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DNA Methylation Affecting the Expression of Murine Leukemia Proviruses

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The endogenous, vertically transmitted proviral DNAs of the ecotropic murine leukemia virus in AKR embryo fibroblasts were found to be hypermethylated relative to exogenous AKR murine leukemia virus proviral DNAs acquired by infection of the same cells. The hypermethylated state of the endogenous AKR murine leukemia virus proviruses in these cells correlated with the failure to express AKR murine leukemia virus and the lack of infectivity of cellular DNA. Induction of the endogenous AKR murine leukemia virus proviruses with the methylation antagonist 5-azacytidine suggested a causal connection between DNA methylation and provirus expression. Also found to be relatively hypermethylated and noninfectious were three of six Moloney murine leukemia virus proviral DNAs in an unusual clone of infected rat cells. Recombinant DNA clones which derived from a methylated, noninfectious Moloney provirus of this cell line were found to be highly active upon transfection, suggesting that a potentially active proviral genome can be rendered inactive by cellular DNA methylation. In contrast, in vitro methylation with the bacterial methylases *MHpa*II and *MHha*I only slightly reduced the infectivity of the biologically active cloned proviral DNA. Recombinant DNA clones which derived from a second Moloney provirus of this cell line were noninfectious. An in vitro recombination method was utilized in mapping studies to show that this lack of infectivity was governed by mechanisms other than methylation.

The role of DNA methylation in the control of vertebrate gene expression has been a very active area of research in the past several years. Of particular interest has been the postreplication methylation that produces 5-methylcytosine (5-^{m}C). This modification occurs generally in the dinucleotide 5′-CpG-3′ and to a lesser extent in 5′-CpC-3′, giving rise to the methylated dinucleotides 5′-^{m}CpG-3′ and 5′-^{m}CpC-3′, respectively (54). 5-^{m}C is by far the most common methylated residue found in vertebrate DNA; estimates of its frequency vary around 1 to 3% of the nucleotides in the genomic DNA (16, 54). Thus, it can be estimated that there are approximately 10^{8} 5-^{m}C bases per diploid mammalian cell.

Several restriction endonucleases whose recognition sequences contain dinucleotide 5′-CpG-3′ or 5′-CpC-3′ do not cleave these sequences if 5-^{m}C is present (7, 16). In recent years, as recombinant DNA clones of a variety of mammalian genes have become available, these restriction endonucleases have been used to analyze the methylation state of the particular sequences contained in the cloned gene as they exist in the DNA of germ line cells and different somatic tissues of an animal (13, 28, 41, 53). Other experiments have compared the methylation of integrated sequences of DNA tumor viruses in cultured cell lines with the expression of viral gene products (15, 50). Virtually all of the evidence suggests an inverse correlation between DNA methylation and gene expression. However, the details of this transcriptional control, its mechanism of action, and its physiological role in differentiation and development are not currently understood.

In our laboratory, we have been studying murine leukemia proviruses as models for the regulation of mammalian gene expression. The proviral genomes of murine retroviruses have

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several features which are advantageous for such experiments. The nondefective retroviruses, such as the AKR murine leukemia virus (AKR-MuLV), Moloney murine leukemia virus (M-MuLV), and mouse mammary tumor virus, can infect susceptible cells and produce multiple, exogenously acquired proviruses at many possible integration sites in the host cell genomic DNA (12, 46, 48). Once established in a clone of infected cells, the number and location of the proviral integrations are stably maintained in the derived cell line (48). We have observed M-MuLV-infected rat kidney (NRK) cell lines containing as few as 3 M-MuLV proviruses, whereas other lines contain 20 or more (48). The exogenously acquired proviruses carry their own transcriptional promoter within terminal repeat sequences (4) and appear not to depend upon integration next to host cell promoters. However, the expression of the provirus may still be sensitive to influences of chromatin structure, DNA modification, and *cis*-acting control elements in the flanking host cell sequences. Cell lines containing exogenously acquired murine proviruses thus represent ideal reagents for studying the cellular transcriptional controls that act at various chromosomal locations, since virtually identical genomes are being regulated in a common intracellular environment, each closely linked to a different set of cellular sequences.

Murine proviruses may also exist endogenously in the mouse germ line, in which case they are vertically transmitted like other Mendelian loci in the mouse chromosomal complement. Such germ line integrations occur naturally in the case of mouse mammary tumor virus (3) and AKR-MuLV (37) and have been produced in the laboratory for M-MuLV (24). The endogenous murine leukemia proviruses provide additional examples of cellular control of proviral transcription. Generally, the endogenous proviruses are expressed spontaneously in only a few rare embryo cells. However, a larger fraction of embryo cells can be induced in culture with agents such as 5-bromodeoxyuridine and 5-iododeoxyuridine (6, 27).

We describe here the role of 5-^{m}C in the expression of two groups of murine leukemia proviruses, the endogenous AKR-MuLV proviruses in AKR embryo cells and the exogenous M-MuLV proviruses in an unusual clone of infected NRK cells.

MATERIALS AND METHODS

Cells and virus assays. Cell lines NIH/3T3 and BALB/3T3 are continuous lines of mouse fibroblasts from NIH/Swiss and BALB/c mice, respectively. NRK cells are a continuous line of rat kidney cells. Of the cloned lines of M-MuLV-infected NRK cells, cell lines NRK1, NRK3, NRK4, and NRK5 were derived by Fan and Paskind (17), and cell line CP1 was derived by E. Rothenberg. The establishment of the AKR2B cell line from AKR mouse embryo fibroblasts has been described by Rowe et al. (38). Cell lines from clones of AKR2B cells infected with ecotropic, N-tropic AKR-MuLV were derived by Steffen et al. (47). All cell lines were obtained from stocks kept at the Center for Cancer Research, Massachusetts Institute of Technology. All cell lines except NRK cells and M-MuLV-infected NRK cells were grown in Dulbecco modified Eagle medium containing 10% calf serum; NRK cells and M-MuLV-infected NRK cells received 10% heat-inactivated fetal bovine serum.

MuLV-producing cells were assayed by the UV-XC plaque assay (40) or by the presence of virion-bound reverse transcriptase activity in culture supernatants (19). Infectious DNA assays (transfections) were performed by the calcium phosphate coprecipitation method (20), as modified for MuLV transfections by Smotkin et al. (43, 44).

Preparation and analysis of DNA. The preparation of high-molecular-weight cellular DNA, its analysis by restriction endonuclease digestion, 1% neutral agarose gel electrophoresis, and transfer to nitrocellulose filters, and hybridization to DNA bound to filters have all been described previously (45–48). Two types of hybridization probes were employed in these analyses. Plasmid DNAs were labeled with ^{32}P by nick-translation to a specific activity of 5×10^7 to 2×10^8 cpm/μg using the procedure of Rigby et al. (35). The preparation of an unselected AKR-MuLV or M-MuLV ^{32}P-labeled cDNA probe from virion RNA by an exogenous reverse transcription reaction was done by the method of Steffen et al. (46). This cDNA had a specific activity of 1×10^8 to 5×10^8 cpm/μg. A selected, AKR-MuLV-specific cDNA probe was obtained by pre-hybridization to a 1,000-fold excess of M-MuLV virion RNA (1, 46).

