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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

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To understand the molecular mechanisms by which the endogenous murine mammary tumor virus (MuMTV) proviruses are expressed and produce late-occurring mammary tumors in C3Hf mice, we analyzed, by the use of restriction enzymes and the Southern transfer procedure, genomic DNA from normal organs of mammary tumor-bearing and tumor-free mice and from 12 late-occurring C3Hf mammary tumors. We found, by using the restriction enzymes *Eco*RI and *Hin*dIII, that in addition to the preexisting endogenous MuMTV proviruses, new MuMTV-specific proviral DNA was integrated into new sites in the host genome in all 12 of the tumors that we examined. *Pst*I digests of C3Hf tumor DNA revealed that the new proviral DNA found in C3Hf tumors was of endogenous origin. Moreover, the respective sizes of at least one of the new DNA fragments generated by *Eco*RI or *Hin*dIII digestion were the same in at least 50% of the C3Hf tumors analyzed, suggesting that the integration site of this new proviral DNA could be at the same location in the host genome of these tumors. Our results may imply that mammary tumorigenesis in C3Hf mice results from activation of cellular oncogenes by an MuMTV proviral DNA promoter. Specific hypomethylation of MuMTV proviral DNA was detected in the mammary tumors and spleens of C3Hf tumor-bearing mice. Our results indicated that most, if not all, of the hypomethylated MuMTV proviral DNA sequences were derived from the endogenous MuMTV provirus located at the MTV-1 locus, a locus responsible for the production of MuMTV antigens and increased incidence of mammary carcinoma in C3Hf mice. In spleens of non-tumor-bearing mice of ages 3, 6, 9, and 12 months, there was progressive hypomethylation of proviral DNA with increasing age, suggesting a possible correlation between demethylation of MuMTV proviral DNA in the spleens of C3Hf mice and the expression of endogenous MuMTV.

The murine mammary tumor virus (MuMTV) is known to be the milk-borne etiological agent of spontaneous murine mammary adenocarcinomas in several strains of mice. C3H mice express high titers of MuMTV in their milk, and approximately 90% of C3H offspring nursed on C3H mothers develop mammary tumors between the ages of 7 and 10 months (2, 27, 31, 35). Foster nursing of C3H offspring on strains of mice with a low incidence of mammary tumors, whose milk does not contain MuMTV, results in a much lower incidence (22 to 52%) and later development (18 to 24 months) of mammary tumors (1, 2). These late-occurring mammary tumors which develop independently of exogenous MuMTV infection are thought to result from activation of endogenous MuMTV. The endogenous MuMTV is expressed in C3Hf mice as age and parity number increase, and high titers of endogenous MuMTV have been found in milk of high-parity C3Hf mice and also in late-occurring mammary tumors (27).

Work by van Nie and Verstraeten (41) has established that a single dominant gene, MTV-1, located on mouse chromosome 7, is responsible for the expression of MuMTV viral antigens in the milk of C3Hf mice and for the increased incidence of mammary tumor development. All

inbred strains of mice contain endogenous MuMTV proviruses (6); the Heston strain of C3Hf mouse contains three endogenous MuMTV proviruses (6), one of which is located at the MTV-1 locus (26, 40). The molecular mechanism(s) by which endogenous proviruses are expressed and produce tumors in C3Hf mice is unknown at present.

In mammary tumors induced by exogenous MuMTV, mechanisms such as hypomethylation and reintegration of MuMTV proviral DNA have been hypothesized. The finding that in these MuMTV-induced early mammary tumors one or more new MuMTV proviruses are present in addition to endogenous MuMTV proviruses has led to the suggestion that integration of MuMTV proviral DNA at new and specific sites in genomic DNA may be a necessary step for the development of early mammary tumors (5, 12, 15). Furthermore, the newly amplified and actively transcribing viral DNA in these tumors has been shown to be hypomethylated, whereas the nonactive endogenous proviral DNA has been shown to be methylated (4, 13). Preliminary evidence obtained by Cohen and Varmus (7) suggests that the integration of new MuMTV proviral DNA may be involved in the development of late-occurring mammary tumors in C3Hf mice.

Our goal in this study was to determine (i) whether new MuMTV proviral DNA integration is present in all C3Hf late-occurring mammary tumors; (ii) whether such putative reintegration is at specific locations in genomic DNA; and (iii) whether the level of methylation of endogenous MuMTV proviruses differs in normal organs and mammary tumors of C3Hf mice. By using restriction endonucleases and the Southern transfer technique (38), we analyzed the MuMTV-specific endogenous proviral DNA from normal organs of tumor-bearing and non-tumor-bearing C3Hf/He mice and from 12 late-occurring C3Hf mammary tumors. We found new MuMTV proviral DNA integrated in all 12 tumors that we studied, and moreover, in at least 50% of these tumors the integration of this new proviral DNA appears to be at the same site. Specific hypomethylation of MuMTV proviral DNA was detected in all 12 tumors examined. Furthermore, we also observed that the DNA from spleens of both tumor-bearing and non-tumor-bearing C3Hf mice was also hypomethylated. The MuMTV-specific DNA from the spleens of non-tumor-bearing mice was found to be progressively hypomethylated with increasing age.

