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Treatment of mice with 5-azacytidine efficiently activates silent retroviral genomes in different tissues

(toxicity of 5-azacytidine/*in vivo* activation of transcription/recombination of defective proviral genomes)

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ABSTRACT **The drug 5-azacytidine was injected into mice to activate silent retroviral genomes. The Mov-7 and Mov-10 substrains of mice were used, each of which carries a Moloney murine leukemia provirus with mutations in the coding regions at nonidentical positions. These proviral genomes are highly methylated and are not expressed in the animal. A single injection of the drug into postnatal mice induced transcription of the endogenous defective proviral genomes in thymus, spleen, and liver at 3 days after treatment. No viral transcription was detected in the brain of drug-exposed animals. When postnatal Mov-7/Mov-10 F_1 mice were treated with the drug, infectious virus was generated efficiently and resulted in virus spread and viremia in all animals by 3 weeks of age. In contrast, infectious virus was not generated in F_1 mice that had been treated during gestation with up to sublethal doses of the drug. Our results demonstrate that injection of 5-azacytidine can be used to efficiently and reproducibly activate silent genes in different cell populations of postnatal mice.**

The importance of DNA methylation for gene expression has been the subject of a number of recent reviews (1–3). Restriction enzyme analyses have demonstrated that the methylation pattern of specific genes changes during development and that hypomethylation correlates with activation of some (4–7) but not other (8–11) genes. A causal relationship between hypermethylation and gene inactivity has been established for retroviral genomes. In our laboratory, the Moloney murine leukemia virus (Mo-MuLV) has been experimentally inserted into the germ line of mice, and a number of mouse strains (Mov substrains) carrying a proviral genome at distinct Mendelian loci have been derived (12–14). The proviral genomes in all Mov substrains were highly methylated and biologically inactive in a transfection assay (15). Removal of the methyl groups by molecular cloning rendered the noninfectious proviral genomes highly infectious (16, 17), but infectivity was abolished by *in vitro* methylation of all CpGs, using a methylase from rat liver (18). Infectious virus is regularly activated in mice carrying nondefective proviruses. The mechanism of activation has not been established yet but is suspected to involve demethylation of the proviral genome in a specific population of cells followed by spread of infectious virus (19).

The experiments reported in this paper were performed to study whether silent retroviral genomes could be activated *in vivo* by treating animals with the drug 5-azacytidine, which has been shown in tissue culture to demethylate genes and to induce transcription (20). The two substrains Mov-7 and Mov-10 were used, each of which carries a defective proviral genome (21). Treatment of animals with 5-azacytidine resulted in transcriptional activation of the silent proviruses, and infectious virus was produced in Mov-7/Mov-10 F_1 mice by interaction of the two proviral genomes. Our results are consistent with the hypothesis that demethylation of DNA can induce silent genes in animals.

MATERIALS AND METHODS

Mice. The origin of the Mov-7 and Mov-10 substrains of mice has been described (14). For timed pregnancies, the day of the vaginal plug was counted as day 1 of gestation.

Injection of Mice with 5-Azacytidine. 5-Azacytidine (Sigma) was freshly dissolved in 0.85% NaCl solution at a concentration of 1 mg/ml. Adult animals were injected intraperitoneally and neonatal mice were injected subcutaneously. The total volume injected into mice 3 weeks of age or younger was 25–40 μl.

Radioimmunoassay. The serum of mice was tested for the presence of Mo-MuLV by a competitive radioimmunoassay for p30 as described (22).

RNA Analysis. RNA blot analysis of total RNA was performed as described (23). For hybridization, a cloned total Mo-MuLV probe was used. For nuclease S1 mapping, a 515-base-pair (bp) fragment from the *Sau*3A site at 95 bp to the *Sau*3A site at 610 bp of the Mo-MuLV genome (24) was prepared from the 5′ end of pMov-3 (16) and was used in the nuclease S1 mapping analysis as described (25) except that hybridization was done at 56°C. A protected fragment of 161 bp is indicative of transcription initiation at the 5′ long terminal repeat cap site.