Isolation of recombinant DNA clones. We attempted to obtain molecular clones of all of the M-MuLV proviruses in the DNA of one cloned, infected NRK cell line, NRK5. Because the restriction endonuclease *Eco*RI does not cleave M-MuLV viral DNA sequences (59), the cloning vector which we used was the bacteriophage lambda vector Charon 4A (9), which has the capacity to carry *Eco*RI insertions within the size range of approximately 8 to 22 kilobase pairs (kb). All of the six M-MuLV proviruses in NRK5 DNA are contained in *Eco*RI fragments which are in this size range (48).

Bacteriophage Charon 4A was propagated essentially as described by Blattner et al. (9), except that *Escherichia coli* LE392 was used as the host. Our previously described (55) method for preparing phage DNA was used not only for the Charon 4A vector, but also for any subsequently derived recombinants.

To increase the representation of M-MuLV proviral DNAs carried in a Charon 4A recombinant library, fragments of NRK5 DNA were digested with *Eco*RI and size-selected by agarose gel electrophoresis. DNA fragments between 10 and 27 kb long were recovered from the agarose by dissolving the agarose slices in saturated sodium iodide and binding the DNA to fine glass powder (56). A parallel experiment with ^{3}H-labeled NRK DNA demonstrated that this range of sizes contains 15% of the total *Eco*RI-digested NRK

DNA by mass and 5% of the total number of *Eco*RI fragments generated by complete digestion of NRK DNA (data not shown). The Charon 4A vector DNA was also digested with *Eco*RI, and the vector arms were separated from the two internal bacteriophage *Eco*RI fragments by agarose gel electrophoresis as described above.

The ligation of DNA for in vitro bacteriophage packaging was performed in a volume of 25 μl. Approximately 2 μg of the gel-eluted *Eco*RI-cleaved NRK5 DNA to be inserted into the vector was coprecipitated in ethanol with an amount of vector arms equivalent to 2 μg of total Charon 4A DNA. The ethanol precipitates were collected in small (0.5-ml) Eppendorf vials and dissolved in 17 μl of 1 mM Tris-hydrochloride (pH 7.6)–1 mM EDTA (sodium salt). Then 5 μl of 5× L buffer (300 mM Tris-hydrochloride, pH 7.6, 50 mM $MgCl_2$, 75 mM dithiothreitol, 5 mM spermidine hydrochloride, 1 mg of gelatin per ml) was added, followed by 2.5 μl of 10 mM ATP and, finally, 0.5 μl of T4 DNA ligase (New England Biolabs). Ligation mixtures were incubated at 16°C for 16 to 24 h. After ligation, the ligase was inactivated at 67°C for 5 min, and the DNA was packaged into intact phage particles by the in vitro encapsidation procedure of Blattner et al. (8). Recombinant bacteriophage were plated and screened as previously described (55) by using the in situ hybridization procedure of Benton and Davis (2) and an M-MuLV viral DNA probe. Approximately 10 μg of size-selected NRK5 DNA was used in several separate ligation and packaging experiments. In total, we screened 2×10^6 independent recombinant DNA clones. This number of recombinant bacteriophage plaques represents a library which has 100 times the estimated sequence complexity of the population of gel-enriched NRK5 DNA insertions.

In vitro methylation. Hamilton Smith generously provided the *MHha*I and *MHpa*II methylases and the conditions for the in vitro methylation reaction. Reactions were performed in 50-μl volumes containing 5 μl of a ^{3}H-labeled *S*-adenosylmethionine stock solution (50 μM; 10.5 Ci/mmol; in 0.1 N HCl; stored frozen at −20°C) and 5 μl of 10× methylase cocktail (0.1 M Tris base, 0.5 M Tris-hydrochloride, pH 7.5, 0.2 M 2-mercapto-ethanol, 0.1 M EDTA [sodium salt], with the remaining volume consisting of DNA (usually 1 μg), enzyme, and water. The reaction mixtures were incubated for 2 h at 37°C, and the reactions were stopped by the addition of 200 μl of NIH 3T3-cell carrier DNA (150 μg/ml), phenol extraction, chloroform-isoamyl alcohol (24:1, vol/vol) extraction, and ethanol precipitation. For titrating the activities of the methylases on λ836 DNA, serial fivefold dilutions of the enzyme stock solutions were made. A sample of the reaction mixture was taken before the addition of the carrier DNA and was digested with 10 U of the corresponding restriction endonuclease per μg. The extent of cleavage was monitored by 1% neutral agarose gel electrophoresis.

RESULTS

5-^{m}C in endogenous AKR-MuLV proviruses. Our interest in the role of DNA methylation in the control of expression of murine leukemia proviruses originally developed from a series of experiments designed to map AKR-like endogenous proviruses in the genomic DNAs of various inbred and feral mice. Part of the restriction endonuclease map for AKR-MuLV viral DNA previously derived in this laboratory (47) is shown in Fig. 1A. This mapping information was utilized to examine the genomic DNAs of AKR and other mouse strains for specific internal restriction fragments of the AKR-MuLV viral DNA which presumably would be indicative of the presence of AKR-MuLV proviruses.

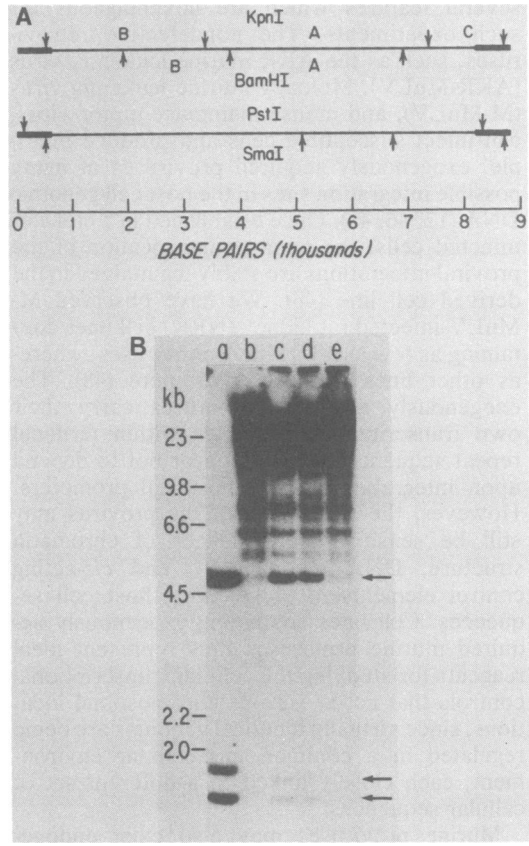

FIG. 1. *Sma*I restriction digests of AKR-MuLV viral and proviral DNAs. (A) Restriction sites in the AKR-MuLV viral DNA map. The sites for the enzymes shown have been published previously (47). (B) Autoradiograph of a Southern blot filter hybridized with an AKR-MuLV cDNA probe. All lanes contained *Sma*I digests. Lanes b through e were each loaded with 10 μg of DNA. Lane a, Unintegrated, linear AKR-MuLV viral DNA (47); lane b, AKR2B DNA; lane c, AKR2B/IID2 DNA, a cloned line of AKR-MuLV-infected AKR2B cells; lane d, AKR2B/IID5 DNA, another AKR-MuLV-infected cloned cell line of AKR2B cells; lane e, a second preparation of AKR2B DNA. Arrows indicate the positions of migration of the internal *Sma*I fragments.

