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Mouse Mammary Tumor Virus Proviral Sequences Congenital to C3H/Sm Mice Are Differentially Hypomethylated in Chemically Induced, Virus-Induced, and Spontaneous Mammary Tumors

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C3H/Sm mice have lost the exogenous milk-borne mouse mammary tumor virus (MMTV) characteristic of the C3H strain and have a very low (1.5%) incidence of spontaneous mammary tumors, yet they are highly susceptible to mammary carcinogenesis by either chemical carcinogens or infection with the milk-borne virus. We have analyzed the MMTV proviral DNA content of normal tissues and of spontaneous, virus-induced, and chemically induced mammary tumors by restriction endonuclease digestion and Southern blot analysis. Although the results clearly showed additional MMTV sequences in the virus-induced tumor which are not present in normal liver DNA, none of the spontaneous or chemically induced tumors could be shown to contain either newly acquired exogenous or amplified endogenous MMTV sequences. Interestingly, mammary tumors arising in C3H/Sm mice treated simultaneously with infectious MMTV (C3H) and dimethylbenz[*a*]anthracene (DMBA) possessed new exogenous MMTV DNA even though no quantitative change in tumor production was observed when these mice were compared with C3H/Sm mice treated with DMBA alone (Smith et al., Int. J. Cancer **26**:373–379, 1980). Our data indicate that the endogenous MMTV proviral units are extensively methylated in normal tissues, such as livers and normal nonlactating mammary glands. In the absence of MMTV (C3H), we found that in the rare, spontaneously occurring C3H/Sm mammary tumors, certain endogenous MMTV sequences were specifically hypomethylated. Hypomethylation of endogenous MMTV sequences was also noted in the chemically induced mammary tumors, even though radioimmune competition assays for MMTV gp52 and p28 are negative (Smith et al., Int. J. Cancer **27**:81–86, 1981). Our results support the conclusion that amplification of endogenous MMTV sequences is not intrinsic to C3H/Sm mouse mammary tumors arising spontaneously or after induction by chemicals. On the other hand, integration of exogenous MMTV DNA into the genome was a constant feature of mammary tumors developing in MMTV (C3H)-infected C3H/Sm mice, even when DMBA was used as the carcinogen. Hypomethylation of some endogenous MMTV sequences is characteristic of C3H/Sm mammary tumors, whether spontaneous or induced by chemicals, which suggests that these sequences are located in actively transcribing regions of the tumor cell genome.

C3H/Sm mice (previously designated C3H/StWi) spontaneously lost their exogenous milk-borne mouse mammary tumor virus (MMTV), MMTV (C3H) or MMTV (S), in 1958 and became a low-mammary-cancer subline (22). Unlike other MMTV (C3H)-free C3H sublines, however, they do not ordinarily express MMTV antigens or complete virions although they actively transcribe their endogenous MMTV proviral sequences into RNA (24). The incidence of spontaneous mammary cancer in uninfected C3H/Sm mice is only 1.5%, the tumors developing in very old, retired breeders at an average age of 23 months. C3H/Sm mice are susceptible to mammary tumorigenesis by the chemical carcinogens 7,12-dimethylbenz[*a*]anthracene (DMBA) and urethane, but they are resistant to the development of an increased rate of mamma-

ry tumorigenesis when exposed to excess hormonal stimulation by implants of 20% diethylstilbestrol or by pituitary isographs (24, 25). The C3H/Sm subline is susceptible to the reintroduction of exogenous MMTV, whereupon it becomes a stable, high-mammary-cancer C3H subline (22–24). The latter subline has been maintained for over 14 generations without losing the exogenous virus. Simultaneous infection of C3H/Sm mammary glands with exogenous MMTV and exposure to DMBA do not lead to an increased tumor risk in virgin females over those treated with the chemical alone (23). Chemical induction of mammary neoplasia in C3H/Sm virgins was accompanied by the appearance of mammary ductal hyperplasia, whereas virus-induced neoplasia is commonly associated with alveolar hyperplasia. Both types of dysplasia were present in the glands of virgin females simultaneously exposed to exogenous MMTV and DMBA, suggesting separate pathways to neoplasia for chemicals and mammary tumor virus.

