JOURNAL OF VIROLOGY, Jan. 1984, p. 66–71
0022-538X/84/010066-06$02.00/0

Vol. 49, No. 1

Mouse Mammary Tumor Virus: Specific Methylation Patterns of Proviral DNA in Normal Mouse Tissues

WEI-SHAU HU, THOMAS G. FANNING, AND ROBERT D. CARDIFF*

Department of Pathology, School of Medicine, University of California, Davis, California 95616

Received 18 July 1983/Accepted 4 October 1983

The methylation state of endogenous mouse mammary tumor virus (MuMTV) proviral DNA was examined in normal mouse tissues. DNAs from various tissues were cleaved with the methylation-sensitive enzymes *Hha*I and *Hpa*II and analyzed by Southern blotting. Tissue-specific MuMTV proviral DNA methylation patterns were found in the BALB/c, C3H, C57BL, GR/A, and GR-Mtv-2$^-$ mouse strains. MuMTV proviral DNA was hypomethylated in DNAs from the spleens and testes of all strains examined. The GR/A mouse strain, which was most thoroughly studied, also showed hypomethylation of MuMTV proviral DNA in bone marrow and placental tissues. Analysis of RNAs extracted from GR/A liver, mammary tumor, testes, placenta, and spleen tissues demonstrated that MuMTV proviral hypomethylation need not reflect significant proviral transcription.

The timing and extent of gene expression in eucaryotes may be controlled, at least in some degree, by the presence or absence of modified bases in DNA. Specifically, a number of investigators have postulated that cytosine methylation may play a significant role in determining which cellular genes will be transcribed and at what times (4, 11, 26, 28). 5-Methylcytosine may control gene transcription by one or more mechanisms: the base may modulate protein-DNA interaction (4, 11, 26, 28) or induce (or stabilize) localized conformational alterations of the DNA (1). The strong correlation between DNA hypomethylation and gene expression which has been demonstrated in a number of systems (4, 11, 26, 28) lends credence to the concept that 5-methylcytosine plays an important role in transcription. However, the correlation between DNA hypomethylation and transcriptional activity has not been demonstrated in all cases (15, 20, 25).

Endogenous and acquired mouse mammary tumor virus (MuMTV) proviral DNA is less methylated in mammary tumors than the endogenous proviruses in mouse liver (3, 6, 10, 12, 14). Since MuMTV-specific RNA is easily detected in mammary tumors but not in livers (33), hypomethylation of MuMTV proviral DNA has been taken as prima facia evidence for MuMTV transcriptional activity (3, 6, 10, 12, 14).

Although the correlation between MuMTV proviral hypomethylation and gene expression is demonstratively true in mammary tissue, it does not appear to be valid in general. We have found hypomethylated endogenous MuMTV proviral DNA in mouse tissues which contain little or no MuMTV RNA. However, our data do not exclude the possibility that hypomethylation of MuMTV DNA is a necessary prerequisite for proviral gene expression.

MATERIALS AND METHODS

Animals. BALB/c, C57BL, C3H, and GR/A mice were originally obtained from the Cancer Research Laboratory, Berkeley, Calif. or the Jackson Laboratory, Bar Harbor, Maine. GR-*Mtv*-2$^-$ mice were originally provided by Jo Hilgers, The Netherlands Cancer Institute, Amsterdam.

Nucleic acid extraction. DNA was extracted from mouse tissues by the proteinase K-sodium dodecyl sulfate-phenol-chloroform method as described previously (13). RNA was extracted as follows. Fresh tissue was homogenized in an ice-jacketed Waring blender in a buffer containing 10 mM Tris-hydrochloride (pH 8), 10 mM EDTA, 40 mM NaCl, and 100 to 200 μg of proteinase K per ml. The homogenate was passed through four layers of cheesecloth to remove large debris, made 0.5% in sodium dodecyl sulfate, and extracted several times with phenol-chloroform. The partially deproteinized sample was ethanol precipitated, dissolved in buffer, and again extracted with phenol-chloroform. The sample was ethanol precipitated and dissolved in 20 mM sodium acetate (pH 5)–10 mM $MgSO_4$, and the nucleic acid concentration was determined by measuring the optical density at 260 nm. The sample was then digested with DNase (RNase free; Miles Laboratories, Elkhorn, Ind.) under conditions recommended by the supplier. The digests were stopped by freezing, and a sample was subjected to electrophoresis in a 2% agarose gel containing 1 μg of ethidium bromide per ml. In addition to the mouse RNAs, at least one lane of the gel contained a known amount of purified *Escherichia coli* rRNA. The gel was photographed under shortwave UV light, and each lane of the photographic negative was scanned with a Gilford 2000 spectrophotometer (13, 14). The densitometer scans of each lane were compared with that of the *E. coli* rRNA standard to arrive at a best estimate of the RNA concentration in each sample. Only those samples showing no RNA degradation were used in the experiments reported here.