Among the first cells from which DNA was made for use in this study were AKR2B cells, an established line of AKR embryo cells. AKR2B

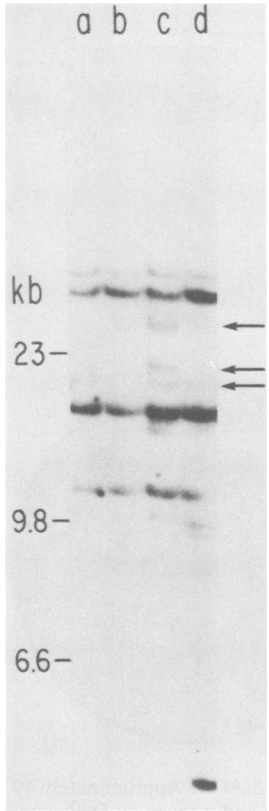

FIG. 2. *Eco*RI and *Eco*RI-*Sma*I restriction digests of AKR-MuLV proviral DNAs: autoradiograph of a Southern blot filter hybridized with a selected AKR-MuLV cDNA probe. Each lane was loaded with 20 μg of DNA. Lane a, AKR2B DNA digested with *Eco*RI; lane b, *Eco*RI-digested AKR2B DNA digested with *Sma*I; lane c, AKR2b/IID2 DNA digested with *Eco*RI; lane d, *Eco*RI-digested AKR2B/IID2 DNA digested with *Sma*I. The *Eco*RI patterns of the three endogenous AKR-MuLV proviruses in AKR2B embryo fibroblasts have been described previously (46). Three *Sma*I-sensitive exogenous AKR-MuLV proviruses in AKR2B/IID2 DNA are indicated by arrows. The band of hybridization near the bottom of lane d is the largest of the three internal *Sma*I-generated fragments of AKR-MuLV viral DNA present in Fig. 1.

cells are generally negative for the expression of AKR-MuLV but are known to contain two to three endogenous AKR-MuLV proviruses (46). When DNA from AKR2B cells was analyzed for diagnostic internal AKR-MuLV DNA fragments, the *Bam*HI-A and *Bam*HI-B fragments, the *Kpn*I-A and *Kpn*I-B fragments, and the large *Pst*I fragment were easily detected (47), but none of the three internal *Sma*I-generated fragments was detected (Fig. 1B, lanes b and e). However, the three diagnostic *Sma*I fragments were present when DNAs from cloned lines of AKR2B cells exogenously infected with AKR-MuLV were examined (Fig. 1B, lanes c and d). These infected lines produced AKR-MuLV virus and had acquired new exogenous AKR-MuLV proviruses (47). Since *Sma*I is one of the restriction endonucleases which cannot cleave methylated DNA, these results suggest the presence of 5-^{m}C in the endogenous, but not the exogenous, AKR-MuLV proviral DNAs.

Additional evidence for this hypothesis was obtained by analyzing *Eco*RI digests and *Eco*RI-*Sma*I double digests of DNAs from AKR2B cells and AKR-MuLV-infected AKR2B cells. Because *Eco*RI has no sites within AKR-MuLV viral DNA (47), individual AKR-MuLV proviruses can be resolved by an analysis of *Eco*RI digests of AKR2B DNAs. This is because the *Eco*RI sites that flank the proviruses are found at varying distances from the integrated viral DNAs. Hence, the endogenous and exogenous AKR-MuLV proviruses can be distinguished by comparing the *Eco*RI pattern of proviral DNAs in the clones of infected AKR2B cells with the pattern in uninfected parent cells. Furthermore, the sensitivity of an individual provirus to *Sma*I digestion can be tested by monitoring the disappearance of its respective *Eco*RI fragment after a second digestion with *Sma*I. As shown in Fig. 2, this analysis demonstrated that the endogenous AKR-MuLV proviruses are *Sma*I resistant and therefore relatively hypermethylated, whereas all of the apparent exogenously acquired AKR-MuLV proviruses are *Sma*I sensitive and hypomethylated. Because only exogenous proviruses appear to be transcriptionally active, as judged by the expression of virus,

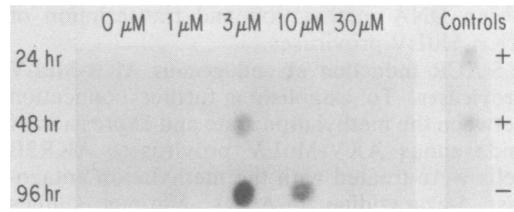

FIG. 3. Kinetics and concentration dependence of AKR-MuLV induction by 5-ACR. Rapid reverse transcriptase assays were performed as described previously (19). The positive controls used were supernatants from cells producing AKR-MuLV and M-MuLV. The negative control was phosphate-buffered saline. The molarities refer to the concentration of 5-ACR. Cells were seeded on the same day, and the times refer to the number of hours of 5-ACR treatment before the assay. Low levels of signal (not apparent on the figure) were detected at 48 h at a concentration of 10 μM and at 96 h at a concentration of 30 μM. Upon treatment with 30 μM 5-ACR, approximately 50% of the cells were killed by 48 h.

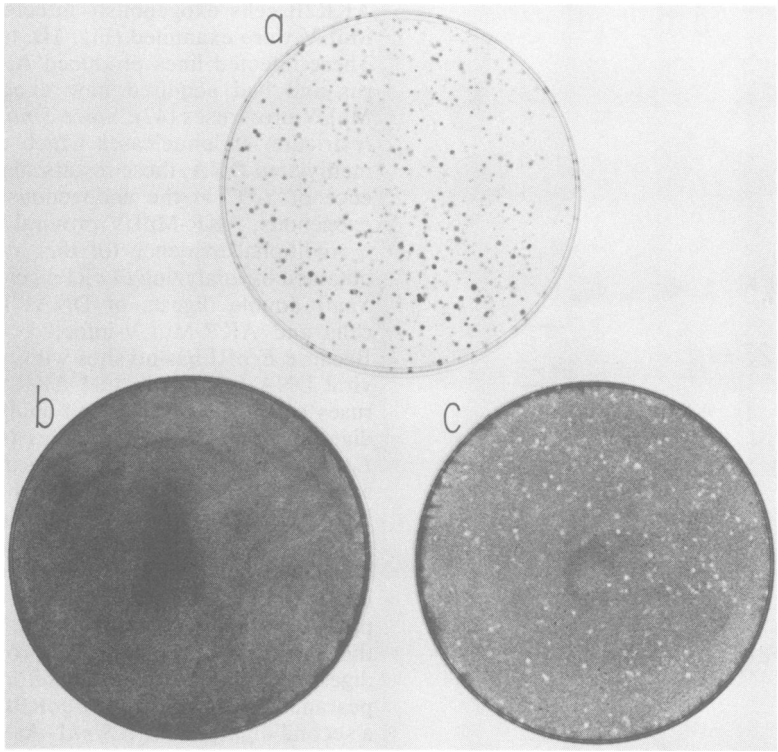

FIG. 4. Infectious center assay of AKR embryo cells induced by 5-ACR. Approximately 10^3 AKR2B cells were seeded onto 15-cm dishes on the first day. The cells on plates a and b received Dulbecco modified Eagle medium, whereas the cells on plate c received Dulbecco modified Eagle medium supplemented with 3 μM 5-ACR. All of the plates of cells were refed with Dulbecco modified Eagle medium on the third day, and on the following day XC plaque assays were performed on plates b and c. On day 7 of the experiment plates b and c were fixed and stained in the usual manner, and the colonies on plate a were stained with a solution containing 1% crystal violet and 20% ethanol.

these results support an inverse correlation between DNA methylation and transcription of AKR-MuLV proviruses.