Mammary tumor virus antigens were present in all of the tumors developing in MMTV (C3H)-infected, DMBA-treated glands, but only sporadically and at much lower levels than in those tumors arising spontaneously or induced by chemicals alone (23, 25). In the latter case, in most of the positive chemically induced tumors (10 of 32), only the MMTV *gag* protein, p28, was detected. The presence of p28 in the absence of detectable *env* gene products suggested noncoordinate expression of the MMTV germline sequences endogenous to C3H/Sm mice. Although virus particles were uniformly present in DMBA-induced, MMTV (C3H)-infected mammary tumors, they were never observed by electron microscopy in C3H/Sm spontaneous tumors or in those induced by chemicals alone. MMTV RNA levels in C3H/Sm mammary tumors induced by chemicals alone were quantitatively equivalent to or less than those observed in normal resting or lactating C3H/Sm mammary glands (24, 25). Conversely, 10- to 100-fold-higher MMTV RNA levels were found in the normal glands and tumors of DMBA-treated, MMTV (C3H)-infected C3H/Sm mice.

In the present study, we have examined the restriction patterns of the DNA isolated from spontaneous, virus-induced, and chemically induced C3H/Sm mammary tumors. These restriction patterns were compared with those of normal C3H/Sm tissues and mammary tumors of C3H/He mice. The studies were performed to determine to what extent MMTV proviral information was amplified in mammary tumors induced by chemicals in the presence and absence of hormones and exogenous MMTV. We also sought to determine whether differential regulation of MMTV proviral sequences occurs in any of the tumors, using hypomethylation of MMTV-specific sequences as an indicator.

MATERIALS AND METHODS

Animals. The mice were bred and maintained in a closed colony. All experimental mice were kept as virgins, four to six in a cage, given water and food ad libitum, and housed in a temperature- and light cycle-controlled room. DMBA (Calbiochem, San Diego, Calif.) and urethane were administered to C3H/Sm virgins in the presence and absence of chronic hormonal stimulation of the mammary gland by pituitary isografts as reported elsewhere (25). Urethane was administered one time per week for 8 to 10 weeks (20 mg/mouse) by intraperitoneal i.p. injection. A 1.0-mg amount of DMBA, dissolved in 0.2 ml of cottonseed oil, was fed intragastrically once weekly until the appropriate dosage was achieved (for these experiments 2 and 6 mg of DMBA were administered).

Tumor incidence and the distribution of MMTV *env*(gp52) and *gag*(p28) antigens in these tumors have been reported elsewhere (25).

Mammary tumors from each of the experimental groups were partitioned and frozen for testing MMTV DNA content and immunological studies. Spontaneous tumors which had developed in very old C3H/Sm females served as non-experimental controls. Mammary tumors which developed in C3H/Sm breeders infected with MMTV (C3H) were used as positive controls (23).

MMTV cDNA. MMTV (C3H) virions were obtained from the supernatant fluids of the C3H mammary tumor cell line Mm5mt/ci (19). Virus was concentrated and purified from the supernatant fluids of mammary tumor cultures by ammonium sulfate precipitation and a series of discontinuous and linear sucrose density gradients as previously described (8). A 5-μg amount of MMTV (C3H) 60 to 70S RNA was dissolved in 250 μl of a solution containing 2 mM dithiothreitol; 25 mM Tris-hydrochloride (pH 8.0); 25 mM KCl; 4 mM $MgCl_2$; 3 μg of actinomycin D per ml; 0.05 mM each of dATP, dGTP, and dTTP; 2 mg of calf thymus primer per ml; 345 U of avian myeloblastosis virus polymerase per ml; and 400 μCi of [^{32}P]dCTP (410 Ci/mmol; Amersham Corp., Arlington Heights, Ill.). The solution was incubated 90 min and extracted with a 1:1 mixture of chloroform-phenol. The aqueous phase was loaded onto a column (1.5 by 22 cm) of Sephadex G-50, the MMTV cDNA peak was identified, and the solution was precipitated in 2 volumes of ethanol.