MATERIALS AND METHODS

Mice. C3H and C3Hf mice were obtained from the breeding colonies which we maintain at the Institute for Medical Research, Camden, New Jersey.

DNA extraction. Tissue was minced into homogenization buffer (0.3 M sucrose, 10 mM NaCl, 1.5 mM $MgAc_2$, 10 mM Tris [pH 8.0], 1 mM dithiothreitol, 0.1% Nonidet P-40) and homogenized with a motor-driven Teflon-glass Dounce homogenizer. Nuclei were sedimented at 1,500 rpm in a Sorvall centrifuge and then washed two to three times with homogenization buffer. Nuclei were resuspended in TNE (0.1 M NaCl, 0.01 M Tris [pH 7.5], 0.001 M EDTA) and deproteinized with self-digested pronase and sodium dodecyl sulfate (SDS) (1 mg/ml and 1%, respectively) at 37°C for at least 12 h. DNA was extracted with equal volumes of phenol-chloroform and dialyzed extensively against 5.0 mM Tris-hydrochloride (pH 7.4)-0.1 mM ETDA. DNA concentration was determined by optical density.

Digestion of DNA with restriction endonucleases. Restriction endonucleases *Eco*RI, *Hin*dIII, *Pst*I, *Hpa*II, and *Msp*I were purchased from Bethesda Research Labs, Rockville, Md., and Boehringer Mannheim, St. Laurent, Quebec, Canada. The digestion mixtures used for these enzymes were those suggested by the supplier. To ensure complete digestion of the DNA, a 5- to 10-fold excess of enzyme was used. Phage lambda DNA and phage ΦX174 replicative form DNA were used to monitor the extent of cleavage.

Gel electrophoresis and DNA transfer. DNA samples digested with restriction endonucleases were electrophoresed in 1% agarose (Seakem) gels containing 0.05 M Tris-acetate (pH 8.0), 0.02 M sodium acetate, 0.018 M NaCl, and 0.002 M EDTA (19). Lambda DNA cleaved with *Hin*dIII or ΦX174 replicative form DNA cleaved with *Hae*III were used as molecular weight references and were visualized under UV light after ethidium bromide staining. Gels were prepared for transfer to nitrocellulose filters by using a previously described minor modification (11) of the methods described by Southern (38).

Preparation of DNA probes for hybridization. (i) $cDNA_{rep}$ probe. A ^{32}P-labeled cDNA representative ($cDNA_{rep}$) probe was synthesized as previously described (11) in a reaction catalyzed by avian myeloblastosis virus DNA polymerase, with oligomers of calf thymus DNA as primers (14). The MuMTV RNA used in the reactions was prepared from MuMTV C3H virions which were obtained from the Frederick Cancer Research Center, Frederick, Md., through the Research Resources, Biological Carcinogenesis Branch, National Cancer Institute, Bethesda, Md.

(ii) Cloned LTR-specific probe. A ^{32}P-labeled long terminal repeat (LTR)-specific probe was prepared by nick translation (34) of an 0.9×10^6-dalton *Pst*I DNA fragment specific to the LTR region of the MuMTV genome. The cloned LTR-DNA was kindly provided by J. Majors and H. Varmus of the University of California, San Francisco.

Hybridization conditions. (i) $cDNA_{rep}$ probe. Nitrocellulose filters were treated for a minimum of 6 h at 41°C in hybridization buffer containing 50% formamide, 5× Denhardt buffer (0.02% each of bovine serum albumin, polyvinylpyrrolidone, and Ficoll) (8), 0.1% SDS, 200 μg of single-stranded sheared salmon sperm DNA per ml, 3× SSC (1× SSC - 0.15 M NaCl plus 0.015 M sodium citrate), and 200 μg of mouse liver RNA per ml. The hybridization buffer was then removed, and fresh hybridization buffer which contained approximately 2.0×10^6 cpm of [^{32}P]dCTP-

incorporated $cDNA_{rep}$ was added. Hybridization was done at 41°C for 48 h. Filters were washed extensively at 68°C in 2× SSPE (1× SSPE, 0.15 M NaCl plus 0.01 M Na_2PO_4 plus 0.001 M trisodium EDTA [pH7.0]) containing 1% SDS and then at 55°C in 0.2× SSPE containing 0.1% SDS to remove non-specifically adsorbed radioactivity. The filters were wrapped in plastic wrap and exposed to Kodak RP-Royal X-Omat film at −70°C in the presence of a Dupont Cronex Lightning Plus intensifying screen (39).

(ii) Nick-translated cloned LTR probes. Nitrocellulose filters were kept at 68°C for 15 min to remove the previously hybridized ^{32}P-labeled $cDNA_{rep}$ probe. These washed unlabeled nitrocellulose filters were then prehybridized at 68°C for 4 h in hybridization buffer containing 50 μg of sheared salmon sperm DNA per ml, 4× SSC, 0.01 M EDTA, 0.05% bovine serum albumin, 0.05% Ficoll, and 0.05% polyvinylpyrrolidone. This hybridization buffer was removed, and fresh hybridization buffer which contained 2.5×10^6 cpm of ^{32}P-labeled nick-translated LTR probe was added. Nitrocellulose filters were hybridized at 68°C for approximately 18 h, washed extensively as described above to remove non-specifically adsorbed radioactivity, and exposed to film at −70°C.