Cells and Infectious Center Assays. Fibroblasts were obtained from lungs and kidneys of adult animals and were immortalized by transformation with simian virus 40. The cells were treated with freshly prepared 5-azacytidine solution at a final concentration of 25 or 50 μM when they were 20%–30% confluent. After 24 hr, the medium was changed and the cells were grown to confluency and dispersed by trypsin treatment. Serial dilutions of the cells were plated together with 10^6 3T3 cells in the presence of 4 μg of Polybrene per ml (Aldrich) onto new 10-cm tissue culture dishes and were grown for 2–4 days. Cultures originating from each dilution were passaged individually 4 times and parallel cultures were tested for replicating Mo-MuLV by the XC assay (15) at each passage. If replicating Mo-MuLV was induced, XC plaques were apparent after the first passage of a given serial dilution of 5-azacytidine-treated cells. The frequency of infectious centers was calculated from the lowest number of cells that induced XC plaques.

RESULTS

Spontaneous Virus Activation in Mov-7/Mov-10 F_1 Mice. The two substrains Mov-7 and Mov-10 used for this study do not activate infectious virus during their lifetime (Table 1).

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Abbreviations: Mo-MuLV, Moloney murine leukemia virus; bp, base pair(s).

Table 1. Spontaneous activation of infectious virus in Mov-7, Mov-10, and Mov-7/Mov-10 F_1 mice

Genetic locus	Expression of viremia at age			Infectivity of cloned provirus in transfection assay*
	4 wk	4–6 mo	10–14 mo	
Mov-7/Mov-7	ND	0/71	ND	–
Mov-10/Mov-10	ND	0/230	ND	–
Mov-7/Mov-10	0/63	3/43	4/43	+†

Mice of the indicated genotypes were tested for the presence of virus in the serum by radioimmunoassay as described (22). ND, not done.
*For comparison, the infectivity of the cloned proviral genomes pMov-7 and pMov-10 in a transfection assay is given (21).
†Cotransfection of pMov-7 and pMov-10.

Molecular cloning of the respective proviral genomes had shown that each carries mutations at nonidentical positions that prevent synthesis of infectious virus in a transfection assay (21). Cotransfection of both clones, however, resulted in production of replication competent virus (Table 1), presumably by recombination between the two defective genomes.

A radioimmunoassay was used to study whether the two defective proviral genomes could interact in Mov-7/Mov-10 F_1 animals to produce infectious virus. Because as few as 10 infectious units will lead to viremia and high serum titers of p30 in 3 weeks (22), this test is highly sensitive to detect qualitatively the synthesis of a single or a few replication competent viruses.

The results in Table 1 show that no p30 was detected in the serum of Mov-7/Mov-7 or Mov-10/Mov-10 mice, confirming previous observations (14). When Mov-7/Mov-10 F_1 animals were tested at 4 weeks of age, none of 63 mice was viremic, whereas viremia had developed in 8%–10% of the animals by 4–6 or 10–14 months of age. These results indicated that spontaneous synthesis of infectious virus did not occur in animals carrying the Mov-7 or Mov-10 provirus alone. Viremia developed, however, in a small fraction of Mov-7/Mov-10 animals late in life, indicating that the two defective proviral genomes can interact and generate replication competent virus with a low frequency. This frequency is increased substantially by treatment with 5-azacytidine as described below.

Postnatal Development of 5-Azacytidine-Treated Mice. Before investigating the effect of 5-azacytidine on virus activation, we first analyzed the toxicity of the drug by treating either pregnant females or mice after birth with different amounts of 5-azacytidine. Table 2 shows that the mean litter size of untreated 129 mice was 6.2 pups, 5.85 of which survived to weaning age. When pregnant females were injected with 40–80 μg of 5-azacytidine on day 14 of gestation, an average of 4.6 pups was born, 3.4 of which survived to weaning age. If the dose of the drug was increased to 400 μg, a decreasing number of pups were born, only a few of which survived to weaning age. Some of the newborns showed various signs of malformation and runting.