5-ACR induction of endogenous AKR-MuLV proviruses. To establish a further connection between the methylation state and expression of endogenous AKV-MuLV proviruses, AKR2B cells were treated with the methylation antagonist 5-azacytidine (5-ACR). Various studies have implicated this drug in the activation of transcriptionally silent genes by causing demethylation of DNA (21, 25, 31, 51). We found 5-ACR to be an effective inducer of AKR-MuLV from AKR2B cells. Figure 3 shows the kinetics and concentration dependence of virus induction after treatment with 5-ACR, as measured by the amount of reverse transcriptase activity in the supernatants of treated cultures (19). Additional experiments demonstrated that the induced retrovirus was infectious on Fv-1^n/Fv-1^n mouse cell monolayers and not infectious on Fv-1^b/Fv-1^b mouse cell monolayers (data not shown); such a pattern is characteristic for AKR-MuLV. Figure 4 shows the result of an infectious center assay for approximately 10^3 AKR2B cells treated with 5-ACR and assayed for virus expression by the XC plaque assay. A large fraction of the cells (as high as 30%) expressed AKR-MuLV upon induction with 5-ACR. These experiments further support a role for DNA methylation in controlling the expression of endogenous AKR-MuLV proviruses.

5-^{m}C in exogenous M-MuLV proviruses. To extend the results obtained with AKR-MuLV, we examined the methylation state of the proviral DNA of another MuLV, M-MuLV. Figure 5 shows the results of an *Eco*RI and *Eco*RI-*Sma*I analysis of the M-MuLV proviruses in DNAs from five clones of NRK cells that chronically produce M-MuLV. The *Eco*RI digestion pattern of the M-MuLV proviruses in these infected NRK clones has been described previously (48). *Eco*RI, which also has no sites in M-MuLV viral DNA (18, 59), was again used to distinguish

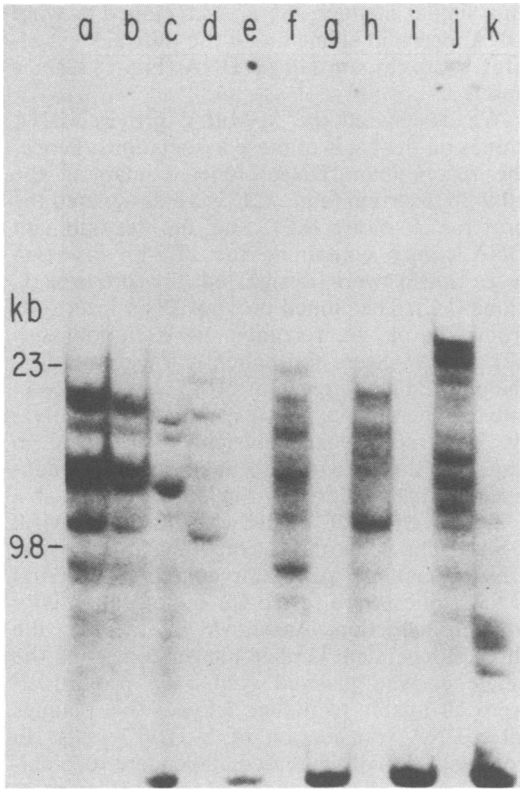

FIG. 5. *Eco*RI and *Eco*RI-*Sam*I restriction digests of M-MuLV proviral DNAs in infected NRK cells. An autoradiograph of a Southern blot filter hybridized with a cloned, unintegrated M-MuLV viral DNA probe was prepared as described in the text. Each lane was loaded with 10 μg of DNA. Lanes a, b, d, f, h, and j contained *Eco*RI digests; lanes c, e, g, i, and k contained *Eco*RI digests and *Sma*I double digests. Lanes a through c, NRK5 DNA; lanes d and e, CP1 DNA; lanes f and g, NRK1 DNA; lanes h and i, NRK3 DNA; lanes j and k, NRK4 DNA. The *Eco*RI patterns of M-MuLV proviruses in these cell lines agree with the pattern previously reported (48).

individual proviral integrations, and the number of M-MuLV proviruses ranged from 3 in CP1 cells to 15 or more in NRK4 cells. Once again, we used sensitivity to *Sma*I digestion to investigate the presence of 5-^{m}C in the proviral DNAs, since *Sma*I has five sites in M-MuLV viral DNA (18).

All of the DNAs in Fig. 5 contain some M-MuLV proviruses which are hypomethylated and *Sma*I sensitive. The largest internal *Sma*I-generated M-MuLV viral DNA fragment appears at the bottom of each of the *Eco*RI-*Sma*I lanes. Special note should be made of cell line NRK5, whose DNA contains six M-MuLV proviruses (Fig. 5, lanes a through c). Only NRK5 DNA was found to have any *Sma*I-resistant M-MuLV proviruses. If the six NRK5 proviruses are designated in order A through F, according to decreasing molecular size, then proviruses B, C, and E are *Sma*I resistant and may be said to be hypermethylated.

Transfection of methylated MuLV proviral DNAs. The DNAs of MuLV-producing cells may be used to transfer the virus-positive phenotype to an NIH/3T3 mouse cell monolayer by the application of a DNA-calcium phosphate coprecipitate (43, 44). A correlation between provirus expression and the infectivity of proviral DNA has been demonstrated in the case of the endogenous and exogenous AKR-MuLV proviruses described previously. Copeland and Cooper (14) have reported that DNA from virus-negative AKR embryo cells containing only the endogenous AKV-MuLV proviruses does not have biological activity upon transfection. In contrast, infectious DNA is obtained from virus-positive AKR-MuLV-infected cell lines containing additional exogenous AKR-MuLV proviruses. We confirmed these reported differences, and our results suggest that the correlation which we observed between the methylation state of a provirus and its expression might also be extended to include the infectivity of the proviral DNA.

The DNAs of all of the M-MuLV-producing cell lines which we have tested are active upon transfection, including all of those used in the experiment shown Fig. 5 (data not shown). This includes NRK5 DNA, which has a specific infectivity in the range of 0.5 to 2.0 PFU/μg. Since three of the M-MuLV proviruses in NRK5 DNA are resistant to *Sma*I digestion and appear to be hypermethylated, we sought to determine the extent to which the biological activity of NRK5 DNA is reduced by *Sma*I digestion. We found that we could obtain no infectivity after NRK5 DNA was digested to completion with *Sma*I, even though the three *Sma*I-resistant proviruses remained intact between their respective flanking *Eco*RI sites. We concluded from the data in Table 1 that the hypermethylated M-MuLV proviruses contain less than 2% of the infectivity in NRK5 DNA.

Although these data from transfection of NRK5 DNA were consistent with the hypothesis that hypermethylation exerts a negative effect on proviral DNA infectivity and expression, we had not yet determined whether any of the three hypermethylated proviruses in NRK5 cells contained complete and potentially active M-MuLV viral genomes. This issue was addressed by molecular cloning of several of these proviruses.