Blot analysis. Cellular DNAs were digested with restriction enzymes (from New England Biolabs, Boston, Mass. or Bethesda Research Laboratories, Bethesda, Md.) and Southern blotted as described previously (13, 14). Dot blot (18) analysis of cellular RNAs was performed with nitrocellulose filters soaked in 10× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) on a Dot Blot apparatus (Bethesda Research Laboratories). All blots, unless otherwise noted, were probed with a mixture of cloned MuMTV *Pst*I fragments (19) that had been labeled by nick translation (27).

RESULTS

Methylation patterns of MuMTV proviral DNA in normal GR/A tissues. Previous studies have compared the methylation patterns of MuMTV proviral DNA in mouse mammary tissues with the patterns exhibited in normal nonmammary

* Corresponding author.

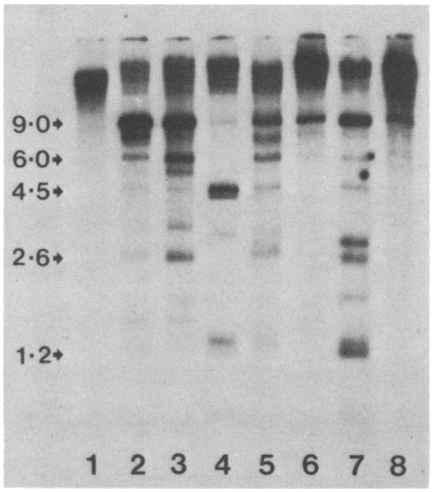

FIG. 1. Hypomethylated endogenous MuMTV proviral DNA in GR/A mouse tissues. Tissue DNAs (7 μg) were digested with the enzyme *Hha*I, electrophoresed into 1% agarose gels, and Southern blotted. The blots were hybridized with labeled MuMTV-specific DNA and autoradiographed. Lane 1, female liver; lane 2, male and female bone marrows (pooled from several animals); lane 3, female spleen; lane 4, testes; lane 5, placenta; lane 6, fetuses (pooled from a single litter); lane 7, mammary tumor; lane 8, female kidney. Arrows denote the sizes of DNA fragments in kilobase pairs.

tissues (3, 6, 10, 12, 14). The normal tissue most commonly used has been liver, although kidney, heart, spleen, and mammary gland tissues have also been investigated. In these studies, only tumor and spleen tissues contained hypomethylated MuMTV proviral DNA.

We surveyed a number of normal nonmammary GR/A mouse tissues for the presence of hypomethylated endogenous MuMTV proviral DNA. Proviral DNA in GR/A spleen, placenta, and mammary tumor tissues appeared to be significantly hypomethylated when digested with the methylation-sensitive restriction enzyme *Hha*I (Fig. 1, lanes 3, 5, and 7). Bone marrow (Fig. 1, lane 2) and testes (Fig. 1, lane 4) also appeared to harbor hypomethylated MuMTV proviral DNA, although the degree of hypomethylation seemed to be less than for spleen and placenta. The patterns exhibited by both the fetus and kidney samples suggested that MuMTV proviral DNA was largely methylated in these tissues, although somewhat less so than proviral DNA in GR/A liver (Fig. 1; compare lanes 6 and 8 with 1).

In addition to the quantitative differences in MuMTV proviral DNA methylation seen in Fig. 1, there were marked qualitative differences. For example, the 2.6-kilobase pair (Kb) and 6.0-Kb MuMTV proviral bands seen in *Hha*I-digested spleen DNA (Fig. 1, lane 3) were not present in *Hha*I-digested testes DNA (Fig. 1, lane 4). These qualitative differences suggest that different MuMTV proviruses are hypomethylated in a tissue-specific fashion, or that different enzyme sites on a given provirus are hypomethylated in a tissue-specific fashion, or both. At present, we have no way of unambiguously differentiating between these two possibilities.