Table 2. Effect of 5-azacytidine on litter size when injected into pregnant females

5-Azacytidine, mg	No. of mothers	Total embryos born	Mean litter size	Total animals weaned	Mean litter size at weaning
None	281	1746	6.2	1645	5.85
0.04–0.08	19	88	4.6	65	3.4
0.1	2	6		5	
0.2	8	36		7	
0.4	10	11		0	

Pregnant females of genetic background 129 were injected intraperitoneally with 5-azacytidine at day 14 of gestation.

Table 3. Survival of mice injected with 5-azacytidine after birth

Age at first injection	No. of mice	
	Injected	Weaned (%)
Day 2	13	0 (0)
Day 3	30	11 (37)
Day 4	75	56 (75)
Day 5	23	22 (96)
Day 12	7	7 (100)

Animals of 129 genetic background were injected with 5-azacytidine beginning at different times after birth. Surviving animals were reinjected at weekly intervals. The amount of 5-azacytidine was 20–25 μg for the first and 40–60 μg for subsequent injections.

The age of the first drug injection after birth was important for survival of the animals. Table 3 shows that none of the pups injected with 25 μg of 5-azacytidine at 2 days of age survived, while an increasing fraction of animals tolerated the drug when the first injection was at 3, 4, or 5 days of age, respectively. Additional subsequent injections were well tolerated once the animals had survived the first treatment for 5 or 6 days.

5-Azacytidine Treatment After Birth, but Not During Gestation, Activates Endogenous Mo-MuLV. The effect of 5-azacytidine on activation of endogenous Mo-MuLV genomes was analyzed in Mov-7/Mov-7, Mov-10/Mov-10, and Mov-7/Mov-10 F_1 animals that had been treated either after birth or during gestation. Postnatal mice were treated with between one and five drug injections, the first injection being administered between 3 and 5 days of age. All animals were treated for viremia between 3 and 8 weeks of age. Table 4 shows that none of the 24 Mov-7/Mov-7 animals and 4 of 22 Mov-10/Mov-10 animals developed viremia after three injections of 5-azacytidine. In contrast, all of the Mov-7/Mov-10 F_1 animals that were injected either once or three times with the drug developed viremia by 3 weeks of age. In contrast, injection of the drug into pregnant females at day 14 of gestation failed to activate infectious virus in the resulting Mov-7/Mov-10 F_1 adult animals (Table 5), even when a highly toxic dose of 100 μg was administered (compare Table 2).

The results in Table 4 indicate that infectious virus is produced in all F_1 animals carrying both the Mov-7 and the Mov-10 locus after a single dose of 5-azacytidine given after birth. In animals carrying the Mov-7 or the Mov-10 proviral locus alone, none or only a low frequency of virus production was observed late in life. Injection of a sublethal dose (0.1 mg) of the drug into pregnant females, however, failed to generate infectious virus in the F_1 offspring (Table 5).

5-Azacytidine Induces Correctly Initiated Proviral Transcripts in Mice. To investigate the effect of 5-azacytidine on retroviral genome transcription, Mov-7/Mov-7 and Mov-

Table 4. Development of viremia after postnatal injection with 5-azacytidine

Genetic locus	Age at first injection, days	No. of injections	No. of viremic animals when tested at age	
			3–4 wk	6–8 wk
Mov-7/Mov-7	3–5	3–5	ND	0/24
Mov-10/Mov-10	3–5	3–5	ND	4/22
Mov-7/Mov-10 F_1	4	3	11/11	ND
	5	1	13/13	ND
	12	3	7/7	ND
			Total 39/39	

Animals were injected with 5-azacytidine as described in Table 3 and tested at different ages for viremia by radioimmunoassay. ND, not done.

