should act in a *cis*-fashion and exert its effect only on the region of chromatin where DNA is modified. Thus, DNA modification is a likely candidate controlling the expression of endogenous viruses.

In our present study, 5azaCyd is shown to be a powerful inducer of murine endogenous viruses, and at the same time it

strongly inhibits methylation of DNA. The level of the inhibition observed in our study (2% by 5azaCyd at 2 μg/ml) is much higher than that reported by other investigators [18% by 5azaCyd at 2.4 μg/ml (4)]. This may be due to the difference in the cell line used, K-BALB cells by us and C3H10T1/2 cells by Jones and Taylor. Dose response of virus induction and the inhibition of DNA methylation by 5azaCyd indicate close correlation between these two phenonena. Requirement of S phase for the induction of the viruses by 5azaCyd is consistent with the fact that methylation of cytosine residues in DNA occurs on the newly synthesized strand shortly after replication in the sequence C-G (19). Other inducers, BrdUrd and cycloheximide, also induce endogenous viruses only from the cells in S phase (ref. 16 and our present data). The pattern of the induction in K-BALB cells through S phase is not much different among three drugs BrdUrd, 5azaCyd, and cycloheximide within the limit of the resolution of our experiment. The maximum induction frequency coincides with the peak of DNA synthesis regardless of the drug used for the induction.

The biological and biochemical effects of 5azaCyd studied here, endogenous virus induction and the inhibition of DNA modification, are completely blocked by the addition of a 10-fold excess of cytidine. This suggests that 5azaCyd has to be incorporated into nucleic acids. Interestingly, the addition of deoxycytidine to the medium containing 5azaCyd has absolutely no effect on the virus induction and reduces only to a minor extent the level of the inhibition of DNA modification.

When K-BALB cells were incubated in medium containing [^{14}C]cytidine, ^{14}C was found in thymine as well as in cytosine. This indicates that the conversion of cytosine into uridine, which is incorporated into DNA as thymine after a series of metabolic conversions, is an efficient pathway in this cell line. 5azaCyd cannot be methylated in the 5 position and therefore this drug is incorporated into DNA only in the position of cytosine. On the other hand, deoxycytidine was found to be incorporated into DNA only in the position of thymine. Thus, the lack of competition with deoxycytidine for 5azaCyd-induced hypomethylation and virus expression may be due to the inability of deoxycytidine to block incorporation of 5azaCyd into DNA. This is consistent with the report of Jones and Taylor (4), who suggested 5azaCyd to be incorporated into DNA in order to block DNA methylation. It is interesting to note that deoxycytidine was reported not to block the cytotoxic effect of 5azaCyd on L1210 cells (20). Experiments using radioisotope-labeled 5azaCyd should allow determination of the amount of 5azaCyd actually incorporated into DNA.

Recent studies have suggested that DNA methylation plays an important role in controlling gene activity of higher organisms at the transcriptional level. Chicken, rabbit, and human globin genes and chicken ovalbumin genes are found to be undermethylated in tissues where they are normally expressed (21–24). Chromosomally integrated adenovirus and herpes virus DNAs are much more methylated than their free virion DNAs (25, 26). Recent investigation has shown reactivation of the transcriptionally inactive human X chromosome by 5azaCyd (27). Investigations on mouse mammary tumor virus genome show that, in a genetically transmitted viral sequence, part of its genome is methylated, whereas the same sequence was devoid of DNA modification in the provirus acquired via milk-borne infection (28).

Regulation of type C virus gene expression at the transcriptional level has been reported for BALB:virus-1 (29) and for ecotropic AKR virus (30). Our data presented here indicate that mouse endogenous type C virus genome may have part of its genome or flanking cellular sequence methylated. If such is indeed the case, then the viral genome maintained in an unexpressed state could possibly be explained.

Recently, R. A. Weinberg (Massachusetts Institute of Technology) personally communicated to us that he also observed the induction of endogenous virus with 5azaCyd. In our laboratory, we have just found that endogenous virus sequences in mouse cells resist digestion with restriction enzyme *Hpa* II, whereas exogenously infecting viral sequences are sensitive to the same enzyme, indicating that repressed endogenous viral sequences are indeed methylated (unpublished).

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