

DNA methylation and gene expression: Endogenous retroviral genome becomes infectious after molecular cloning

(germ-line integration of Moloney murine leukemia virus/virus expression *in vivo*/removal of methyl groups/infectivity)

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ABSTRACT The Mov-3 substrain of mice carries Moloney murine leukemia virus as a Mendelian gene in its germ line. All mice segregating the *Mov-3* locus activate virus and develop viremia and leukemia. The integrated provirus (i.e., *Mov-3* locus) was molecularly cloned from *Mov-3* liver DNA as a 16.8 kilobase long *EcoRI* fragment. Comparison of the cloned and genomic *Mov-3* specific *EcoRI* fragment by restriction enzyme analysis showed no differences in the size of the fragments, indicating that no major sequence rearrangements occurred during cloning. The genomic and cloned *Mov-3* DNAs were compared for methylation and infectivity. Analysis with *Hha* I showed that the genomic proviral and the flanking mouse sequences were methylated at cytosine residues, in contrast to the cloned *Mov-3* locus. The cloned *Mov-3* locus, however, was highly infectious in a transfection assay (1×10^{-5} plaque-forming unit per viral genome) in contrast to the genomic *Mov-3* DNA ($<10^{-7}$ per viral genome). Our results suggest that genes containing 5-methylcytosine are not expressed after transfection into susceptible cells and that removal of the methyl groups by molecular cloning in prokaryotes leads to expression generating infectious proviral DNA. If gene expression of transfected DNA is controlled by mechanisms that are relevant for gene expression in the animal, this suggests that DNA methylation may play a causative role in eukaryotic gene regulation.

Evidence obtained in several experimental systems suggests that various molecular mechanisms may be involved in gene control of eukaryotic cells (for review, see ref. 1). These mechanisms include gene amplification (2–4), gene rearrangement (5), and gene modification. Methylation of cytosine at the 5 position is the major way by which eukaryotic DNA is modified (6). Recently, restriction enzyme analysis has been used to correlate the extent of methylation of developmentally regulated genes to gene expression. These studies suggested an inverse relationship between gene methylation and gene expression (7–10).

Our approach to study gene regulation involved in development has been to introduce retrovirus genomes into the germ line of mice, thus deriving substrains that transmit the viral information as new Mendelian genes (11–13). Endogenous retroviruses are stable genetic elements and can serve as models for the study of the regulation of normal cellular genes. Substrains of mice, designated *Mov-1* to *Mov-13*, have been described, each carrying the Moloney murine leukemia virus (M-MuLV) genome in its germ line at a different chromosomal position (13). The phenotypes of these mice differ. In the *Mov-3* substrain, which was used for the studies presented here, virus is expressed prior to birth. All *Mov-3* animals develop viremia and, subsequently, leukemia.

To study the structure of the inserted provirus in *Mov-3* animals, the virus genome and its flanking mouse sequences were molecularly cloned. The cloned DNA is highly infectious in a transfection assay, whereas the genomic *Mov-3* DNA is not infectious. The only difference between the cloned and genomic *Mov-3* DNAs appears to be the presence of 5-methylcytosine in the latter.

MATERIALS AND METHODS

Mice. All mice were bred in our colony at the Heinrich-Pette-Institut. Derivation of the substrain of mice that carries the M-MuLV genome at the *Mov-3* locus has been described (12).

Enzymes. Restriction enzymes were obtained from New England BioLabs or Boehringer Mannheim, and reaction conditions used were those recommended by the suppliers. Phage T4 ligase was purchased from New England BioLabs. *Escherichia coli* DNA polymerase I was obtained from Boehringer Mannheim.

Isolation of DNA and Enrichment of M-MuLV Proviral Sequences. High molecular weight DNA was extracted from the livers of 2-month-old *Mov-3* mice as described (14). DNA was digested with *EcoRI*, extracted with phenol, and precipitated with ethanol. Five milligrams of *EcoRI*-digested DNA was fractionated on a 0.5% preparative agarose gel as described (15). Aliquots of the fractionated DNAs were electrophoresed, transferred to nitrocellulose filters according to the method of Southern (16), and hybridized to a ^{32}P -labeled cDNA that was specific for M-MuLV (14). Fractions that hybridized to the M-MuLV cDNA were used for molecular cloning.