Similarities among the profiles shown in Fig. 1 were also apparent. MuMTV proviral DNA fragments of 9 Kb were generated by *Hha*I digestion of many tissue DNAs (Fig. 1, lanes 2, 3, 5, 6, and 7). Since integrated MuMTV proviral DNA is 10 Kb (7), this finding would suggest that only two restriction sites are available for cleavage within, or near, one or more proviral DNAs in these tissues. We have no way of deciding, however, whether these 9-Kb fragments originated from one or more proviruses or whether both sites were located within a provirus, as opposed to one site within a provirus and the other in the flanking host DNA. Parenthetically, all bands seen in Fig. 1 (as well as those in all subsequent figures) arose from enzymatic cleavage of integrated proviral DNA since Southern blot analysis of uncleaved tissue DNAs failed to reveal any MuMTV-specific bands below that of the bulk DNA, which ran at a position of 30 to 50 Kb (data not shown).

The bands seen below the 9-Kb band (Fig. 1, lanes 2 to 5 and 7) suggested the presence of multiple hypomethylated enzyme sites within MuMTV proviral DNA. We have, in fact, demonstrated that this is the case for several of the lower-molecular-weight bands by performing restriction mapping experiments and by using small fragments of cloned MuMTV proviral DNA as probes (data not shown). Since GR/A mice contain five nearly identical copies of MuMTV proviral DNA (13, 17, 22), we have not been able to assign specific fragments to specific proviruses with one exception: the 4.2-Kb proviral fragment present in GR/A mouse testes is missing in GR-*Mtv*-2$^-$ mouse testes and, thus, probably originated from the GR-MTV-2 provirus (see below).

The tissue-specific proviral restriction patterns in Fig. 1 were generated by the restriction enzyme *Hha*I. To corroborate this finding, we have digested GR/A tissue DNAs with the isoschizomer pair *Msp*I (methylation insensitive) and *Hpa*II (methylation sensitive). The *Msp*I-*Hpa*II digests confirmed the results obtained with *Hha*I (Fig. 2) and, in addition, demonstrated that although specific MuMTV proviral DNA sequences were hypomethylated in spleen, testes, and tumor tissues, many other proviral DNA sequences were methylated in these same tissues (Fig. 2; compare lane 2 with 6, 3 with 7, and 4 with 8).

Hypomethylated MuMTV proviral DNAs in spleens of different mouse strains. DNAs derived from GR/A spleens appeared to contain hypomethylated MuMTV proviral DNA (Fig. 1, lane 3; Fig. 2, lane 2). To determine whether this was

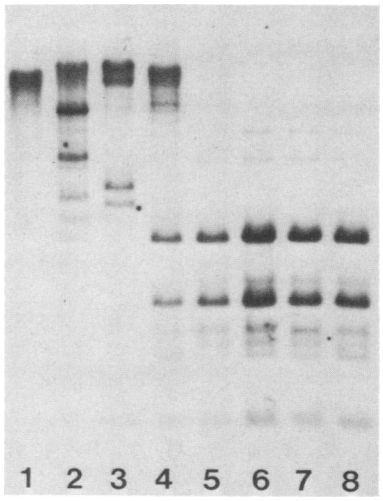

FIG. 2. Hypomethylated endogenous MuMTV proviral DNA in GR/A mouse tissues. Tissue DNAs were digested with either *Hpa*II (lanes 1 to 4) or *Msp*I (lanes 5 to 8) and treated as described in the legend to Fig. 1. Lanes 1 and 5, female liver; lanes 2 and 6, male spleen; lanes 3 and 7, testes; lanes 4 and 8, mammary tumor.

a general phenomenon, DNAs were extracted from the spleens of BALB/c, C57BL, C3H, and GR-*Mtv*-2$^-$ mice and examined for the degree of MuMTV proviral DNA methylation with the enzyme *Hha*I. In all cases, hypomethylated proviral DNA was detected, although both quantitative and qualitative differences were apparent among the strains (Fig. 3).

Figure 3 shows both *Sac*I and *Hha*I digests of mouse spleen DNAs. Since *Sac*I is not inhibited by DNA methylation, these patterns indicate the total amount of MuMTV-specific DNA applied to each lane of the gel (Fig. 3, lanes 6 to 10). Taking into account the overloading of the GR-*Mtv*-2$^-$ lanes (Fig. 3, lanes 4 and 9), it was apparent that spleen DNAs from GR/A and GR-*Mtv*-2$^-$ mice contained more hypomethylated MuMTV proviral DNA than did spleen DNAs from BALB/c, C57BL, and C3H mice (Fig. 3). Since the GR/A strain contains more endogenous MuMTV provirus than do the other strains (24), this finding was not completely unexpected. Nevertheless, the fact that both GR/A and GR-*Mtv*-2$^-$ mice contained more lower-molecular-weight MuMTV restriction fragments did suggest that more *Hha*I enzyme sites were hypomethylated in these two strains.