RESULTS

Presence of newly integrated MuMTV proviral DNA in C3Hf mammary tumors. The restriction enzymes *Eco*RI and *Hin*dIII each cleave once within the full-length C3H MuMTV genome (5, 36). When these enzymes are used to cleave an integrated C3H genome, two DNA fragments are generated which are composed of both viral and cellular sequences. The size of these *Eco*RI- and *Hin*dIII-generated DNA fragments depends on the location of the nearby *Eco*RI or *Hin*dIII sites in the cellular DNA. Similarly sized *Eco*RI and similarly sized *Hin*dIII fragments generated from the genomic DNA of different tumors and containing MuMTV proviral DNA sequences suggest integration of MuMTV proviral DNA at the same location in the DNA of these different tumors.

Figure 1 shows the MuMTV-specific DNA fragments that were generated after *Eco*RI digestions of genomic DNA from 12 C3Hf mammary tumors of 12 individual mice (lanes B to M) and from C3Hf livers of 2 tumor-bearing mice (lanes A and N). Six individual digests were done for each sample, and, although the results from the six digests were always consistent, the clarity of the gels varied. Only the clearest (best) digest for each tumor is shown in Fig. 1. The three endogenous MuMTV proviruses, designated as units I, II, and V (6), and an endogenous 5.8-kilobase (kb) *Eco*RI-generated subgenomic MuMTV DNA fragment (40), all characteristic of the C3H/He strain of mice, are present in both liver and tumor DNA. In addition to the preexisting endogenous MuMTV proviruses, new MuMTV-specific proviral DNA was detected in 11 of the 12 C3Hf mammary tumors after *Eco*RI digestion.

Digests of tumor DNA shown in Fig. 1, lanes C, E, and G to K, revealed the presence of a new *Eco*RI fragment with an approximate size of 13.0 kb (upper arrow). A second new *Eco*RI fragment with an approximate size of 5.0 kb (lower arrow) was seen in seven tumor digests (lanes B, E to I, and K). The remaining digests, with the exception of that in lane M, revealed that new *Eco*RI proviral DNA fragments were integrated at various locations, i.e., new proviral DNA is seen in lane D between the 6.7- and 8.5-kb endogenous fragments and in lane L above the 8.5-kb fragment. The intensity of the newly acquired proviruses that were detected in the tumors of C3Hf mice by hydribization to a ^{32}P-labeled MuMTV-specific $cDNA_{rep}$ probe was always less than the intensity of the preexisting endogenous proviral DNA.

Figure 2 (lanes B to M) shows the MuMTV-specific DNA fragments that were generated

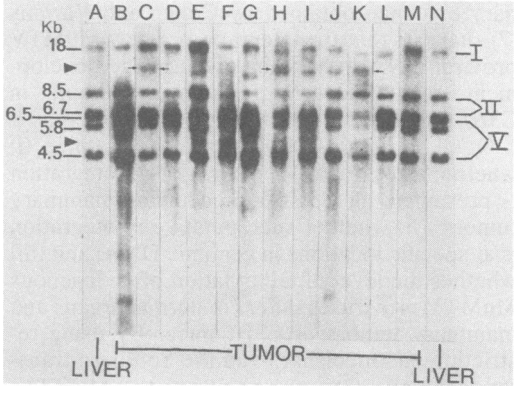

FIG. 1. *Eco*RI restriction pattern of MuMTV-specific proviral DNA in genomic DNA isolated from livers (lanes A and N) and tumors (lanes B to M) of C3Hf mice. The cellular DNA (approximately 5 μg) was digested to completion with the restriction enzyme *Eco*RI, subjected to electrophoresis in a 1.0% agarose gel, transferred to nitrocellulose filters, hybridized to a ^{32}P-labeled MuMTV $cDNA_{rep}$ probe, and autoradiographed as described in the text. The molecular weights were determined for the gel shown in this figure and all the other figures from known molecular weights of marker DNA fragments obtained by *Hin*dIII digestion of lambda DNA and *Hae*III digestion of ΦX174 RF DNA electrophoresed in the same gel and detected by ethidium bromide staining. The molecular weights of the *Eco*RI fragments of C3Hf endogenous proviruses are given on the left. The Roman numerals I, II, and V on the right represent the three endogenous proviruses found in C3Hf mice. The two larger arrows indicate the two common sizes of new proviral DNA present in C3Hf tumors after *Eco*RI digestion. The smaller arrows indicate the similarly sized *Eco*RI fragments of new proviral DNA.