Extraction of high-molecular-weight DNA. DNA was extracted from normal and mammary tumor tissue of C3H/Sm mice as previously described (9).

Oligo(dT)-cellulose selection of MMTV RNA. A 100-μg amount of 70S MMTV viral RNA was boiled for 10 min, rapidly chilled, made 0.5 M NaCl, and loaded onto a column of oligodeoxythymidylic acid [oligo(dT)]-cellulose. The column was extensively washed with the same buffer, and the polyadenylic acid-containing RNA was eluted with 10 mM Tris, pH 7.5. The final yield of polyadenylic acid-containing MMTV RNA was 10 μg. This MMTV-specific RNA was concentrated and used as a template to synthesize MMTV cDNA. This probe was designated 3′ MMTV cDNA (see Fig. 5). The 3′ MMTV cDNA probe

hybridized to all MMTV-containing DNA bands of *Eco*RI-digested normal C3H/Sm DNA, demonstrating that the probe contained MMTV sequences. When the same probe was hybridized to *Pst*I-digested normal C3H/Sm DNA, the 0.8-kilobase (kb) fragment, representing the long-terminal-repeat sequences, reacted best. However, some hybridization was observed to the 1.7-kb fragment representing the envelope region of the endogenous MMTV provirus. Consequently, the 3′ MMTV cDNA probe was enriched in those nucleic acid sequences from the 3′ end of the viral genome.

Restriction endonuclease digestion of DNA. *Eco*RI and *Pst*I restriction endonucleases were obtained from Boehringer Mannheim (Indianapolis, Ind.). *Bgl*II, *Msp*I, and *Hpa*II were purchased from Bethesda Research Laboratories (BRL; Bethesda, Md.), and *Sac*I was purchased from Biotec (Madison, Wis.). Conditions for *Bgl*II, *Sac*I, *Msp*I, and *Hpa*II digestion were those suggested by the manufacturer. *Eco*RI digestions were performed in 100 mM Tris-hydrochloride (pH 7.5)–50 mM NaCl–5 mM $MgCl_2$–100 μg of bovine serum albumin per ml. *Pst*I reaction buffer consisted of 6 mM $MgCl_2$, 6 mM 2-mercaptoethanol, and 100 μg of bovine serum albumin per ml. *Eco*RI restriction digestions at 5 to 10 U of enzyme per μg of DNA were incubated for 4 h at 37°C, whereas *Pst*I restriction digestions at 2 U/μg of DNA were incubated for 2 h at 37°C. *Msp*I and *Hpa*II restriction digestions at 2 U/μg of DNA were incubated at 37°C for 4 h. *Bgl*II utilized 1 U/μg of DNA and 4 h for digestion, whereas *Sac*I used 2 U/μg of DNA and 4 h. A 10-μg amount of DNA was loaded per lane. Lambda bacteriophage DNA (New England Biolabs, Beverly, Mass.), digested with *Eco*RI, was used to provide marker bands for molecular weight determinations.

Gel electrophoresis, DNA transfer, and hybridization. After restriction endonuclease digestion, DNAs were adjusted to 20% glycerol and electrophoresed in 0.8% agarose (BRL) containing 0.02 M sodium acetate and 0.32 mM Tris, pH 8.05. Bromophenol blue was added as tracking dye, and gels were electrophoresed at 35 V until the dye migrated 13 to 16 cm. Ethidium bromide (5 μg/ml) staining was used to visualize the lambda marker DNAs after electrophoresis. DNA was transferred to nitrocellulose filter paper by the method of Southern (26). Gels were treated with denaturing solution (1.5 M NaCl, 0.05 M NaOH) for 20 min, neutralized (3.0 M NaCl, 0.5 M Tris-hydrochloride, pH 7.5) for 40 min, and equilibrated with the 6× SSC transfer buffer (SSC = 0.15 M NaCl, 0.015 M sodium citrate) for 10 min. After the DNAs had been transferred, the filters were dried in a vacuum oven at 80°C for 4 h.