Table 5. Development of viremia in Mov-7/Mov-10 F_1 animals treated with 5-azacytidine during gestation

5-Azacytidine, mg	Viremic animals/ total tested
0.04–0.08	0/42
0.1	0/5

Pregnant females were treated with 5-azacytidine at day 14 of gestation as described in Table 2. All animals were tested for viremia between 4 and 6 weeks of age.

10/Mov-10 mice were treated once or three times with the drug beginning at 4 days of age. RNA was isolated from various organs at 7 days of age—i.e., 3 days after a single injection with 5-azacytidine or 24 days after three consecutive drug injections. The RNA was subjected to nuclease S1 mapping analysis using as a probe the 515-bp *Sau*3A fragment depicted in Fig. 1. The presence of a nuclease S1-resistant fragment of 161 bp is indicative of viral transcripts that are initiated at the promoter in the 5′ long terminal repeat. Fig. 1 shows that correctly initiated viral transcripts were detected in spleen, thymus, and liver but not in brain of drug-treated Mov-7/Mov-7 and Mov-10/Mov-10 animals. Viral RNA was not regularly detected in the thymus (compare lane h), because drug treatment causes a rapid and severe depletion of cells from this organ. No viral transcripts were seen in untreated Mov-7 and Mov-10 controls or in drug-treated 129 control mice. The nuclease S1 analysis in Fig. 1 does not distinguish between RNA transcribed from the endogenous Mo-MuLV genomes and RNA transcribed from superinfecting infectious virus, which is activated at a low frequency in 5-azacytidine-treated Mov-10/Mov-10 animals (compare Table 4). To ensure that transcription of only the endogenous Mo-MuLV was measured, the serum of all animals in Fig. 1 was analyzed for the presence of viremia by the radioimmunoassay. None of the animals used for the experiments in Fig. 1 showed evidence for virus spread. The results in Fig. 1, therefore, indicate that the endogenous Mo-MuLV genomes at the Mov-7 and Mov-10 loci, respectively, become derepressed in several organs 3 days after a single injection with 5-azacytidine. Similar results were obtained with animals tested at 3 weeks of age after three consecutive drug injections (data not shown).

We furthermore attempted to detect drug-induced DNA demethylation by Southern blot analysis of DNA extracted from different organs of 5-azacytidine-treated animals. Using the methylation-sensitive restriction enzymes *Hha* I and *Hpa* II (26), we failed to detect demethylation of the proviral genomes (data not shown). This suggests that demethylation may occur in a fraction of cells that is too small to be detected by our assay.

5-Azacytidine Efficiently Induces Provirus Transcription in Tissue Culture Cells. The tissue culture cell lines were established from Mov-7/Mov-7, Mov-10/Mov-10, and Mov-7/Mov-10 F_1 animals to study the effect of 5-azacytidine on proviral genome activation *in vitro*. The cells were treated for 24 hr with the drug at 25 or 50 μM, and 5 days later RNA was extracted and an infectious center assay was performed.

Fig. 2 shows that RNA from drug-treated Mov-7/Mov-10 F_1 hybrid as well as from drug-treated Mov-7/Mov-7 and Mov-10/Mov-10 cells protected the 161-bp-long probe fragment against nuclease S1 digestion, indicating that transcription of the Mo-MuLV genomes was initiated at the 5′ promoter (compare Fig. 1). No transcripts were seen in drug-treated 129 control cells or in untreated cells from Mov mice. The three cell lines induced Mo-MuLV-specific RNA to a different extent after treatment with 5-azacytidine, as indicated by the different intensities of the 161-bp fragment. Mov-7/Mov-10 F_1 hybrid cells contained the highest concentration of viral transcripts, ≈50% of which was transcribed in Mov-7/Mov-7 cells and only 10% in Mov-10/Mov-10 cells. A lower concentration of viral transcripts in Mov-10/Mov-10 cells was seen in two independently derived cell lines treated with 25 μM or 50 μM 5-azacytidine (lanes h–m). The high concentration of viral transcripts in Mov-7/Mov-10 F_1 cells is possibly caused by the generation of infectious virus, which is followed by virus replication and virus spread (see below). RNA blot analyses showed that drug-treated