Cloning of M-MuLV proviral DNAs from NRK5 cells. A library of 2×10^6 recombinant Charon 4A bacteriophage was prepared from

TABLE 1. *Eco*RI and *Sma*I sensitivities of NRK5 DNA infectivity[a]

Enzyme digest	PFU obtained in transfection experiment:			Specific infectivity (PFU/μg)
	I	II	III	
None	19	12	20	0.6
*Eco*RI	13	17	ND[b]	0.5
*Eco*RI + *Sma*I	0	0	0	<0.02

[a] Each transfection experiment was performed with 30 μg of DNA. The specific infectivity for the *Eco*RI + *Sma*I double digest was calculated by dividing 1 PFU by the total amount of digested DNA transfected.

[b] ND, Not done.

gel-enriched *Eco*RI-digested NRK5 DNA and screened with an M-MuLV viral DNA probe as described above. The analysis of the NRK5 DNA prepared by this size selection procedure is shown in Fig. 6, lane b. Each of the six M-MuLV proviruses from NRK5 cells appeared to have been recovered with approximately equal yield. Six recombinant proviral clones were isolated from the screenings and were verified by their restriction endonuclease maps to contain integrated M-MuLV genomes (Fig. 7). Although each of the recombinant bacteriophage plaques was believed to be generated independently, the proviral DNA insertions were of only two sorts; i.e., three of the clones contained a proviral DNA insertion of 10.9 kb with the restriction endonuclease map shown in Fig. 7a, and the other three clones contained a proviral DNA insertion of 17.2 kb with the restriction map shown in Fig. 7b. Both groups contained the proviral DNA insertion in both orientations relative to the Charon 4A vector arms. Thus, these six proviral DNA clones appeared to represent only two of the six M-MuLV proviruses in NRK5 DNA, and we had obtained no recombinant DNA clones for any of the remaining four proviruses. A similar difficulty in obtaining molecular clones for some M-MuLV proviruses has been described by others (1), who also used the Charon 4A vector system.

To associate the cloned M-MuLV proviral DNAs with their respective proviruses in the NRK5 genomic DNA, a small amount (3 to 5 pg) of *Eco*RI-digested DNA from the 10.9-kb clone λ21 was mixed with *Eco*RI-digested DNA from uninfected NRK cells and loaded onto an agarose gel for electrophoresis. An equal amount of *Eco*RI-digested NRK5 DNA was loaded onto the same gel in an adjoining lane. When these two lanes were blotted with an M-MuLV viral DNA probe, the 10.9-kb insertion was found to align with the smallest of the six M-MuLV proviruses in NRK5 DNA (Fig. 6, lanes c and d). In a similar manner, the 17.2-kb cloned proviral DNA insertion aligned with the third largest M-MuLV provirus in NRK5 DNA (Fig. 6, lanes e and f).

We designated the M-MuLV proviral DNA clones on the basis of these associations; hence, the recombinant DNA clones containing the 10.9-kb insertion (e.g., λ21) were designated the provirus F clone (λF), and the recombinant DNA clones containing the 17.2-kb insertion (e.g., λ836) were designated the provirus C clone (λC). The cloned proviral DNA insertions from Charon 4A recombinant bacteriophages λ21 and λ836 were subsequently transferred into the plasmid cloning vector pBR322 at the *Eco*RI site to generate plasmids pF and pC, respectively. The recombinant plasmids were used to complete the derivation of the restriction endonuclease maps shown in Fig. 7.

Transfection of cloned M-MuLV proviral DNAs. The Charon 4A recombinant proviral DNA clones and pBR322 recombinant proviral DNA clones were tested for biological activity upon transfection. As shown in Table 2, the three independent lambda phage clones and the single derived plasmid containing provirus F were all unable to induce XC-positive plaques after DNA transfection of NIH/3T3 cells. In contrast, each of the three independent recombinant lambda phage clones and the single derived

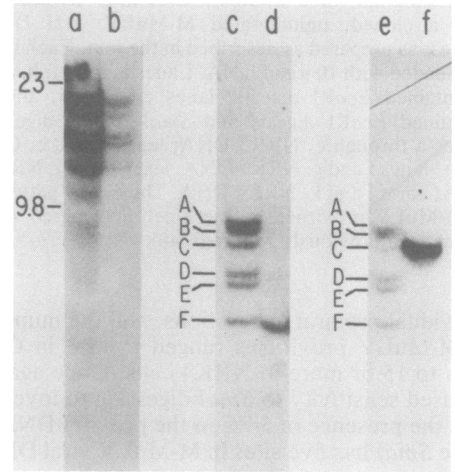

FIG. 6. Association of cloned M-MuLV proviral DNAs with proviruses in the NRK5 cell line. Autoradiographs of three separate Southern blot filters hybridized with a cloned, unintegrated M-MuLV viral DNA probe are shown. All lanes contained *Eco*RI digests. Lanes a, c, and e, 10 μg of NKR5 DNA; lane b, size-selected NRK5 DNA eluted from an agarose gel; lane d, 10 μg of NRK DNA carrier plus 3 to 5 pg of *Eco*RI-digested λ21 DNA; lane f, 10 μg of NRK DNA carrier plus 3 to 5 pg of *Eco*RI-digested λ836 DNA.