Virus-specific DNA sequences were detected by hybridization of the filters to ^{32}P-labeled DNA complementary to MMTV (C3H) 70S RNA (cDNA). Filters were soaked in 3× SSC at 65°C for 30 min and then prehybridized in 3× SSC–0.1% sodium dodecyl sulfate–100 μg of sonicated, denatured dog DNA per ml–Denhardt buffer (0.2% each of bovine serum albumin, Ficoll, and polyvinylpyrrolidone) for 4 h at 65°C. [^{32}P]cDNA was added at 4×10^6 cpm per ml and hybridized for 15 h. After incubation, the filters were washed with 3× SSC–0.1% sodium dodecyl sulfate–Denhardt solution at 5-min intervals until the wash buffer contained only 100 cpm of ^{32}P per ml. The filters were then washed with 0.3× SSC–0.1% sodium dodecyl sulfate at 15-min intervals as indicated above. Filters were air dried and were autoradiographed with Kodak X-OMAT R film.

RESULTS

Search for amplification of MMTV proviral DNA in C3H/Sm mammary tumors. We wished to determine whether DNA sequences related to MMTV were amplified in either chemically induced or spontaneously arising mammary tumors in C3H/Sm mice. Because the restriction endonuclease *Eco*RI cleaves most known MMTV genomes at a single internal site (6, 11, 12, 21), the size of the MMTV-containing DNA fragment will differ for different viral integration sites, depending on the location of the nearest *Eco*RI sites in the host flanking sequences. High-molecular-weight DNA was extracted from tumors or normal tissues, digested with *Eco*RI, and electrophoresed in agarose gels, and the sizes of the MMTV-containing fragments were analyzed by Southern blot hybridization. Figure 1 shows the results obtained for seven C3H/Sm tumors (one MMTV induced, two spontaneous, one DMBA induced, one induced by a combination of DMBA and hormone treatment via pituitary isografts, and two in animals infected with MMTV (C3H) and treated with DMBA). All three mammary tumors that developed in animals exposed to the milk-borne, exogenous MMTV (C3H), regardless of whether they were exposed to chemicals or not, showed additional DNA bands (Fig. 1, lanes f, g, and h, see arrows) when compared with normal C3H/Sm tissues (Fig. 1, lanes a, b, and c).

As expected, a mammary tumor from a C3H/He mouse also showed additional bands (Fig. 1, lane 1) not present in the C3H/He liver (Fig. 1, lane k). The MMTV-specific DNA profiles obtained with the two spontaneous mammary tumors (Fig. 1, lanes d and e), the chemically induced tumor (Fig. 1, lane i), and the tumor induced by combined hormone and chemical treatment (Fig. 1, lane j) are identical to the DNA profiles obtained with normal and lactating mammary glands. Parenthetically, both the pattern and the number of endogenous bands in the C3H/Sm liver (Fig. 1, lane a) differ from those of the C3H/He liver (Fig. 1, lane k). The additional bands in C3H/Sm confirm the observation of Cohen and Varmus (6) of an additional endogenous MMTV provirus in this C3H subline.

The restriction endonuclease *Pst*I cuts the MMTV genome at multiple internal sites (4, 6, 14, 18, 21). *Pst*I digests of murine DNA have been useful in determining which endogenous proviral units are present in different inbred mouse strains (6). All C3H/Sm mice examined contained *Pst*I restriction fragments of 5.1, 4.8,