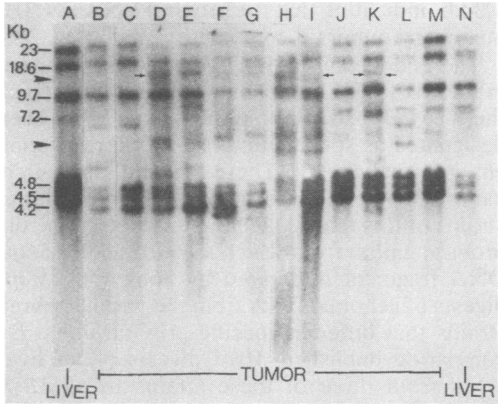

FIG. 2. *Hin*dIII digestion pattern of MuMTV-specific proviral DNA in genomic DNA isolated from livers (lanes A and N) and tumors (lanes B to M) of C3Hf mice. The cellular DNA was digested to completion with the restriction enzyme *Hin*dIII and analyzed as described in the legend to Fig. 1. The molecular weights given are for *Hin*dIII fragments of endogenous proviral DNA found in all organs of the C3Hf mouse. The two larger arrows indicate the two common sizes of new proviral DNA present in C3Hf tumors after *Hin*dIII digestion. The smaller arrows indicate the similarly sized *Hin*dIII fragments of new proviral DNA.

after *Hin*dIII digestion of genomic DNA from 12 C3Hf late-occurring mammary tumors from 12 individual mice and 2 C3Hf livers (lanes A and N) from 2 tumor-bearing mice. The tumors and livers digested with *Hin*dIII were the same as those digested with *Eco*RI. Six prominent MuMTV-specific DNA bands (23.0, 18.6, 9.7, 4.8, 4.5, and 4.2 kb) and one less prominent band (7.2 kb) were always seen in *Hin*dIII digests of liver from these mice and were considered to be MuMTV-specific proviral DNA endogenous to C3Hf mice. We cannot at present explain why the 7.2-kb band is so much less intense than the other six endogenous proviral DNA bands. In addition to the MuMTV-specific proviral DNA present endogenously, one can detect new MuMTV-specific proviral DNA in all 12 C3Hf mammary tumors after *Hin*dIII digestion.

Digests of tumor DNA shown in Fig. 2, lanes D to I and K, revealed the presence of a new *Hin*dIII fragment with an approximate size of 14.0 kb (upper arrow). A second new *Hin*dIII fragment ranging in size from 6.0 to 6.5 kb (lower arrow) was seen in nine tumor digests, as shown in lanes D to K and M. One of these nine tumors, which contains a new *Hin*dIII DNA fragment of 6.5 kb (Fig. 2, lane M), did not appear to contain new MuMTV-specific proviral DNA after *Eco*RI digestion (Fig. 1, lane M). Other new *Hin*dIII-generated proviral DNA fragments of approximate sizes 5.3 and 7.6, 5.7, 7.0, 5.5 and 8.9, 5.3, 5.5, and 5.9 and 6.9 kb can be seen in lanes K, H, I, C, E, D, B, and L, respectively. The intensity of the bands of newly acquired proviruses that were detected in the tumors of C3Hf mice after *Hin*dIII digestion and hybridization to a ^{32}P-labeled MuMTV-specific $cDNA_{rep}$ probe was often less than the intensity of the six prominent bands of preexisting endogenous proviral DNA.

A comparison of the *Eco*RI and *Hin*dIII digests revealed that after *Eco*RI digestion, five C3Hf tumors (Fig. 1, lanes E, G, H, I, and K) each contained two new DNA fragments of approximately 13.0 and 5.0 kb; after *Hin*dIII digestion, these same tumors (Fig. 2, lanes E, G, H, I, and K) also contained at least two DNA fragments with an approximate size of 14.0 and 6.1 to 6.5 kb, respectively. The two DNA fragments generated by *Eco*RI and *Hin*dIII digestion may represent the presence of one new provirus in these tumors. Thus, data from both *Eco*RI and *Hin*dIII digests of genomic DNA of C3Hf tumors hybridized to a MuMTV-specific $cDNA_{rep}$ probe indicated that new MuMTV-specific proviral DNA was present in all C3Hf mammary tumors studied and that the newly integrated proviral DNA might be present at integration sites common to several C3Hf mammary tumors.

Endogenous origin of new proviral DNA of C3Hf mammary tumors. DNA fragments generated from the internal regions of MuMTV proviruses by *Pst*I digestion can be used to distinguish endogenous proviruses from those introduced by exogenous infection in certain inbred strains of mice (5, 11), one of which is the C3H/He strain. The *Pst*I-generated 3.9- and 1.1-kb DNA fragments are characteristic of the proviral DNA of the exogenous milk-borne strain of C3H MuMTV (5). Therefore, we used the restriction enzyme *Pst*I to determine whether the MuMTV proviral DNA inserted at new locations in the DNA of 12 C3Hf mammary tumors was of endogenous or exogenous origin. Figure 3 depicts the *Pst*I digests of genomic DNA obtained from C3H and C3Hf mammary tumors, livers, and spleens. In the C3H mouse, the MuMTV-specific 3.9- and 1.1-kb *Pst*I fragments characteristic of exogenous infection are clearly seen in the mammary tumor, but not in the liver or spleen. C3Hf mice did not contain the 3.9- or 1.1-kb DNA fragments in their late-occurring tumors or in their livers or spleens. Therefore, the new MuMTV-specific proviral DNA which we have detected in C3Hf mammary tumors must represent endogenous MuMTV.