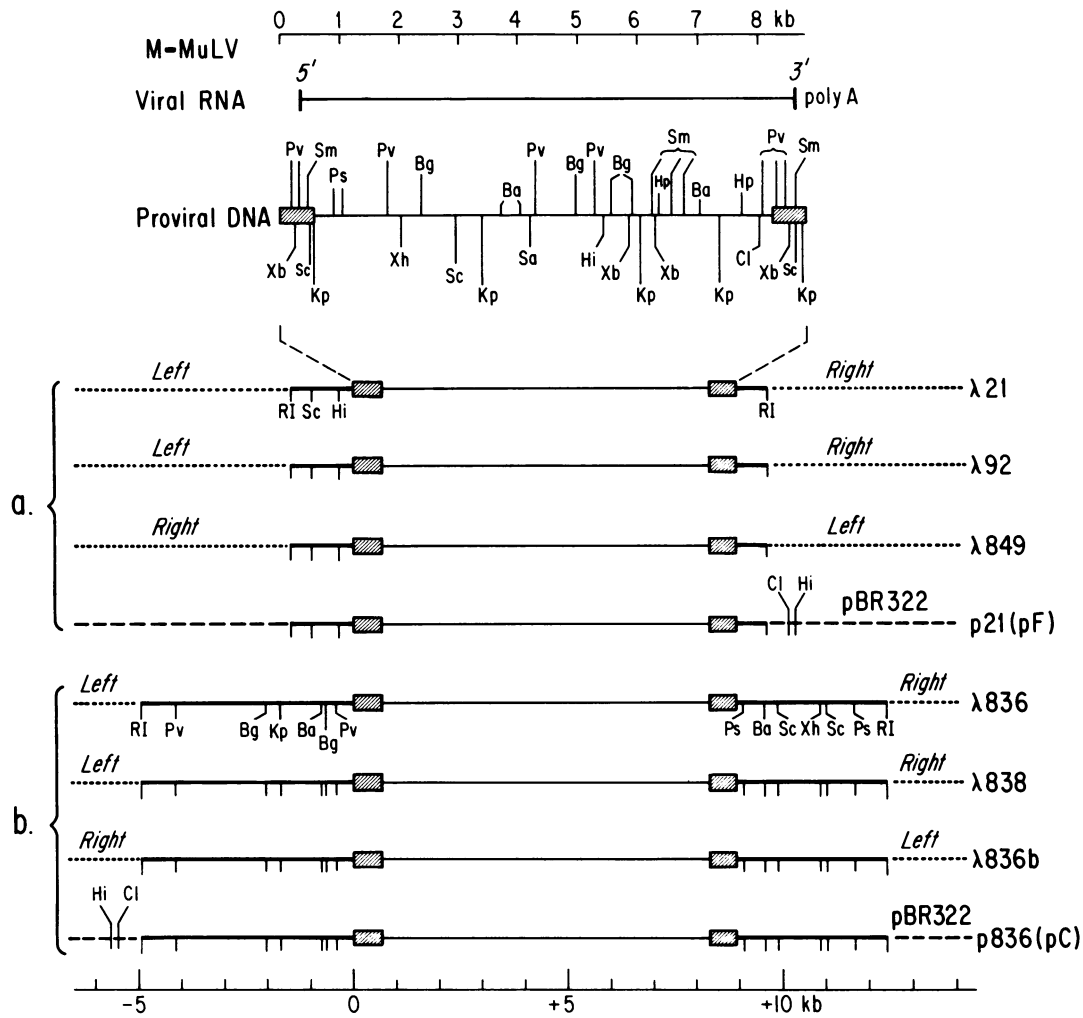

FIG. 7. Physical maps of the cloned M-MuLV proviral DNAs. Abbreviations: Ba, *Bam*HI; Bg, *Bgl*II; Cl, *Cla*I; RI, *Eco*RI; Hi, *Hin*dIII; Hp, *Hpa*I; Kp, *Kpn*I; Ps, *Pst*I; Pv, *Pvu*II; Sa, *Sal*I; Sc, *Sac*I; Sm, *Sma*I; Xb, *Xba*I; Xh, *Xho*I; poly A, polyadenylic acid.

plasmid clone containing provirus C were highly active upon transfection. A dilution experiment performed with clone λ836 DNA demonstrated that cloned provirus C DNA was active with single-hit kinetics and that the specific DNA infectivity was approximately 12 PFU/ng (data not shown).

Because we believed from our earlier experiments that the hypermethylated proviruses of NRK5 cells, including provirus C, did not have infectious DNA, we sought to reconcile this with the infectivity of cloned provirus C DNA. The occurrence of infectious provirus C clones demonstrated that at least one of the three hypermethylated proviruses contains complete and nondefective M-MuLV viral DNA sequences. The most obvious and perhaps only differences between the proviral DNA sequences in NRK5 DNA and the cloned proviral DNA sequences are the differences in secondary DNA modifications, particularly DNA methylation. The 5-^{m}C bases in the rat cellular DNA are replaced by unmethylated cytosine residues as a result of the molecular cloning procedures. We speculate that the resulting demethylation of the provirus was the likely cause of the increased infectivity of provirus C clone DNA.

In vitro methylation of infectious cloned proviral DNA. To explore further the effect of 5-^{m}C bases on the DNA infectivity of M-MuLV proviral genomes, the infectious cloned proviral DNA was methylated with bacterial methylases

TABLE 2. Transfection of cloned M-MuLV proviral DNAs[a]

Recombinant DNA clone	Provirus from which clone was derived	Amt of DNA (ng)	Total PFU	PFU/ng
836	C	20	250	12.5
p836 (pC)	C	10	162	16.2
836b	C	25	196	7.8
838	C	25	225	9.0
21	F	500,50,5	0	<0.002
p21 (pF)	F	200	0	<0.005
92	F	200	0	<0.005
849	F	500	0	<0.002

[a] Cloned DNAs were tested for biological activity by using the UV-XC plaque assay on transfected NIH/3T3 cell monolayers. λ836, 836b, and 838 are chimeras of Charon 4A and provirus C. p836 is a plasmid containing the inserted sequences of lambda phage 836. Similarly, 21, 92, and 849 represent independent lambda phage strains carrying provirus F. p21 is a plasmid containing the inserted sequences of lambda phage 21.

*MHpa*II and *MHha*I; these enzymes methylate the internal cytosine bases of the tetranucleotide sequences 5'-CCGG-3' and 5'-GCGC-3', respectively. By titration, we determined for each enzyme a minimum concentration of the methylation reaction which left the methylated cloned proviral DNA resistant to any detectable cleavage by the corresponding restriction endonuclease (*Hpa*II or *Hha*I) (data not shown). The infectious cloned proviral DNA was then methylated with 4 times the minimum concentration of *Hpa*II or *Hha*I or 20 times the minimum concentration of *Hpa*II. The completeness of methylation was ensured by subsequent challenge by endonucleases *Hpa*II and *Hha*I (Table 3). The biological activity of the in vitro methylated DNA was then tested by transfection (Table 3). We found only a slight, but reproducible, reduction in the DNA infectivity of the provirus C clone after each of the in vitro methylations which we used. Therefore, methylation of DNA per se does not greatly suppress the infectivity of M-MuLV DNA; rather, the presence of methyl groups at critical sites in the provirus seems to be an important determinant of transcriptional control. These critical sites were apparently not altered by treatment with the two methylases used.

In vitro recombination of cloned M-MuLV proviral DNAs. Although the NRK5 cells from which the proviruses were cloned released competent M-MuLV particles, neither of the isolated molecular clones could be derived from the proviruses responsible for this virus production. As discussed above, provirus C is apparently silent in these cells by virtue of imposed methyl groups. The second provirus, provirus F, also appears not to be active in specifying infectious particles, a conclusion which we draw from the lack of infectivity of clones pF and λF. We sought to investigate the possibility that the defect in this provirus might be located in other types of control elements, including those which might reside in the flanking rat cellular sequences. To study this possibility, restriction endonuclease fragments containing 5' M-MuLV and 3' M-MuLV sequences of the infectious and noninfectious cloned proviral DNAs were ligated pairwise in a manner simulating genetic recombination. The four enzymes used were *Xho*I, *Sal*I, *Hin*dIII, and *Cla*I, which have single cleavage sites in M-MuLV viral DNA conveniently spaced at 2.0, 3.85, 5.4, and 8.05 kb, respectively, from the left end of the viral genome (Fig. 7). The results of this analysis (Fig. 8) placed the defect for DNA infectivity of the provirus F clone entirely within the structure encoding sequences on the 3' side of the *Xho*I site and to the 5' side of the *Hin*dIII site. The absence of DNA infectivity in either of the recombinant proviral DNAs resulting from recombination at the *Sal*I site suggests that mutations are present in the provirus F clone on both sides of the site. Thus, the lesion in this provirus is not associated in any apparent way with sequences that regulate provirus expression; rather, the lesions appear to be in structure-encoding sequences.