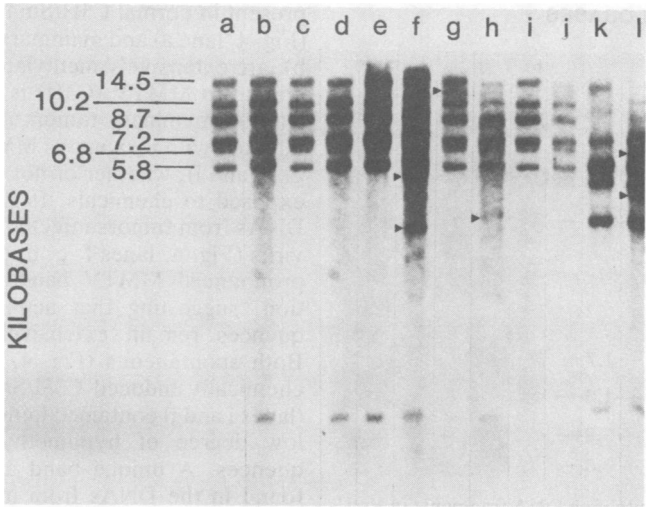

FIG. 1. MMTV-containing DNA fragments of *Eco*RI-digested C3H/Sm tissues. Ten micrograms of DNA was digested, electrophoresed on 0.8% agarose gels, transferred to nitrocellulose filter paper by the method of Southern (26) and then hybridized to 5×10^5 cpm of MMTV [^{32}P]cDNA per ml, representing the entire viral genome. Lane a, Normal C3H/Sm liver. Lane b, Normal C3H/Sm mammary gland. Lane c, Lactating C3H/Sm mammary gland. Lanes d and e, Spontaneous C3H/Sm mammary tumors. Lane f, Mammary tumor arising in a C3H/Sm mouse infected with MMTV (C3H). Lanes g and h, Mammary tumor arising in C3H/Sm mice infected with MMTV (C3H) and exposed to DMBA. Lane i, Mammary tumors arising in DMBA-treated C3H/Sm mouse. Lane j, Mammary tumor arising in a DMBA-treated C3H/Sm mouse exposed to high levels of hormones via implantation of pituitary isografts. Lane k, Normal C3H/He liver. Lane l, Spontaneous mammary tumor arising in a C3H/He mouse.

3.9, 1.7, 1.3, 0.9, and 0.8 kb. Of interest is the fact that C3H/Sm mice contained *Pst*I fragments of 3.9 and 0.9 kb which are absent in the C3H/HeN liver (Fig. 2, lane m). However, since the 0.9-kb MMTV specific fragment is present only once per haploid genome, it is difficult to resolve routinely on autoradiographs unless they are overexposed. The 3.9- and 0.9-kb fragments are markers for the endogenous MMTV Unit IV and the proviral DNA of the highly infectious MMTV (C3H) (Fig. 2, lanes g and n). Consequently, unlike other C3H strains of mice, C3H/Sm mice have acquired, as an endogenous provirus, a MMTV variant bearing the *Pst*I restriction site of a highly oncogenic virus.

Digestion of DNAs from normal or neoplastic C3H/Sm tissues indicates the absence of a 4.3-kb *Bgl*II fragment characteristic of the MMTV (C3H) variant (6, 13).

This fragment was found in MMTV (C3H) virus-induced C3H/Sm and C3H/HeJ mammary tumors and in cat cells infected by MMTV (C3H). The *Bgl*II data, like the *Eco*RI data, indicated that no amplification of the MMTV proviral content occurred in the spontaneous and chemically induced mammary tumors of C3H/Sm mice.

The absence of an 8.0-kb band in *Sac*I digests of C3H/Sm DNA is yet another distinction between the Unit IV provirus in these mice and the MMTV (C3H) DNA. The *Sac*I data (not shown) were consistent with the *Eco*RI and *Bgl*II results in showing no additional bands in any of the tumors, with the exceptions of the C3H/Sm virus-induced tumor and the C3H/HeJ tumor. Both of these MMTV (C3H)-induced mammary tumors contain a very prominent 8.0-kb band. Again, as with the *Eco*RI and *Bgl*II results, it was noted that there was significant variation in the sizes of the MMTV fragments from DNAs of C3H/Sm and C3H/HeJ mice, which reflects differences in the flanking sequences of their respective MMTV proviruses.