An alternative possibility was that MuMTV proviruses in GR/A and GR-*Mtv*-2$^-$ mice have more *Hha*I sites per se than do proviruses in the other strains. Although we cannot rule out this possibility, it seems unlikely in view of the high sequence homologies exhibited by MuMTV proviruses (33). Thus, enzymes with short recognition sequences (such as *Hha*I and *Msp*I-*Hha*II, which cleave at four base pair sequences) would be expected to show conservative patterns; indeed, this appeared to be the case (see Fig. 2 and Fig. 5).

Significantly, no differences (either qualitative or quantitative) were detected between the GR/A mouse strain and the congenic line GR-*Mtv*-2$^-$ (Fig. 3). These two lines differ only in the presence (GR/A) or absence (GR-*Mtv*-2$^-$) of the highly oncogenic GR-MTV-2 provirus (13, 17, 22). Thus, it seems reasonable to assume that all of the *Hha*I restriction fragments seen in GR/A spleen samples (Fig. 1 and 2) contain sequences from proviruses other than GR-MTV-2.

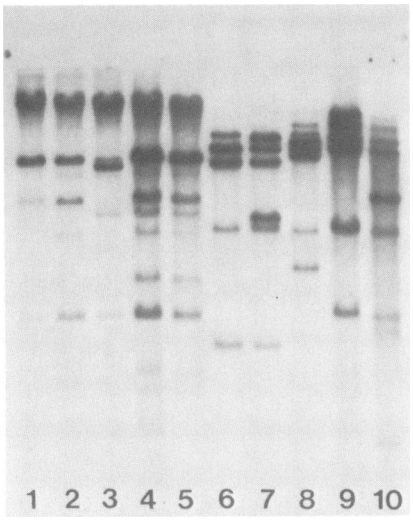

FIG. 3. Hypomethylated endogenous MuMTV proviral DNA in spleens of different mouse strains. Tissue DNAs were digested with either *Hha*I (lanes 1 to 5) or *Sac*I (lanes 6 to 10) and treated as described in the legend to Fig. 1. Lanes 1 and 6, female BALB/c; lanes 2 and 7, female C57BL; lanes 3 and 8, female C3H; lanes 4 and 9, male GR-*Mtv*-2$^-$; lanes 5 and 10, female GR/A.

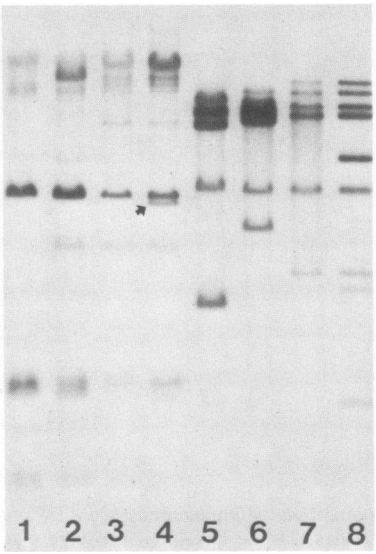

FIG. 4. Hypomethylated endogenous MuMTV proviral DNA in testes of different mouse strains. Tissue DNAs were digested with either *Hha*I (lanes 1 to 4) or *Sac*I (lanes 5 to 8) and treated as described in the legend to Fig. 1. Lanes 1 and 5, BALB/c; lanes 2 and 6, C3H; lanes 3 and 7, GR-*Mtv*-2$^-$; lanes 4 and 8, GR/A. The arrow identifies the 4.2-Kb fragment present in GR/A mice that is missing in GR-*Mtv*-2$^-$ mice.

Hypomethylated MuMTV proviral DNAs in testes of different mouse strains. DNAs derived from the testes of male mice belonging to different strains contained hypomethylated MuMTV proviral DNA (Fig. 4). When the differences in total amounts of DNA loaded per lane were taken into account, it appeared as though roughly equivalent amounts of hypomethylated MuMTV proviral DNAs were present in the testes of BALB/c, C3H, GR-*Mtv*-2$^-$, and GR/A mice (Fig. 4).

Interestingly, the MuMTV proviral patterns exhibited after *Hha*I cleavage of testes DNAs appeared to be very similar between the different mouse strains (Fig. 4, lanes 1 to 4). To test this further, the samples were digested with the isoschizomer pair *Msp*I (methylation insensitive) and *Hpa*II (methylation sensitive). The different strains exhibited very similar *Msp*I profiles (Fig. 5, lanes 5 to 8) indicating that *Msp*I restriction sites are highly conserved between the MuMTV proviruses in each strain.