TABLE 3. Transfection of in vitro methylated cloned proviral DNA[a]

Methylase used	Concn (U/μg)	Amt of methylated 836 DNA transfected (ng)	Total PFU	Fraction of control
None	0	20	250	1.0
*MHpa*II	10	20	200	0.8
*MHpa*II	10	2	22	0.8
*MHpa*II	50	20	170	0.7
*MHha*I	15	20	200	0.8
*MHha*I	15	2	28	0.9
*MHpa*II (*Hpa*II cut)	10	10	82	0.7
*MHha*I (*Hha*I cut)	15	10	90	0.7

[a] One unit of methylase was the amount of enzyme required to incorporate 1 pmol of methyl groups from *S*-adenosylmethionine per h into DNA; 2.5 U of *MHpa*II per μg of DNA was the minimum amount of enzyme required to methylate all of the *Hpa*II sites of λ836, and 3.75 U of *MHha*I per μg of DNA was the minimum amount of enzyme required to methylate all of the *Hha*I sites of λ836. *Hpa*II cut and *Hha*I cut refer to experiments in which the in vitro methylated DNA was digested with an excess of the corresponding restriction endonuclease before transfection.

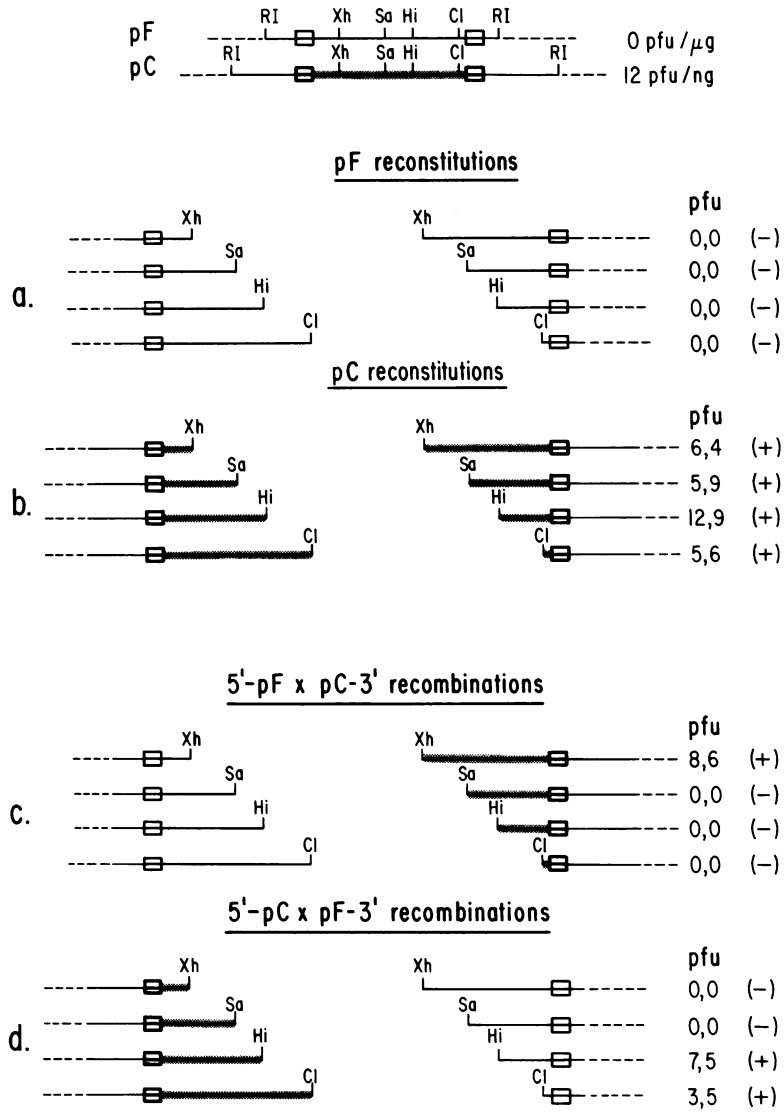

FIG. 8. In vitro recombination of cloned M-MuLV DNAs: results of transfections performed with in vitro recombined pF and pC DNAs. The numbers of plaque-forming units are the total numbers of XC plaques obtained from 1 μg of DNA gel eluted as 5' and 3' ends of the cloned proviral DNAs and ligated. Although other arrangements of the ligated restriction fragments were generated by this method, only those structures producing complete, potentially infectious proviral genomes are shown. Abbreviations: Cl, *Cla*I; RI, *Eco*RI; Hi, *Hin*dIII; Sa, *Sal*I; Xh, *Xho*I.

DISCUSSION

Models involving 5-^{m}C in the regulation of vertebrate gene expression are attractive for several reasons. Modification by methylation of the DNA double helix at particular sequences is a plausible and sufficiently precise means for controlling transcription. This could be achieved either by directly affecting critical nucleic acid-protein interactions or by indirectly altering the local structure of chromatin. Furthermore, the methylation pattern is generally replicated in a semiconservative manner during cell division (34), so that each daughter cell could receive an inherited set of transcriptionally active and inactive genes. Perpetuation of the methylation pattern is believed to be accomplished by the activity of a maintenance DNA methylase, which rapidly methylates the newly synthesized DNA strand at sites corresponding to 5-^{m}C residues in the parent strand. Therefore, the

methylase is said to act on half-methylated sites and is not believed to methylate completely unmethylated sites (34). Occasional demethylations, whether programmed or random, would be expected to be inherited stably. Since sperm DNA is known to be much more extensively methylated than DNA from somatic tissues (28, 53), the idea has developed that the programmed demethylation of individual genes could be included among the molecular genetic events which direct many developmental and differentiation processes (23, 36).

A key experimental point relating to these models is the effect on gene expression of 5-^{m}C bases in the DNA sequence. When restriction endonucleases sensitive to differences between methylated and unmethylated DNAs are used to map 5-^{m}C residues in particular genes, the presence of methylated cytidine nucleotides has usually been correlated with transcriptional repression.

We have observed that the endogenous, vertically transmitted AKR-MuLV proviruses in AKR embryo cells are hypermethylated relative to the proviruses acquired by exogenous infection. A similar result has been reported by Cohen (12) for vertically transmitted mouse mammary tumor virus proviral sequences, which are hypermethylated relative to mouse mammary tumor virus proviruses acquired by milk-borne infection. In both cases, cells containing only the endogenous proviruses are negative for virus expression, whereas exogenously acquired proviruses are expressed. Other endogenous proviruses whose expression is known to be suppressed have recently been found to be hypermethylated. These include the endogenous chicken provirus specified by the *ev*-1 locus (21), as well as several germ line integrations of M-MuLV obtained by infection of preimplantation mouse embryos (49). If DNA methylation is a means by which suppression of certain genes is maintained during embryogenesis, we surmise that the methylation of endogenous proviruses may be a means of avoiding the inappropriate or pathogenic release of viral gene products in developing embryos.

AKR embryo cells and other cells containing endogenous murine leukemia proviruses spontaneously begin expressing virus at a very low frequency. Treatment of these cells in culture with 5-bromodeoxyuridine or 5-iododeoxyuridine induces a larger fraction (up to 5%) to express virus (27). Induction by 5-bromodeoxyuridine and 5-iododeoxyuridine appears to involve transcriptional derepression of endogenous proviral sequences (6) and may require the incorporation of the drug into newly synthesized DNA (52). 5-Bromodeoxyuridine and 5-iododeoxyuridine are halogenated at the same position of the pyrimidine ring which is methylated in 5-^{m}C. We speculate that induction by these drugs may be related to altering recognition of the methylation state of the endogenous provirus (J. Hoffmann, Ph.D. thesis, Massachusetts Institute of Technology, Cambridge, 1981).