Molecular Cloning. The EK2 certified vector λ Charon 4A and host *E. coli* DP50 supF and *E. coli* strain LE392 were obtained from F. Blattner. Lysogenes used for preparation of packaging mixtures, BHB 2688 and BHB 2690, were generously provided by B. Hohn. Plasmid pBR322 was provided by H. Boyer, and *E. coli* χ 1776, by R. Curtiss. Purification of λ 4A arms was performed after digestion with *EcoRI* as described (17) and used for ligation and packaging. Partially purified proviral DNA and vector DNA were adjusted to 36 and 120 $\mu\text{g}/\text{ml}$, respectively, and incubated with T4 DNA ligase for 24–36 hr at 8°C.

The resultant recombinant DNA was packaged *in vitro* into phage particles (18) and plated onto *E. coli* DP50 supF in a 20 \times 20 cm plastic dish (Nunc) so that 50,000–100,000 plaques would arise. Nitrocellulose replicas of the plastic dish were prepared (19) and hybridized with ^{32}P -labeled representative M-MuLV cDNA. Plaques containing recombinant phage were located by autoradiography. Samples (2 ml) of phage lysates were prepared from the unpurified plaques and the recombinant phage DNA was analyzed by restriction enzyme analysis and

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Abbreviations: M-MuLV, Moloney murine leukemia virus; kb, kilobase(s).

by filter hybridization (16). Plaques that contained M-MuLV were further purified by additional cycles of plaque purification until the number of recombinant phage was $>95\%$. A final plaque was then picked and used to prepare the recombinant phage DNA. The M-MuLV proviral DNA insert was recloned into the *EcoRI* site of plasmid pBR322 and grown in *E. coli* χ 1776. Subfragments of the proviral DNA and its flanking mouse sequences were subcloned in pBR322 and amplified in *E. coli* HB101. Construction and growth of recombinant plasmids and phages were conducted under L2/B2 conditions as specified by the Zentrale Kommission für Biologische Sicherheit of the Federal Republic of Germany.

Transfection Assays. Infectivity of DNA was tested by a modification (20) of the calcium phosphate coprecipitation method of Graham and van der Eb (21). Thirty micrograms of high molecular weight DNA from liver of BALB/c mice was used as carrier for transfection of NIH 3T3 cells with cloned DNA. Infectious centers were scored by the XC plaque assay (22) 1 week after transfection.

RESULTS

Cloning of a Cellular DNA Fragment Containing Integrated M-MuLV. The integrated M-MuLV genome of Mov-3 mice had previously been mapped by restriction enzyme analysis on an 18-kilobase (kb)-long *EcoRI* fragment (12). In order to enrich for fragments containing proviral DNA, *EcoRI*-digested liver DNA was fractionated on a preparative agarose gel. The fractions containing the provirus were identified by hybridization with a probe specific for M-MuLV. We estimate that a 20- to 30-fold enrichment of DNA was achieved by this procedure.

The enriched proviral DNA fragments were ligated to the arms of λ 4A, packaged *in vitro*, and plated on *E. coli* DP50 supF. In a typical experiment, about 100,000 plaques were present on one agar plate. About 40 plaques hybridized with a representative M-MuLV cDNA. Ten of these clones were picked, and hybrid phage DNA was prepared from small lysates and analyzed by digestion with *Sac I*. DNA from seven plaques contained the 2.6- and 5.6-kb *Sac I* fragments characteristic of

the M-MuLV genome (13, 23). The result indicated that about 70% of the plaques that hybridized with a representative M-MuLV probe contained hybrid phage DNA with a complete M-MuLV genome. We assume that the remaining 30% contained DNA from other endogenous viruses that have sequence homologies with M-MuLV. DNA was isolated from one of the purified plaques, and the DNA insert containing proviral and mouse sequences was separated from the vector arms by *EcoRI* digestion and recloned in the plasmid pBR322. All further experiments described were performed with DNA from the plasmid clone designated "pMov-3".