Presence of hypomethylated endogenous MuMTV proviral DNA sequences in mammary tumors of C3Hf mice. Newly integrated exoge-

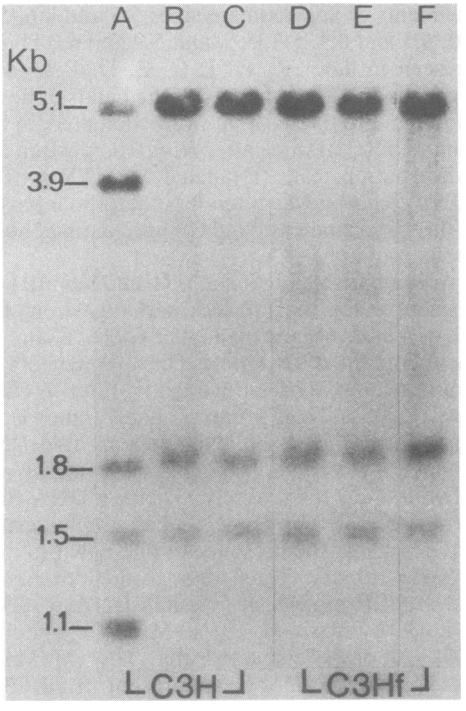

FIG. 3. *Pst*I restriction pattern of MuMTV-specific proviral DNA isolated from (A) C3H mammary tumor, (B) C3H liver, (C) C3H spleen, (D) C3Hf mammary tumor, (E) C3Hf liver, and (F) C3Hf spleen. The cellular DNA (approximately 5 μg) was digested to completion with *Pst*I and analyzed as described in the legend to Fig. 1.

nous MuMTV proviral DNA is hypomethylated in early mouse mammary tumors, but endogenous proviral DNA remains extensively methylated in both mammary tumors and uninfected normal organs such as liver (4, 13). To determine whether hypomethylated endogenous MuMTV proviral DNA exists in late-occurring C3Hf mammary tumors and not in normal organs, we used the restriction enzyme isoschizomers *Msp*I (which cleaves at the sequence 5'CCGG regardless of the methylation state of the internal cytosine residue) (42) and *Hpa*II (which does not cleave at the sequence 5'CCGG if the internal cytosine is methylated) (24) to analyze the extent of methylation of MuMTV proviral DNA in C3Hf mammary tumors, livers, and spleens. The results are shown in Fig. 4. *Msp*I digestion of the DNA from all three tissues generated eight virus-specific fragments, which range in size from 1.3 to 6.8 kb. From the results of the *Hpa*II digestion, it is clear that all viral DNA fragments in C3Hf liver are extensively methylated, whereas *Hpa*II digestions of C3Hf tumor DNA generate four virus-specific bands of 4.5, 3.3, 1.9, and 1.3 kb, indicating that the proviral DNA in C3Hf mammary tumors is hypomethylated.

The endogenous MuMTV proviruses present in the CBA/C3H family of inbred mice, of which the C3H/He strain is a member, have been designated as specific units based on restriction enzyme analysis (6). These proviral DNA units vary in number among the various inbred mouse strains of this family, and one can determine the proviral unit(s) from which a *Hpa*II and/or *Msp*I DNA fragment is derived by comparing *Msp*I digests of genomic DNA from the various inbred strains that differ in specific proviral units. By comparing published *Msp*I digests (4) of liver DNA from three of these strains to an *Msp*I digest of C3H/He liver DNA, we have determined that the 4.5-kb DNA fragment could be derived only from the MuMTV proviral DNA unit V and that the 3.3-, 1.9-, and 1.3-kb fragments could be derived from MuMTV unit V or

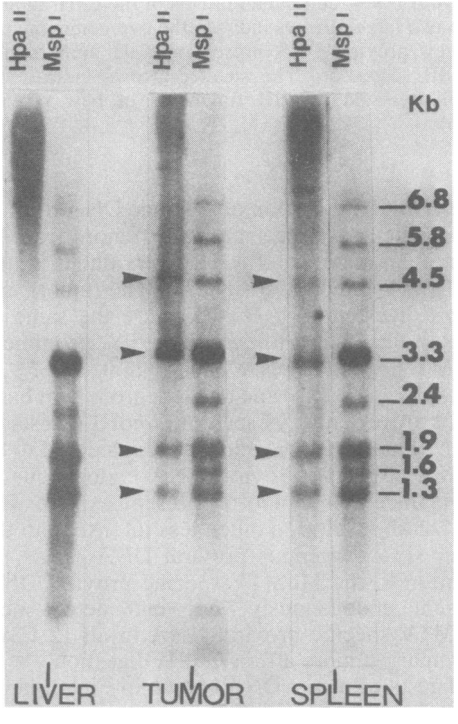

FIG. 4. *Hpa*II and *Msp*I restriction patterns of MuMTV-specific proviral DNA in genomic DNA isolated from the liver, mammary tumor, and spleen of a C3Hf mouse. The mammary tumor in this figure is the tumor seen in lane G of Fig. 1 and 2. The cellular DNA (approximately 5 μg) was digested to completion with either *Hpa*II or *Msp*I and analyzed as described in the legend to Fig. 1. Molecular weights denote the MuMTV-specific DNA fragments derived from *Msp*I digestion. The arrows identify the MuMTV-specific DNA fragments present after *Hpa*II digestion.