The MuMTV proviral patterns generated by *Hpa*II were also very similar (Fig. 5, lanes 1 to 4). This indicates that the methylation patterns of MuMTV proviruses in mouse testes are strongly conserved among the different strains. The patterns are less strongly conserved in mouse spleens (Fig. 3) or in the different tissues of a single individual (Fig. 1 and 2). As yet, we have no explanation for this interstrain conservation of MuMTV proviral DNA methylation patterns in mouse testes.

The profiles shown in Fig. 4 demonstrate the only difference in MuMTV proviral methylation between GR/A and GR-*Mtv*-2$^-$ mice that we have encountered to date. GR/A (Fig. 4, lane 4) and GR-*Mtv*-2$^-$ (Fig. 4, lane 3) differ in a 4.2-Kb MuMTV proviral DNA fragment that is present in the GR/A testes sample but absent in the GR-*Mtv*-2$^-$ sample.

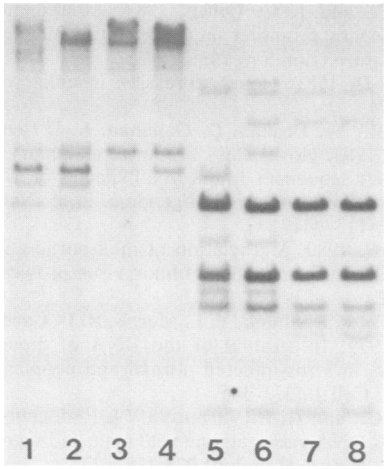

FIG. 5. Hypomethylated endogenous MuMTV proviral DNA in testes of different mouse strains. Tissue DNAs were digested with either *Hpa*II (lanes 1 to 4) or *Msp*I (lanes 5 to 8) and treated as described in the legend to Fig. 1. Lanes 1 and 5, BALB/c; lanes 2 and 6, C3H; lanes 3 and 7, GR-*Mtv*-2$^-$; lanes 4 and 8, GR/A.

The two mouse lines differ, in terms of MuMTV proviruses, only in respect to the GR-MTV-2 provirus, which is missing in the GR-*Mtv*-2$^-$ line. Thus, we have provisionally assigned the 4.2-Kb band to GR-MTV-2.

MuMTV proviral transcription in GR/A mouse tissues. Are hypomethylated MuMTV proviral DNAs transcriptionally active? RNA extracted from two GR/A mammary tumors contained significant quantities of MuMTV-specific RNA. When compared with controls (Fig. 6, rows 1 to 3), 0.64% of the total RNA from tumor A (Fig. 6, row 6) was found to hybridize with an MuMTV-specific probe, whereas 2.5% of the total RNA from tumor B (Fig. 6, row 7) hybridized with the probe. In addition, 0.33% of the total RNA extracted from GR/A prelactating mammary gland tissue (Fig. 6, row 16) hybridized with the MuMTV-specific probe. These results are in agreement with the observations of others that GR/A tumors and prelactating and lactating mammary gland tissues contain significant amounts of MuMTV RNA (33).

Little or no MuMTV RNA could be detected in the livers of either male or female GR/A mice (Fig. 6, rows 4 and 5), again confirming results previously obtained by other investigators (33). Similarly, little or no MuMTV RNA was detected in GR/A male spleens, female spleens, testes, placenta, a pool of fetuses, or in female thymus tissues (Fig. 6, rows 9 to 15 respectively).

Spleens and testes of GR/A mice contained hypomethylated MuMTV proviruses (Fig. 1 and 4). Since these same tissues lacked detectable quantities of MuMTV RNA (Fig. 6), we conclude that hypomethylation of MuMTV proviral DNA does not indicate significant transcriptional activity per se (i.e., transcription on a par with that in tumors).

DISCUSSION

We have shown that certain normal nonmammary mouse tissues contain hypomethylated endogenous MuMTV proviral DNA. Additionally, we have established that hypomethylated proviruses in nonmammary tissues are either sparingly transcribed or not transcribed at all. This result is at odds with many (3, 6, 10, 12, 14, 21) but not all (15) observations demonstrating a correlation between MuMTV DNA hypomethylation and gene expression in mammary tissues and cultured cells.

MuMTV proviruses are present in the genomes of all inbreed strains of mice and are also found in many feral mice (8, 24). The viruses are known to be a causative agent in mammary neoplasia, but no other biological functions have been assigned to them (2). The recent discovery of healthy feral mice lacking MuMTV sequences would suggest that viral genetic information is superfluous for normal mouse development (5, 8, 9). Thus, why should apparently nonessential DNA sequences exhibit tissue-specific methylation patterns? This question is especially cogent in view of the well-established correlation between hypomethylation and transcriptional activity (4, 11, 26, 28) and the lack of such a correlation for MuMTV.