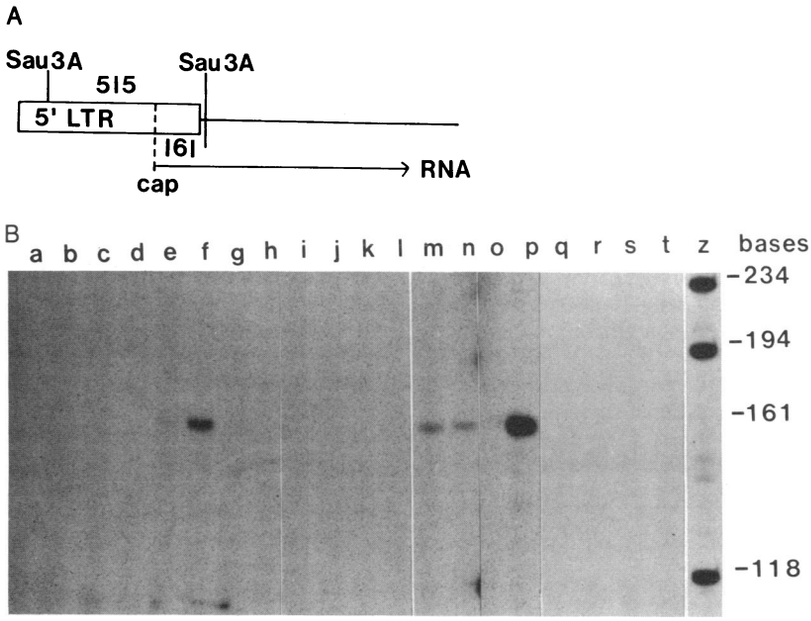

FIG. 1. (*A*) S1 nuclease mapping scheme. A 515-bp-long *Sau*3A fragment from the 5′ long terminal repeat (LTR) of the Mo-MuLV provirus was used as a radioactive probe. A 161-bp-long fragment is protected from S1 nuclease digestion when hybridized to Mo-MuLV RNA. (*B*) S1 nuclease analysis of RNA isolated from various organs of untreated and 5-azacytidine-treated Mov-7, Mov-10, and 129 control mice. RNA was isolated at 7 days of age—i.e., 3 days after a single injection with 5-azacytidine (for details see *Results*). Lanes a–h, liver, spleen, brain, and thymus, respectively, from untreated (lanes a–d) and 5-azacytidine-treated (lanes e–h) Mov-7 mice. Lanes i–p, liver, thymus, brain, and spleen, respectively, from untreated (lanes i–l) and 5-azacytidine-treated (lanes m–p) Mov-10 mice. Lanes q–t, liver, thymus, brain, and spleen, respectively, from 5-azacytidine-treated 129 mice. Lane z, end-labeled marker fragments (ϕX174RF digested with *Hae* III).