5-ACR is another cytidine analog which can be incorporated into DNA. 5-ACR is believed to block methylation at the site of incorporation as a result of the presence of nitrogen in place of carbon at the fifth position of the pyrimidine ring. Treatment of mouse cells with 5-ACR produces hypomethylation of genomic DNA (25). Hypomethylation produced by treatment of cells with 5-ACR has been associated with the appearance of new differentiated phenotypes in mouse cell cultures (25, 51) and with the reactivation of genes on an inactive human X chromosome (29).

We have found 5-ACR to be a potent inducer of AKR-MuLV from AKR embryo cells. The kinetics of induction are similar to those obtained with halogenated pyrimidines, but a larger fraction of cells is induced, suggesting that 5-ACR is a more effective inducer. We believe that the induction of the endogenous AKV-MuLV proviruses strongly supports a causal connection between DNA methylation and provirus expression. Recently, we have learned that induction with 5-ACR of endogenous murine leukemia proviruses has also been demonstrated by Niwa and Sugahara (31).

Although maintenance DNA methylases of vertebrates methylate cytosine residues at hemimethylated sites during cell division, the de novo DNA methylation of unmethylated sites seems to be quite rare (34). We suppose that the methylation of endogenous retroviral DNA sequences represents such de novo methylation, since the germ line infections which presumably gave rise to the endogenous proviruses originated with unmethylated reverse transcribed viral DNA. The earliest germ line insertion producing an endogenous AKV-MuLV provirus precedes the development of the AKR mouse strain. However, additional germ line insertions, which produce endogenous AKV-MuLV proviruses at new integration sites, are occurring continuously at a low incidence (39). The experimental insertions of M-MuLV into the germ line of BALB/Mo mouse strains by Jaenisch et al. (10, 24) provide further examples of de novo methylation of endogenous proviruses.

We appear to have discovered evidence for de novo methylation of retroviral DNA in somatic cell culture. The M-MuLV-infected NRK5 cells contain three hypermethylated M-MuLV proviruses and three hypomethylated M-MuLV proviruses, as determined by the sensitivity of the proviral DNAs to digestion with *Sma*I. Genomic

DNAs from single-cell subclones of NRK5 cells obtained over a period of 2 years showed no alteration in the *Eco*RI pattern of the M-MuLV proviruses or their respective methylation states (data not shown). This suggests that de novo methylation of proviral DNA probably occurred in the NRK5 parent cell shortly after infection.

Whereas three of the six M-MuLV proviruses in NRK5 DNA are hypermethylated, we detected no *Sma*I-resistant proviral DNAs among more than 30 other M-MuLV proviruses in four other clones of M-MuLV-infected NRK cells. This unusual nature of the NRK5 cells remains unexplained. Since the chromosomal locations of the M-MuLV proviruses in NRK5 cells are not known, the possibility of linkage among the three hypermethylated proviruses cannot be excluded, but this seems unlikely. One possibility is that the production of hypermethylated proviruses by exogenous infection is dependent on the timing during the cell cycle when integration occurs.

We find all of the detectable DNA infectivity of the M-MuLV proviruses in NRK5 DNA associated with hypomethylated proviruses. If we assume the correlation of DNA infectivity and virus expression obtained with AKR-MuLV proviruses (14), our results suggest that the hypermethylated proviruses in NRK5 DNA are transcriptionally inactive.

There is evidence that the chromatin structure of transcriptionally inactive genes may be more resistant to the endonucleolytic activity of DNase I than the chromatin structure of transcriptionally active genes (57). Breindl et al. (10) studied the DNase I sensitivity of endogenous M-MuLV proviral sequences of BALB/Mo mice in embryonic tissues and somatically acquired M-MuLV proviral sequences in lymphatic target tissues. These authors reported that the expression of these M-MuLV proviral sequences is correlated with their preferential DNase I digestibility. We have made preliminary observations which indicate that the three hypermethylated M-MuLV proviruses in NRK5 DNA are all relatively DNase I resistant compared with the hypomethylated M-MuLV proviruses which coexist in the common intracellular environment. Thus, this finding is in agreement with the recent report of Groudine et al. (21), who found that the transcriptionally active endogenous avian retroviral gene sequences at the *ev*-3 locus are preferentially DNase I sensitive compared with the inactive *ev*-1 locus. These authors also found that transient exposure of *ev*-1-containing cells to 5-ACR resulted in the hypomethylation and transcriptional activation of *ev*-1, as well as the acquisition of a new DNase I-hypersensitive site in the *ev*-1 chromatin.

Perhaps the most direct indication of the role of methylation comes from the cloned proviral DNAs. Both of the proviruses represented in clones appear to be complete, in that they contain direct terminal repeats (11) and the proper array of restriction endonuclease sites (18). Moreover, in agreement with other workers (30, 32), we found no similarity in the physical maps of the cellular sequences adjacent to the two proviruses, a result confirmed by direct sequencing of the flanking rat DNA (42). Of the two proviruses represented in clones, one was infectious and the other was noninfectious. This result agreed with similar findings with provirus clones of spleen necrosis virus (32), feline leukemia virus (30), and AKR-MuLV (26). Our finding of an infectious clone of provirus C was especially useful for the present analysis, since it showed that the act of cloning converted a previous noninfectious provirus into a provirus that was biologically highly active. We believe that the essential difference between the uncloned and cloned proviruses is the array of methyl groups lost during the passage of cloned DNA through bacteria.

An analysis of the defects of the noninfectious clone carrying provirus F yielded results that were not germane to regulation of expression. Using an in vitro recombination technique similar to that used by other workers (5), we found that this provirus contains two apparent point mutations, each capable of inactivating the provirus. These mutations lie in the region encoding the *gag-pol* precursor polyprotein.

In vitro methylation of the infectious cloned proviral DNA with bacterial methylases only slightly reduces its activity upon transfection. The two bacterial methylases used in our study methylate only 2 of the 16 possible 5′-CpG-3′ dinucleotides and none of the 5′-CpC-3′ dinucleotides. Other investigators have reported variations in the amount of reduction of biological activity upon DNA transfer of cloned herpesvirus (33) and chicken (58) thymidine kinase genes after in vitro methylation with a bacterial methylase. The perpetuation of the methylation state after introduction of the in vitro methylated DNA into the recipient cells has been recognized as one significant variable in these experiments (58). Long-term perpetuation of methyl groups is likely not relevant to the present experiments since the plaque assay used reflects a biological activity of the transfected DNA which is expressed in the first 1 or 2 days after transfection. We conclude that the inability to inactivate provirus C by in vitro methylation is due to the fact that the methylases used did not affect critical control sequences in the proviral DNA. The sites that we did methylate can apparently be so modified without having any effect on provirus expression.

After completion of the manuscript for this paper, a report whose results parallel closely those described here appeared. In this report, Harbers et al. (22) show that the normally noninfectious DNA of an endogenous M-MuLV provirus becomes infectious upon molecular cloning. Our results are in good agreement with this finding.

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