Based upon the results obtained in other systems, we offer three proposals to explain our results with MuMTV proviruses: (i) genes near MuMTV proviruses are hypomethylated and transcribed, with MuMTV proviruses acting as "hitch-hikers"; (ii) hypomethylated MuMTV proviral DNA cannot be transcribed because a "critical site" remains methylated; or (iii) hypomethylated MuMTV proviral DNA cannot be transcribed because a transcriptional "trigger" is not present.

The hitch-hiker proposal assumes that MuMTV proviruses are integrated near genes that are transcriptionally active. It is known that large regions of chromatin undergo conformational alterations around active genes (16, 34) and that nontranscribed sequences within these active regions contain hypomethylated DNA (30). Endogenous MuMTV proviruses do not appear to be clustered in the mouse genome (31). Thus, for the hitch-hiker proposal to be correct, one would need to postulate that at least one MuMTV provirus is present near an active gene(s) in each mouse line. This hypothesis is reasonable if, at the time of germ line infection, MuMTV preferentially integrated in active (ex-

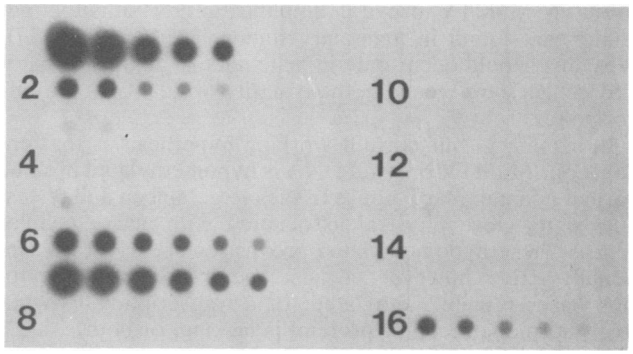

FIG. 6. MuMTV-specific RNA in GR/A mouse tissues. Tissue RNAs were extracted and analyzed by the dot blot procedure as described in the text. Rows 1 to 3 are purified MuMTV RNA extracted from C3H virions produced by the Mm5mt/cl cell line. Each row has the most concentrated sample on the left (spot 1) and proceeds with serial twofold dilutions to the right. Thus, row 1 begins with 100 ng of MuMTV RNA for spot 1 and proceeds (left to right) to 50, 25, 12.5, and 6.3 ng; row 2 begins with (spot 1) 5 ng of MuMTV RNA; and row 3 begins with (spot 1) 0.5 ng of MuMTV RNA. The remaining rows begin with (spot 1) 1.2 μg of total tissue RNA. Row 4, male liver; row 5, female liver; row 6, mammary tumor A; row 7, mammary tumor B; row 8, *E. coli* rRNA; row 9, male spleen; row 10, female spleen; row 11, testes from mouse no. 1; row 12, testes from mouse no. 2; row 13, placenta; row 14, a pool of fetuses; row 15, female thymus; row 16, prelactating mammary gland.

pressed) regions of the genome and if these regions are expressed in a tissue-specific fashion.

The hitch-hiker proposal may explain the hypomethylated state of endogenous proviruses in normal nonmammary mouse tissues, but it does not explain our failure to detect MuMTV RNA in the same tissues. The critical site proposal assumes that hypomethylated MuMTV proviral DNA is not transcribed because one or more cytosine bases remain methylated, thus blocking a function necessary for transcription initiation. Support for such a proposal comes from recent work with both Adenovirus and MuMTV where it has been demonstrated that the methylation of a select number of cytosines markedly affected transcription (21, 32). Certainly, our results with endogenous, nontranscribed MuMTV proviral DNA demonstrate that although some cytosines are not methylated, many others remain methylated (Fig. 2 and 5).

The trigger proposal assumes that although hypomethylated MuMTV proviruses are fully capable of being transcribed, they are not because some component of the transcriptional machinery is either missing or prevented from operating. Since MuMTV transcription is known to be hormonally controlled (29), the trigger could be a hormone or hormone receptor protein. If by some mechanism the trigger were prevented from exercising its effect, MuMTV proviral DNA would remain transcriptionally silent despite its hypomethylated state.