Unit II or both. The presence of these four DNA fragments (one of which, the 4.5-kb fragment, is specific only to unit V) in *Hpa*II digests of C3Hf tumor DNA (Fig. 4) strongly suggests that unit V, located at the MTV-1 locus, is hypomethylated in its entirety in C3Hf tumors. However, we cannot rule out the possibility that specific sequences of unit II are also hypomethylated.

The LTR region of MuMTV contains a hormone-responsive promoter for transcription of viral genes (22). We considered the possibility that, if only specific regions of units II and V were being hypomethylated, these regions might contain LTR sequences and the MuMTV promoter and would therefore play a role in the transcription of nearby cellular oncogenes (3). We also recognized that if unit V was hypomethylated in its entirety, then two hypomethylated fragments should contain LTR-specific sequences. Hybridization of an *Hpa*II digest of C3Hf tumor DNA to a ^{32}P-labeled, nick-translated cloned probe specific to the LTR region of MuMTV (Fig. 5) revealed that the two hypomethylated fragments of 4.5 kb (specific to unit V) and 3.3 kb (which may be derived from either unit II or V) contain MuMTV LTR-specific sequences. Hybridization of the MuMTV LTR-specific probe to *Msp*I digests (Fig. 5) revealed that DNA fragments of 5.8, 2.4, and 1.6 kb also contain LTR-specific sequences which are not hypomethylated in C3Hf tumors. Furthermore, both liver and lung of C3Hf tumor-bearing mice contained no hypomethylated MuMTV DNA fragments.

Age-dependent hypomethylation of MuMTV proviral DNA in the spleens of C3Hf mice. In our initial experiments, we used DNA extracted from spleens of C3Hf tumor-bearing mice as a control in restriction enzyme digestions. These initial experiments, however, yielded the unexpected finding that, after hybridization to an MuMTV-specific cDNA$_{rep}$ probe, *Hpa*II digests of C3Hf spleen DNA of tumor-bearing animals resulted in the same hypomethylated DNA fragments (4.5, 3.3, 1.9, and 1.3 kb) as that of DNA from C3Hf tumors (Fig. 4). The intensity of the bands representing the hypomethylated fragments from the spleen was often less than that from tumors. Hybridization of *Hpa*II digests of spleen DNA to ^{32}P-labeled MuMTV LTR-specific probe revealed the same two hypomethylated fragments of 4.5 and 3.3 kb that were detected with this probe in C3Hf tumor DNA.

In view of this unexpected finding, we determined whether hypomethylation occurs in spleens and other organs of non-tumor-bearing C3Hf mice. *Hpa*II digests of DNA from liver and mammary glands of 3-, 6-, 9-, and 12-month-old C3Hf mice (Fig. 6 and 7) revealed extensive methylation of MuMTV proviral DNA. Although *Hpa*II digests of spleen DNA from C3Hf non-tumor-bearing mice revealed that a large percentage of MuMTV proviral DNA is methylated, we were able to detect the presence of a hypomethylated 3.3-kb fragment in 3-, 6-, and 9-month-old mice, whereas in 12-month-old mice, the 1.9- and 1.3-kb fragments were also hypomethylated.

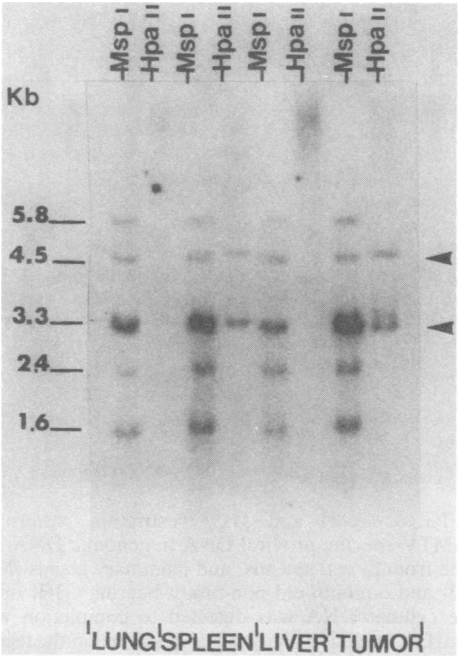

FIG. 5. *Hpa*II and *Msp*I restriction patterns of LTR-specific MuMTV proviral DNA in genomic DNA isolated from lung, spleen, liver, and mammary tumor of a C3Hf mouse. The cellular DNA (approximately 5 μg) was digested to completion with *Hpa*II or *Msp*I and analyzed as described in the legend to Fig. 1, except that hybridization was to a ^{32}P-labeled cloned probe specific to the LTR region of MuMTV. Molecular weights denote the MuMTV-specific DNA fragments which are present after *Msp*I digestion of C3Hf tumor DNA and which contain LTR-specific sequences.