Restriction Enzyme Analysis of the Recombinant M-MuLV Clone. Fig. 1 shows a restriction endonuclease map of the proviral and flanking mouse DNA of pMov-3. The provirus is flanked at the 5' and 3' ends by 1.8 and 6.3 kb of mouse DNA, respectively. Based on estimated molecular weights of the restriction fragments, the total length of the cloned *EcoRI* fragment is about 16.8 kb, a value slightly smaller than previously reported (12). The restriction enzyme sites within the proviral DNA are in agreement with previously published maps (12, 23).

The structures of the M-MuLV-specific *EcoRI* fragment from liver DNA of Mov-3 mice (genomic *Mov-3* DNA) and the cloned *EcoRI* fragment of pMov-3 were compared by restriction analysis with *Pst I*, *BamHI*, and *HindIII*. The restriction fragments obtained from the genomic DNA and the cloned DNA were identical in length (Fig. 2), indicating that the structure of the genomic *EcoRI* fragment had not been grossly rearranged during cloning.

To show further that pMov-3 was derived from the DNA corresponding to the *Mov-3* locus, a ^{32}P -labeled probe was prepared by nick-translation from the *EcoRI*-*Pst I* cellular fragment at the 5' end of pMov-3. This probe was hybridized to Southern blots of *EcoRI*-digested DNA from Mov-3 mice and ICR mice from which the Mov-3 substrain originally had been derived (12). *EcoRI*-digested DNAs from C57BL, 129, BALB/c, Mov-1, and Mov-2 mice were also included in the analysis. The results are shown in Fig. 3. The probe hybridized predominantly with an 8-kb fragment present in ICR mouse DNA (lane c). The

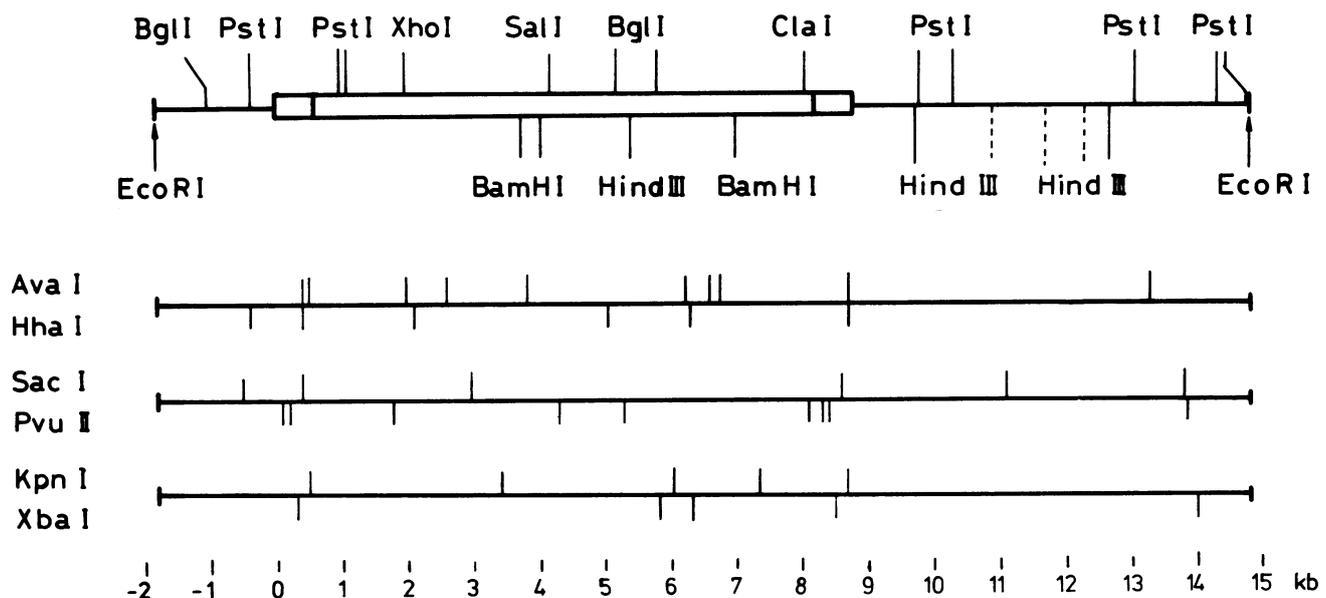


FIG. 1. Restriction enzyme map of the cloned *Mov-3* locus. The M-MuLV provirus spanning from 0 to 8.9 kb on the map is indicated by a double line. Adjacent cell sequences are indicated by a single line. The map was derived by cleavage of the cloned DNA with various restriction enzymes (singly or in combinations) followed by agarose gel electrophoresis. The total fragment patterns were determined by ethidium bromide staining and those that contained viral sequences were identified by blot-transfer hybridization with M-MuLV cDNA. Additional *Hha I* cleavage sites, which have not been accurately mapped, are present in the region between 0.4 and 2.1 kb.