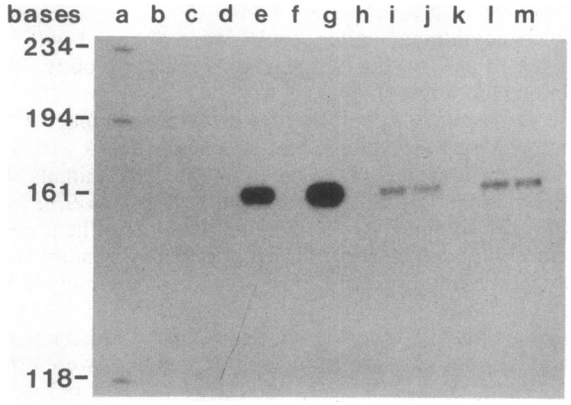

FIG. 2. S1 nuclease analysis of RNA isolated from untreated and 5-azacytidine-treated tissue culture cell lines derived from 129, Mov-7, Mov-10, and Mov-7/Mov-10 F_1 animals. The cells were treated for 24 hr with 25 μM or 50 μM 5-azacytidine, and 5 days later RNA was isolated. For nuclease S1 mapping, 20 μg of each RNA was hybridized to the radioactive DNA fragment. Lanes b and c, 129 cells untreated (lane b) and treated with 25 μM 5-azacytidine (lane c). Lanes d and e, Mov-7 cells untreated (lane d) and treated with 25 μM 5-azacytidine (lane e). Lanes f and g, Mov-7/Mov-10 cells untreated (lane f) and treated with 25 μM 5-azacytidine (lane g). Lanes h–m, two independently derived Mov-10 cell lines, untreated (lanes h and k), treated with 25 μM 5-azacytidine (lanes i and l), and treated with 50 μM 5-azacytidine (lanes j and m). Lane a, end-labeled marker fragments (ϕX174RF digested with *Hae* III).

Mov-7/Mov-10 F_1 cells produced similar amounts of 35S and 21S viral RNA as a fibroblast culture chronically infected with Mo-MuLV. Similarly, drug-treated Mov-7/Mov-7 and Mov-10/Mov-10 cells produced both viral RNA species, but in lower concentrations (data not shown).

The infectious center assay was performed on parallel cultures and indicated that 1 of 100–1000 Mov-7/Mov-10 F_1 cells produced infectious virus after drug treatment (Table 6). No virus production was found in untreated Mov-7/Mov-10 F_1 cells or in drug-treated Mov-7/Mov-7, Mov-10/Mov-10, or in 129 control cells. The low frequency of infectious centers after 5-azacytidine treatment in Mov-7/Mov-10 F_1 cells contrasts the high concentration of viral transcripts. This suggests that both defective proviral genomes are activated rather efficiently, but that recombination between the two defective genomes, which is necessary to generate an infectious virus, occurs only with a low frequency.

DISCUSSION

The effect of 5-azacytidine on expression of the two retroviral genomes carried in Mov-7 and Mov-10 mice was studied by monitoring induction of infectious virus in the animal and by analyzing proviral transcription in various animal tissues. Replication competent virus cannot be synthesized in either of the two substrains alone, because each provirus carries two or one structural defects, respectively, in their coding region (17, 27). Previous results had demonstrated, however, that infectious virus was synthesized in 3T3 cells that were cotransfected with both plasmid clones carrying the proviral genomes, which indicated that the genetic lesions in the two proviral genomes were at nonidentical positions (21).

Table 6. Induction of infectious centers by treatment of cells in tissue culture with 5-azacytidine

Genotype of cells	Concentration of 5-azacytidine	Frequency of infectious centers in XC assay
Mov-7/Mov-7	0, 25, 50 μM	$<10^{-6}$
Mov-10/Mov-10		
Mov-7/Mov-10	0	$<10^{-6}$
	25 μM	10^{-2}–10^{-3}

Cells of different genotypes were treated with 5-azacytidine and tested for infectious centers.

A single injection of homozygous Mov-7 or Mov-10 animals with 5-azacytidine induced correctly initiated viral transcripts in spleen, thymus, and, to a lesser extent, in liver after 3 days (Fig. 1). No proviral transcripts were seen in organs of untreated animals or in brain of treated animals. Because the proviral copies carried in these mice are alone unable to specify synthesis of infectious virus, the viral RNA must have been transcribed from the endogenous Mov-7 or Mov-10 proviral copy, respectively, and could not have been the result of replication and spread of infectious virus. The low frequency of viremia in drug-treated homozygous Mov-10 animals could be due to interaction of the Mov-10 provirus with an endogenous murine virus followed by recombination to generate infectious virus (see below). Alternatively, the drug may induce a reversion in the single lesion (21) of the Mov-10 provirus. No generation of infectious virus was seen in drug-treated homozygous Mov-7 animals, the Mo-MuLV genome of which carries two mutations (21).