DISCUSSION

C3Hf mice which develop mammary tumors at a lower incidence and later in life than C3H mice do so independently of infection by exogenous milk-borne MuMTV. High-parity C3Hf mice do, however, contain high titers of endogenous MuMTV in their milk and mammary tumors. Our studies have determined that, in addition to the preexisting endogenous MuMTV proviruses, new MuMTV-specific proviral DNA was integrated into new sites in the host genome in all 12 C3Hf tumors that we analyzed. Moreover, when the respective sizes of two of the

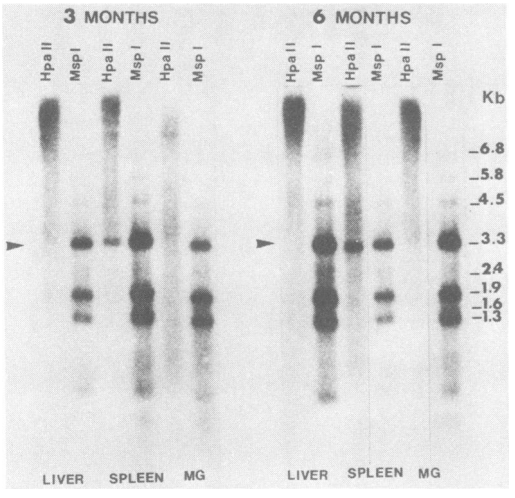

FIG. 6. *Hpa*II and *Msp*I restriction pattern of MuMTV-specific proviral DNA in genomic DNA isolated from livers, spleens, and mammary glands (MG) of 3- and 6-month-old non-tumor-bearing C3Hf mice. The cellular DNA was digested to completion with *Hpa*II or *Msp*I and analyzed as described in the legend to Fig. 1. Molecular weights denote the MuMTV-specific DNA fragments derived from *Msp*I digestion. The arrows identify the MuMTV-specific DNA fragments present after *Hpa*II digestion.

new DNA fragments resulting from *Eco*RI or *Hin*dIII digestion of different tumors were compared, the size of at least one new proviral DNA fragment appeared to be the same in at least half of the C3Hf tumors examined, suggesting that this newly acquired proviral DNA may be integrated at the same site in the cellular genome of these different tumors. Because no transforming gene has been detected in the genome of either exogenous or endogenous MuMTV, our results suggest that the mode of tumor induction by MuMTV may depend on activation of cellular oncogenes by a proviral DNA promoter, as is the case in bursal lymphoma induction by avian leukosis virus (18, 32, 33). Recently, it has been shown that exogenous C3H MuMTV proviral DNA is integrated in similar sites in a number of different C3H early mammary tumors (R. Nusse and H. Varmus, personal communication). More definitive studies, such as the isolation of specific RNA transcripts containing both viral and cellular sequences, are necessary to establish the role of newly integrated endogenous MuMTV in the induction of mammary tumors in C3Hf mice.

If integration of new proviral DNA is necessary for C3Hf mammary tumor development, and if this integration results from endogenous MuMTV infection, then the late appearance of C3Hf mammary tumors in C3Hf mice could be explained by the time needed for enough virus to be produced for infection and proviral DNA reintegration.

The intensities of the bands representing the newly acquired proviruses that were detected in many of the C3Hf tumors examined with an MuMTV-specific $cDNA_{rep}$ probe were found to be less than the intensity of the preexisting endogenous proviral DNA, indicating that fewer copies of newly integrated proviral DNA per tumor mass were present than there are copies of each preexisting endogenous provirus. Work from many laboratories (21, 25, 37, 43) has shown that a sizeable population of non-tumorigenic cells is present in mammary tumors. Recent work by MacInnes et al. (23) and Morris et al. (29) has also shown that tumor cells within the same mammary tumor mass may be heterogeneous with respect to the integration of MuMTV proviral DNA sequences and that the integration site of proviral DNA is more important with respect to tumorigenic potential than the number of integrated proviral DNA copies, since many non-tumorigenic cells also contain new proviral DNA integrations. New proviral DNA fragments not detectable in DNA from original tumor cells were detected in tumors caused when the original tumors cells were injected into mice, suggesting selection of tu-

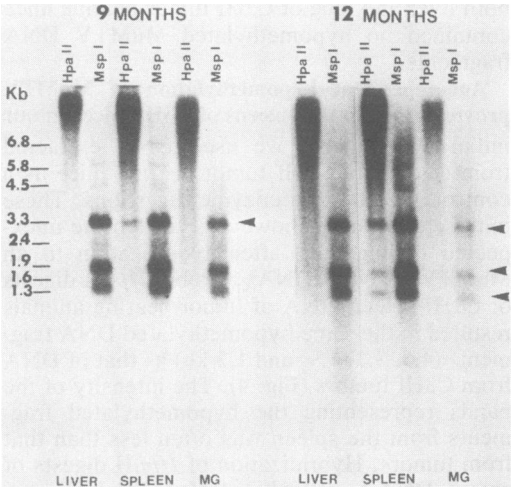

FIG. 7. *Hpa*II and *Msp*I restriction pattern of MuMTV-specific proviral DNA in genomic DNA isolated from livers, spleens, and mammary glands (MG) of 9- and 12-month-old non-tumor-bearing C3Hf mice. The cellular DNA (approximately 5 μg) was digested to completion with *Hpa*II or *Msp*I and analyzed as described in the legend to Fig. 1. Molecular weights are given for the MuMTV-specific DNA fragments derived from *Msp*I digestion. The arrows identify the MuMTV-specific DNA fragments present after *Hpa*II digestion.

morigenic cells in tumor development (29). The probability of detecting certain new proviral DNA fragments therefore depends on the percentage of cells in the tumor which contain these specific new integrations. Thus, detection of new proviral DNA is made more difficult than it would be if each cell of the tumor mass contained the same copy number of newly integrated proviral DNA as it did of preexisting endogenous proviruses. The lower intensity of the bands representing the newly acquired proviruses relative to those bands representing preexisting endogenous proviruses may be a result of mixed, but limited, populations of cells within the tumors and may therefore explain why we are unable to detect common integration sites for new proviral DNA in all of the C3Hf tumors that we analyzed. Also, proviral DNA fragments would not be detected by the methods used in our study if the newly inserted DNA has *Eco*RI or *Hin*dIII fragments which are the same size as those of the already existing endogenous proviruses. Therefore, it is difficult as yet to determine exactly how many new proviruses are present in C3Hf mammary tumor DNA.