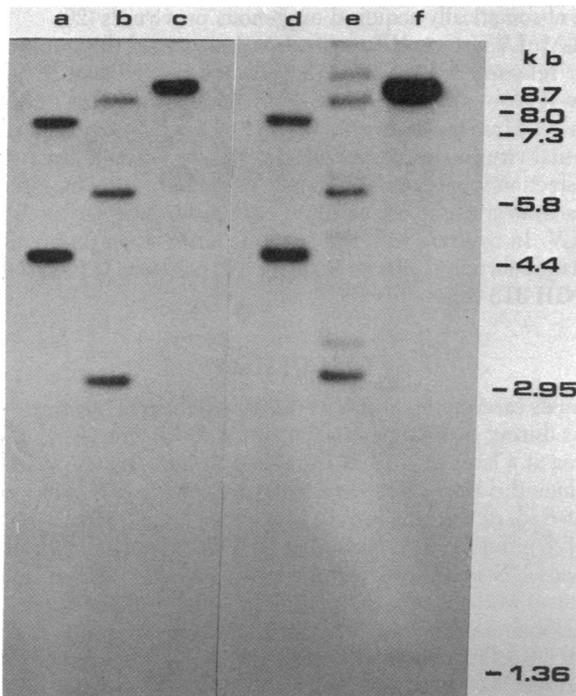


FIG. 2. Comparison of the cloned and genomic *Mov-3* locus DNAs by Southern blot analysis. The cloned *EcoRI* fragment of pMov-3 was cleaved with *HindIII* (lane a), *BamHI* (lane b), and *Pst I* (lane c). An enriched fraction containing the genomic M-MuLV-specific *EcoRI* fragment of DNA from the livers of *Mov-3* mice was digested with the same enzymes: *HindIII* (lane d), *BamHI* (lane e), and *Pst I* (lane f). After electrophoresis on 0.8% agarose gel, the DNA was transferred to a nitrocellulose filter and hybridized to a ^{32}P -labeled cDNA that was specific for M-MuLV (14). The *BamHI* digest of the genomic fraction (lane e) was not complete, and additional fragments resulting from partial digestion are visible. *HindIII* and *EcoRI* fragments of λ wt DNA were used as length markers (24).

intensity of hybridization to this fragment was decreased by about 50% in *Mov-3* DNA and a new fragment, 16.8 kb, corresponding to the size of the *Mov-3 EcoRI* fragment was detected (lane d). Because the DNA was isolated from an animal heterozygous at the *Mov-3* locus, this result indicates that pMov-3 is derived from the *Mov-3* locus of *Mov-3* mice and that no large deletions in the cellular DNA occurred during virus integration. The same 8-kb-long sequence found in ICR DNA also was present in the DNAs of all other mice included in the analysis. However, M-MuLV had not integrated into the proximity of this or related sequences in *Mov-1* and *Mov-2* mice. Fig. 3 also shows that the 5' flanking mouse sequence of pMov-3 hybridized—although to a lesser extent—to several other *EcoRI* fragments of the mouse DNA. This indicates that sequences closely related to the 5' flanking cellular sequences of the integrated provirus are reiterated many times within the mouse genome.

***Mov-3* Locus in Genomic DNA is Methylated.** As expected for a DNA molecule that has been propagated in bacteria, the cloned *Mov-3* locus is digested by *Hha I*, a restriction enzyme that is inhibited when the internal cytosine residue in the recognition sequence G-C-G-C is methylated (25, 26). Fig. 4B shows an autoradiogram of the fragments obtained after digestion of pMov-3 with *Hha I*. Most of the *Hha I* restriction sites have been mapped and their locations are shown on the restriction map in Fig. 1.