We have found hypomethylated MuMTV proviruses in many tissues (e.g., bone marrow, thymus, and lymph nodes) associated with the lymphatic system (Fig. 1 and unpublished data). If hypomethylation is a prerequisite for MuMTV proviral gene expression, could it be that these proviruses are occasionally expressed? The recent finding of MuMTV proviral amplification in some GR/A male mouse leukemias (23) is intriguing in this respect. Given the hypomethylated state of MuMTV proviruses in GR/A lymphoid tissue, the inadvertant transcription of these proviruses may result in MuMTV proviral amplification, a situation not unlike that found in mammary tumors (7, 10, 12–14, 17). Why this should occur only in male mice is unknown, but it may reflect a difference in the quantity or quality of various hormones.

In summary, our current working hypothesis is that endogenous MuMTV proviral DNA is hypomethylated in some normal nonmammary mouse tissues (e.g., spleen and testes) due to its close physical association with active cellular genes. These hypomethylated proviruses are not transcriptionally active, however, because one or more critical cytosine bases remain methylated, or a transcriptional trigger (e.g., hormone receptor protein) is lacking, or both.

ACKNOWLEDGMENTS

We thank G. Heidecker, V. Pathek, L. J. T. Young, and D. Morris for suggestions and discussion.

This work was supported by Public Health Service grant 5R01-CA21454 from the National Cancer Institute.

LITERATURE CITED

1. **Behe, M., and G. Felsenfeld.** 1981. Effects of methylation on a synthetic polynucleotide: the B-Z transition in poly(dG-m^5dC) · poly(dG-m^5dC). Proc. Natl. Acad. Sci. U.S.A. **78**:1619–1623.
2. **Bentvelzen, P., and J. Hilgers.** 1980. Murine mammary tumor virus, p. 311–355. *In* G. Klein (ed.), Viral oncology. Raven Press, New York.
3. **Breznik, T., and J. C. Cohen.** 1982. Altered methylation of endogenous viral promoter sequences during mammary carcinogenesis. Nature (London) **295**:255–257.
4. **Brown, D. D.** 1981. Gene expression in eukaryotes. Science **211**:667–674.
5. **Callahan, R., W. Drohan, D. Gallahan, L. D'Hoostelaere, and M. Potter.** 1982. Novel class of mouse mammary tumor virus-related DNA sequences found in all species of *Mus*, including mice lacking the virus proviral genome. Proc. Natl. Acad. Sci. U.S.A. **79**:4113–4117.
6. **Cohen, J. C.** 1980. Methylation of milk-borne and genetically transmitted mouse mammary tumor virus proviral DNA. Cell **19**:653–662.
7. **Cohen, J. C., P. R. Shank, V. L. Morris, R. D. Cardiff, and H. E. Varmus.** 1979. Integration of the DNA of mouse mammary tumor virus in virus-infected normal and neoplastic tissues of the mouse. Cell **16**:333–345.
8. **Cohen, J. C., and H. E. Varmus.** 1979. Endogenous mammary tumor virus DNA varies among wild mice and segregates during inbreeding. Nature (London) **278**:418–423.
9. **Cohen, J. D., V. L. Traina, T. Breznik, and M. B. Gardner.** 1982. Development of a mouse mammary tumor virus-negative strain: a new system for the study of mammary carcinogenesis. J. Virol. **44**:882–885.
10. **Drohan, W. N., L. E. Benade, D. E. Graham, and G. H. Smith.** 1982. Mouse mammary tumor virus proviral sequences congenital to C3H/Sm mice are differentially hypomethylated in chemically induced, virus-induced, and spontaneous mammary tumors. J. Virol. **43**:876–884.
11. **Ehrlich, M., and R. Y.-H. Wang.** 1981. 5-Methylcytosine in eukaryotic DNA. Science **212**:1350–1357.
12. **Etkind, P. R., and N. H. Sarkar.** 1983. Integration of new endogenous mouse mammary tumor virus proviral DNA at common sites in the DNA of mammary tumors of C3Hf mice and hypomethylation of the endogenous mouse mammary tumor virus proviral DNA in C3Hf mammary tumors and spleens. J. Virol. **45**:114–123.
13. **Fanning, T. G., J. P. Puma, and R. D. Cardiff.** 1980. Selective amplification of mouse mammary tumor virus in mammary tumors of GR mice. J. Virol. **36**:109–114.
14. **Fanning, T. G., A. B. Vassos, and R. D. Cardiff.** 1982. Methylation and amplification of mouse mammary tumor virus in normal, premalignant and malignant cells of GR/A mice. J. Virol. **41**:1007–1013.
15. **Feinstein, S. C., S. R. Ross, and K. R. Yamamoto.** 1982. Chromosomal position effects determine transcriptional potential of integrated mammary tumor virus DNA. J. Mol. Biol. **156**:549–565.
16. **Garel, A., and R. Axel.** 1976. Selective digestion of transcriptionally active ovalbumin genes from oviduct nuclei. Proc. Natl. Acad. Sci. U.S.A. **73**:3966–3970.
17. **Hynes, N. E., B. Groner, H. Diggelmann, R. Van Nie, and R. Michalides.** 1980. Genomic location of mouse mammary tumor virus proviral DNA in normal mouse tissue and in mammary tumors. Cold Spring Harbor Symp. Quant. Biol. **44**:1161–1168.
18. **Kafatos, F. C., C. W. Jones, and A. Efstratiadis.** 1979. Determination of nucleic acid sequence homologies and relative concentrations by a dot hybridization procedure. Nucleic Acids Res. **7**:1541–1552.
19. **Majors, J. E., and H. E. Varmus.** 1981. Nucleotide sequences at host-proviral junctions for mouse mammary tumor virus. Nature (London) **289**:253–258.
20. **McKeon, C., H. Ohkubo, I. Pastan, and B. de Crombrugghe.** 1982. Unusual methylation pattern of the alpha-2(1) collagen gene. Cell **29**:203–210.
21. **Mermod, J.-J., S. Bourgeois, N. Defer, and M. Crepin.** 1983. Demethylation and expression of murine mammary tumor proviruses in mouse thymoma cell lines. Proc. Natl. Acad. Sci. U.S.A. **20**:110–114.
22. **Michalides, R., R. Van Nie, R. Nusse, N. E. Hynes, and B. Groner.** 1981. Mammary tumor induction loci in GR and DBAf mice contain one provirus of the mouse mammary tumor virus. Cell **23**:165–173.