All Mov-7/Mov-10 F_1 animals developed viremia by 3 weeks of age after a single drug injection after birth (Table 4). The presence of drug-induced infectious virus in F_1 animals is likely to be the result of transcription of both proviral copies in the same cell and synthesis of heterozygous particles, reverse transcription of which would result in generation of a replication competent virus by recombination (28, 29) followed by virus spread and development of viremia (22). The observation that cells transfected with pMov-7 or pMov-10 synthesize viral transcripts but fail to produce stable viral particles (unpublished results) is consistent with such a mechanism of generating the infectious virus. The low frequency of viremia in untreated Mov-7/Mov-10 mice late in life (Table 1) suggests that spontaneous virus expression and recombination is a rare event.

Drug treatment of cells in tissue culture confirmed the results obtained with mice. No infectious virus was induced in Mov-7/Mov-7 or Mov-10/Mov-10 cells, while a low frequency of infectious centers was observed in Mov-7/Mov-10 F_1 cells (Table 6). Correctly initiated viral RNA was transcribed in drug-treated cells of all genotypes (Fig. 2). The extent of viral transcription, however, differed, as the Mov-7 provirus was induced more efficiently than the Mov-10 provirus. A more efficient transcription of the Mov-7, as compared to the Mov-10, proviral genomes has also been observed in previous experiments in which the cloned proviruses were microinjected into *Xenopus* oocytes (30).

Recombination between defective proviral genomes as a common mechanism to generate infectious virus has been documented in tissue culture and in animals. Cotransfection of two cloned defective proviral genomes resulted in replication competent recombinant viruses (21, 31, 32). *In vivo*, enhanced production of ecotropic MuLV was shown to be due to interaction of different loci that were closely linked to ecotropic virus structural genes (33), and recombination between ecotropic and xenotropic endogenous proviruses occurred after treatment of mice with carcinogen (34). More recently, the spontaneous generation of replication competent recombinant virus in HRS/J mice was shown to result from the complementation of genetic defects in two endogenous murine retroviruses (35). Similarly, in chickens recombination between defective endogenous viruses was suggested to cause spontaneous generation of leukosis virus (36).

The drug 5-azacytidine has been shown to inhibit the maintenance methylase and to induce demethylation of

DNA (37, 38). This can result in active transcription of previously silent cellular (39–41) or proviral (20, 42) genes and can be followed by a change of the differentiated state of cells treated with the drug in tissue culture (43, 44). Previous results have shown that the proviral genomes in Mov-7 and Mov-10 mice are highly methylated (15) and are not transcribed (Figs. 1 and 2). Because drug-induced demethylation presumably requires DNA replication, dividing cell populations most likely are the target cells affected by the drug in treated animals. The preferential induction of proviral transcription in spleen, thymus, and liver, which in the newborn mouse consists largely of replicating hemopoietic cells, but not in the brain, consisting mainly of nondividing cells, is consistent with this hypothesis. We do not know, however, whether 5-azacytidine can penetrate the blood–brain barrier.

In contrast to the highly efficient induction of viremia in mice injected with 5-azacytidine after birth, treatment of pregnant females even with 0.1 mg of 5-azacytidine, a drug concentration that is highly toxic to the embryos, failed to generate infectious virus (Table 5). This may suggest that 5-azacytidine can efficiently induce activation of silent genes only in specific cell populations at certain developmental stages. Alternatively, the drug may not have directly affected cells of the embryo, and embryo toxicity may have been due to other undefined effects of drug treatment. Injection of the drug directly into the embryos will clarify this question.

Foreign genes, which are experimentally introduced into embryos and subsequently into the germ line of mice, become *de novo* methylated upon genomic integration and are frequently transcriptionally inactive in the animal (26, 45, 46). Our results demonstrate that injection of 5-azacytidine can efficiently and reproducibly activate silent genes in different cell populations of postnatal animals. Thus, treatment with 5-azacytidine may enable us to manipulate the expression of cellular as well as experimentally inserted genes and to study the effects of gene activation in the living animal.

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