Hha I also was used to study the extent of methylation in genomic *Mov-3* DNA. Total unfractionated liver DNA (the same

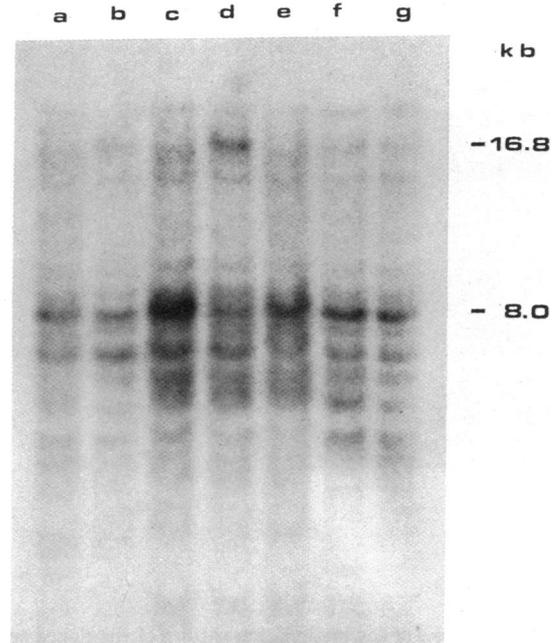


FIG. 3. Identification of the cellular origin of the cloned *EcoRI* fragment of pMov-3. *EcoRI*-cleaved DNAs from different mouse strains were electrophoresed on 0.8% agarose gel, followed by Southern blotting transfer and hybridization to the nick-translated *EcoRI-Pst I* fragment derived from the 5' cellular part of the cloned *Mov-3* DNA. Lanes: a, C57BL; b, 129; c, ICR; d, *Mov-3* substrain; e, *Mov-2* substrain; f, BALB/c; g, *Mov-1* substrain.

as for the molecular cloning) was first digested with *EcoRI* and *HindIII*. Half of the digested DNA was further digested with *Hha I*, and the two DNA digests were coelectrophoresed on an agarose gel. After transfer to a nitrocellulose filter, the DNA fragments were hybridized with a M-MuLV-specific probe. The result is shown in Fig. 4A. Digestion with *EcoRI* and *HindIII* yielded two fragments of 7.3 and 4.4 kb that are characteristic for the *Mov-3* locus (lane b). These two fragments were completely resistant to further digestion with *Hha I* (lanes c and d). Double digestion of pMov-3, however, yielded the expected *Hha I* fragment of 3 kb (lane e; smaller fragments are not seen on this gel). This result clearly indicates that all the *Hha I* sites in the *Mov-3* locus are highly methylated.

Infectivity of Genomic and Cloned M-MuLV *EcoRI* Fragment of *Mov-3* Mice. We examined the biological activity of the cloned M-MuLV *EcoRI* fragment in the DNA transfection assay and compared it with that of the genomic DNA fragment (Table 1). The infectivity was monitored by the XC plaque assay. The cloned fragment was highly infectious with a specific infectivity of about 1×10^{-5} plaque-forming unit per viral genome. No change in infectivity was observed when *EcoRI*-cleaved DNA from liver of *Mov-3* mice (the same DNA used for molecular cloning) was used instead of DNA from BALB/c liver as carrier DNA. The infectivity of the cloned DNA was comparable to the specific infectivity of M-MuLV proviral genomes in NIH 3T3 cells chronically infected with virus. The specific infectivity of uncleaved pMov-3, on the other hand, was found to be about 1/5th of this. The appearance of the XC plaques was indistinguishable from that of plaques induced by M-MuLV. In contrast; when *EcoRI*-cleaved liver DNA from *Mov-3* mice (the same DNA was used for the molecular cloning) was tested in the transfection assay, no XC plaques were found. We conclude that the genomic *EcoRI* fragment is no more than 1/100th as infectious (specific infectivity, $<10^{-7}$ plaque-forming unit per