23. **Michalides, R., E. Wagenaar, J. Hilkens, B. Groner, and N. E. Hynes.** 1982. Acquisition of proviral DNA of mouse mammary tumor virus in thymic leukemia cells from GR mice. J. Virol. **43**:819–829.
24. **Morris, V. L., E. Medeiros, G. H. Ringold, J. M. Bishop, and H. E. Varmus.** 1977. Comparison of mouse mammary tumor virus-specific DNA in inbred, wild and Asian mice, and in tumors and normal organs from inbred mice. J. Mol. Biol. **114**:73–91.
25. **Ott, M.-O., L. Sperling, D. Cassio, J. Levilliers, J. Sala-Trepat, and M. C. Weiss.** 1982. Undermethylation at the 5′ end of the albumin gene is necessary but not sufficient for albumin production by rat hepatoma cells in culture. Cell **30**:825–833.
26. **Razin, A., and J. Friedman.** 1981. DNA methylation and its possible biological roles. Progr. Nucleic Acid Res. Mol. Biol. **25**:33–52.
27. **Rigby, P. W., M. Dieckmann, C. Rhodes, and P. Berg.** 1977. Labelling deoxyribonucleic acid to high specific activity in vitro by nick translation with DNA polymerase I. J. Mol. Biol. **113**:237–251.
28. **Riggs, A. D.** 1975. X inactivation, differentiation and DNA methylation. Cytogenet. Cell Genet. **14**:9–25.
29. **Ringold, G. M., K. R. Yamamoto, J. M. Bishop, and H. E. Varmus.** 1977. Glucocorticoid-stimulated accumulation of mouse mammary tumor virus RNA: increased rate of synthesis of viral RNA. Proc. Natl. Acad. Sci. U.S.A. **74**:2879–2883.
30. **Shen, C.-K. J., and T. Maniatis.** 1980. Tissue-specific DNA methylation in a cluster of rabbit β-like globin genes. Proc. Natl. Acad. Sci. U.S.A. **77**:6634–6638.
31. **Traina, V. L., B. A. Taylor, and J. C. Cohen.** 1981. Genetic mapping of endogenous mouse mammary tumor viruses: locus characterization, segregation, and chromosomal distribution. J. Virol. **40**:735–744.
32. **Vardimon, L., A. Kressmann, H. Cedar, M. Maechler, and W. Doerfler.** 1982. Expression of a cloned adenovirus gene is inhibited by in vitro methylation. Proc. Natl. Acad. Sci. U.S.A. **79**:1073–1077.
33. **Varmus, H. E., N. Quintrell, E. Medeiros, J. M. Bishop, R. C. Nowinski, and N. H. Sarkar.** 1973. Transcription of mouse mammary tumor virus genes in tissues from high and low tumor incidence mouse strains. J. Mol. Biol. **79**:663–679.
34. **Weintraub, H., and H. Groudine.** 1976. Chromosomal subunits in active genes have an altered conformation. Science **193**:848–856.