Our study of the extent of methylation of endogenous proviral DNA in various organs of the C3Hf mouse has revealed that endogenous MuMTV proviral DNA is hypomethylated in both mammary tumors and spleens of tumor-bearing C3Hf mice and that in both tissues the same proviral DNA sequences are hypomethylated. No new hypomethylated DNA fragments (i.e., those not present in *Msp*I digests of C3Hf liver DNA) which would represent junction fragments of newly integrated proviral DNA and host cell DNA were detected in mammary tumor digests. This may be a result of the number of cells in each tumor mass which contain newly integrated proviral DNA, as previously discussed for *Eco*RI and *Hin*dIII digests.

Livers of both C3Hf tumor-bearing and normal mice remain extensively methylated. By determining the endogenous proviral unit(s) from which each hypomethylated DNA fragment could be derived, we found that proviral unit V (MTV-1) could be hypomethylated in every one of its *Hpa*II sites. Two of the hypomethylated fragments (4.5 and 3.3 kb) hybridize to the LTR-specific probe and therefore could represent both the 5′ and 3′ sides of the provirus. Also, two fragments of 1.9 and 1.3 kb hybridize only to the $cDNA_{rep}$ probe and are internal to the genome. The total size of these hypomethylated fragments is 11 kb, suggesting that some cellular sequences are attached to one or both end fragments. As discussed previously, the MTV-1 locus has been shown by a series of backcrosses of C3Hf mice to the virus-negative BALB/c strain to be the single dominant gene of C3Hf mice responsible for the expression of endogenous MuMTV viral antigens and an increased risk of mammary carcinoma.

Hypomethylation of MuMTV proviral DNA in spleens of non-tumor-bearing mice of 3, 6, 9, and 12 months progressed with age; however, we did not detect hypomethylation of proviral DNA sequences in C3Hf mammary glands in mice of identical ages. This result is somewhat unexpected, since, by the age of 12 months and earlier, viral antigens can be detected in the mammary glands of all C3Hf mice. It is possible that the viral antigens expressed in C3Hf milk up to at least 12 months of age are a result of infection by endogenous MuMTV, which results in the integration of hypomethylated endogenous proviral DNA. Detection of the hypomethylated fragments of this new proviral DNA may not be possible by our methods if the percentage of infected cells in a mammary gland population is less than 10% (5) and/or if the number of copies of newly integrated proviral DNA in the normal infected mammary gland cells is very low (4, 5, 13). Similarly, the viral antigens produced by the mammary gland cells may result from hypomethylation of existing endogenous proviral DNA in less than 10% of the cell population and therefore could not be detected by the methods employed in this study. From our results, it appears that specific hypomethylation of existing endogenous proviral DNA is occurring in a large enough number of spleen cells in non-tumor-bearing mice to allow detection.

Splenic tissue and lymphocytes have often been implicated as playing a role in MuMTV infection. Dux and Mühlbock (9, 10) have demonstrated, with mammectomized mice, that MuMTV replicates in organs other than the mammary gland and can be transferred from mammectomized mice by spleen cells to recipient mice, in which mammary tumors will then develop. Hilgers et al. (20) reported the presence of MuMTV viral antigens in the spleens of C3Hf mice; others have reported that lymphocytes carry cell-bound MuMTV activity (16, 17, 28, 30). However, no mature MuMTV particles have been detected as yet in lymphoid tissue.

We cannot at present determine the source of MuMTV endogenous virus which infects the mammary glands of C3Hf mice. Our results indicate that the MTV-1 locus, which is responsible for viral antigen production and increased mammary carcinoma in C3Hf mice, is hypomethylated in both C3Hf mouse mammary tumors and spleens. The possibility exists that hypomethylation of proviral DNA and subsequent endogenous virus production is occurring in C3Hf mammary glands, and these mature virus particles reinfect the same C3Hf mammary

glands. Alternatively, the hypomethylated MuMTV proviral DNA observed in spleens early in the lifetime of C3Hf mice, and which becomes progressively demethylated as the mouse ages, may produce a low level of endogenous MuMTV which infects the mammary glands of C3Hf mice. Whatever the source of the endogenous C3Hf MuMTV, the C3Hf mammary tumor contains new MuMTV proviral DNA inserted into host genome locations that may be common to many C3Hf tumors. Our results are consistent with a promoter insertion mechanism for mammary tumor induction; that is, the viral promoter of the newly inserted provirus allows the transcription of a nearby cellular oncogene which is involved in mammary tumorigenesis in C3Hf mice.

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