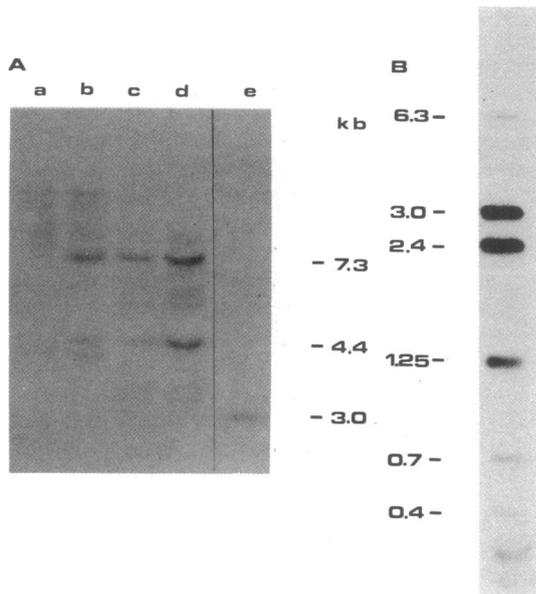


FIG. 4. Analysis of the methylation pattern of the cloned and genomic *Mov-3* locus DNAs by digestion with *Hha* I. (A) Unfractionated high molecular weight DNAs were digested with various enzymes. After electrophoresis on 0.8% agarose gel, the DNA was transferred to a nitrocellulose filter and hybridized to a ^{32}P -labeled cDNA that was specific for M-MuLV (14). The completeness of the *Hha* I digestion was monitored by addition of simian virus 40 DNA. Lanes: a, 15 μg of ICR DNA digested with *Hind*III/*Eco*RI; b, 15 μg of *Mov-3* DNA digested with *Hind*III/*Eco*RI; c, 15 μg of *Mov-3* DNA digested with *Hind*III/*Eco*RI/*Hha* I; d, same as c but with 20 μg of DNA; e, 200 μg of p*Mov-3* digested with *Eco*RI/*Hha* I. (B) p*Mov-3* (200 μg) digested with *Eco*RI/*Hha* I; 1% agarose gel was used for the separation of fragments, and the filter was hybridized with a representative M-MuLV cDNA.

viral genome) as p*Mov-3*. Related studies in our laboratory have demonstrated that occasionally DNA from liver of *Mov-3* mice (3 months and older) shows low infectivity due to the pres-

Table 1. Infectivity of genomic and cloned M-MuLV provirus DNA of *Mov-3* mice

DNA	Infectivity per μg viral DNA $\times 10^{-5}$	Specific infectivity, plaque-forming unit per viral genome $\times 10^5$
Mov-3 mouse liver:		
<i>Eco</i> RI-digested	<0.05	<0.01
p<i>Mov-3</i>:		
Undigested	1.2	0.17
<i>Eco</i> RI-digested + BALB/c carrier DNA	5.7	0.8
<i>Eco</i> RI-digested + <i>Mov-3</i> mouse carrier DNA	5	0.7
Control:		
NIH 3T3 cells productively infected with M-MuLV	6	0.9

Each DNA was tested at least twice in two independent transfection assays. A DNA sample used for transfection contained 30 μg of *Eco*RI-cleaved *Mov-3* liver DNA or 0.5–1 ng of cloned DNA mixed with 30 μg of BALB/c carrier DNA or 30 μg of *Eco*RI-cleaved or high molecular weight DNA from liver of *Mov-3* mice. The specific infectivity of M-MuLV genomes in infected NIH 3T3 cells was calculated from the total number of M-MuLV copies present as determined by quantitative hybridization (14).

ence of somatically acquired exogenous proviruses (20).

M-MuLV virus is NB-tropic. We determined the tropism of virus released from NIH 3T3 cells transfected with *Eco*RI-cleaved p*Mov-3* DNA. As shown in Table 2, the virus released from cells transfected with p*Mov-3* DNA is NB-tropic, as is its parental virus strain. Moreover, the titer of virus released after transfection was of the same order of magnitude as the titer of virus from a fibroblast cell line chronically infected with M-MuLV. In contrast with the N-tropic AKR virus, the number of plaque-forming units on BALB/c 3T3 cells was 1/1000th that on NIH 3T3 cells.

DISCUSSION

Animals carrying M-MuLV at the *Mov-3* locus (12) activate the virus during embryogenesis (20) and develop viremia and leukemia at a later age. DNA from liver of *Mov-3* mice was used to clone the *Mov-3* locus as a 16.8-kb *Eco*RI fragment that contains 8 kb of flanking mouse sequences in addition to the M-MuLV provirus. Comparison of the cloned and genomic *Mov-3* locus DNAs by restriction enzyme analysis revealed no differences in the size of the restriction fragments. Furthermore, restriction enzyme analyses using a radioactive probe derived from the cellular part of the cloned DNA indicated that no major deletions had occurred in the cellular DNA after proviral integration.

A detailed map of the cloned *Mov-3* locus was established by using various restriction enzymes (Fig. 1). Comparison of the cloned and the genomic *Mov-3* locus DNAs using the methylation-sensitive enzyme *Hha* I revealed a distinct difference: the provirus as well as the flanking sequences contained 5-methylcytosine in all recognition sequences of this enzyme (Fig. 4). This is in agreement with other studies from this laboratory showing that proviral genomes integrated into the germ line of other substrains of mice are highly methylated (20).

The cloned DNA was highly infectious, generating about 1×10^{-5} plaque-forming unit per viral genome. The same specific infectivity was observed when DNA from NIH 3T3 cells productively infected with M-MuLV was used for transfection (Table 1). Uncleaved p*Mov-3* had a lower specific infectivity, which is probably due to the circular supercoiled structure. The infectivity of the cloned DNA clearly indicated that the *Eco*RI fragment comprising the *Mov-3* locus carried all the information needed for the expression of virus in mouse fibroblasts. In contrast, *Eco*RI-cleaved genomic DNA from livers of *Mov-3* mice was not infectious (specific infectivity $<10^{-7}$ plaque-forming unit per viral genome). Because the only difference between the genomic and the cloned *Mov-3* proviral DNA appears to be the pattern of DNA methylation, our results suggest that genes with methylation in the 5 position of cytosine cannot be ex-

Table 2. Tropism of virus released from NIH 3T3 cells transfected with p*Mov-3* DNA

Virus	XC plaque-forming units $\times 10^{-6}$ per ml	
	NIH 3T3	BALB/c 3T3
From transfection		
with p <i>Mov-3</i> DNA	5.9	6.9
M-MuLV	4.9	1.3
AKR	2.8	0.027

NIH 3T3 cells were transfected with 10 μg of p*Mov-3* DNA. After five cell passages, the supernatant of the transfected cells was harvested and titered on NIH 3T3 cells ($F_v - 1^{\text{N}}$) or BALB/c 3T3 cells ($F_v - 1^{\text{B}}$) and analyzed by the XC plaque assay. As a control, N-tropic AKR MuLV was tested in parallel.

pressed after transfection into cells. Removal of the methyl groups by molecular cloning, however, permits expression that generates infectious proviral DNA.

It is unlikely that our observations are an artifact of the transfection system selecting against methylated DNA because it has been shown that methylated and unmethylated DNAs are equally well accepted by the recipient cell (27). It is also unlikely that the lack of infectivity of the genomic *Mov-3* DNA is due to material interfering with the transfection assay because the infectivity of cloned DNA is not affected by the presence of high molecular weight or *EcoRI*-cleaved DNA from liver of *Mov-3* mice. However, our results do not definitely rule out other possibilities that might explain the lack of infectivity of genomic *Mov-3* DNA. For example, it is not proven that methylation is the only modification present in eukaryotic DNA. We also cannot exclude the possibility that DNA propagated in *E. coli* becomes modified, rendering the cloned DNA infectious. Related studies analyzing *in vivo* synthesized M-MuLV genomes, however, underline the importance of DNA methylation for infectivity (20).

M-MuLV is activated in every animal carrying the *Mov-3* locus at a defined stage of embryonal development (12, 20). We assume that demethylation of the *Mov-3* locus leads to activation of the viral genome and to subsequent virus spread. Expression may be initiated in a specific population of cells not yet identified. The endogenous proviruses of mice and chickens that have been cloned so far (28, 29) are not expressed *in vivo*, the only exception being a cloned DNA fragment that is part of the *Mov-1* locus of the *Mov-1* mouse substrain (30).

A correlation between gene activity and methylation has been described for a number of viral (8, 30–34) and nonviral genes (7, 9, 10, 35). Our results suggest that an inactive viral genome can be rendered infectious by removal of methyl groups. Assuming that gene activity of transfected DNA is controlled by similar mechanisms as gene activity in the animal, this suggests that DNA methylation may play a causative role in the control of gene expression during development and cellular differentiation.

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