Differential methylation of MMTV proviral sequences in C3H/Sm mammary tumors. We examined the 5-methylcytosine content of spontaneous and chemically and virally induced C3H/Sm mammary tumors since several investigators have suggested that this base modification may play a role in gene expression during differentiation (13, 20). More importantly, using the restriction endonuclease *Msp*I, which cleaves at the recognition sequence 5'-CCGG and its isoshizomer *Hpa*II, which is inhibited by methylation of the cytosine base of the CpC dinucleotide, Cohen (3) has shown that MMTV proviruses acquired by germline infection are extensively methylated, whereas those acquired by milk-borne infection are not. Figure 3 shows the restriction pattern generated when the

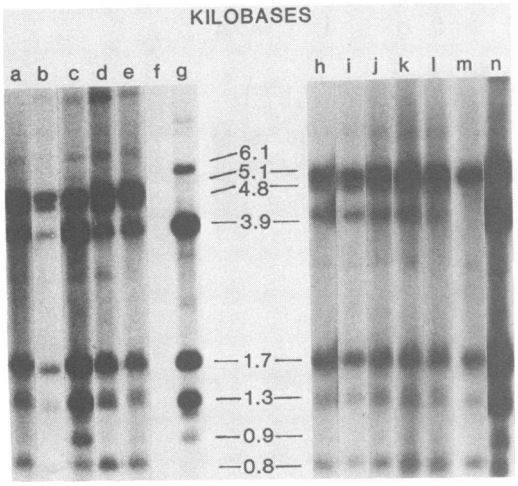

FIG. 2. MMTV-containing DNA fragments in *Pst*I-digested C3H/Sm tissues. Conditions were as described in the legend to Fig. 1. Lane a, Normal C3H/Sm liver. Lane b, Lactating C3H/Sm mammary gland. Lanes c, d, and e, Spontaneous C3H/Sm mammary tumors. Lane f, Normal feline cells. Lane g, Feline cells infected with MMTV (C3H). Lanes h, i, and l, Mammary tumors of C3H/Sm mice treated with urethane and exposed to hormonal stimulation by placing a pituitary isograft under the kidney capsule. Lanes j and k, Mammary tumors arising in C3H/Sm mice treated with DMBA and exposed to hormones stimulation by placing a pituitary isograft under the kidney capsule. Lane m, Normal C3H/He liver. Lane n, Spontaneous C3H/He mammary tumors.

DNAs from C3H/Sm mammary tumors were digested with *Msp*I. C3H/Sm liver (Fig. 3, lane a), normal and lactating mammary glands (Fig. 3, lane b and c), and all of the mammary tumor (Fig. 3, lanes d through j) DNAs contain intense restriction bands at 3.6, 2.3, 1.8, and 0.7 kb.

No additional bands could be seen in DNA from spontaneous (Fig. 3, lanes d and e) or chemically induced (Fig. 3, lanes i and j) mammary tumors. Nevertheless, all three mammary tumors arising in MMTV (C3H)-infected C3H/Sm mice contained an additional MMTV-specific band at 1.6 kb, whether or not they were treated with DMBA. This 1.6-kb MMTV band appears to be a marker for the exogenous MMTV (C3H) because it was also found in the C3H/He mammary tumor and in normal cat cells infected with MMTV (C3H) (data not shown) but not in C3H/He liver DNA (Fig. 3, lane k).

To determine which MMTV fragments were hypomethylated, the DNAs from the same tissues were digested with the restriction endonuclease *Hpa*II, which will not cleave a methylated CpG dinucleotide, and then hybridized with an MMTV cDNA probe representative of the entire MMTV genome. MMTV proviral sequences present in normal C3H/Sm tissues, such as liver (Fig. 4, lane a) and mammary glands (Fig. 4, lane b), are extensively methylated. The 1.6-kb band unique to MMTV (C3H) is clearly hypomethylated in mammary tumors arising in all animals infected with exogenous MMTV (Fig. 4, lanes f, g, h, and l), whether or not the animal had been exposed to chemicals. It should be noted that DNAs from tumors infected with the milk-borne virus (Fig. 4, lanes f, g, h, and l) gave the most pronounced MMTV bands after *Hpa*II digestion, suggesting that acquired exogenous sequences remain extensively hypomethylated. Both spontaneous (Fig. 4, lanes d and e) and chemically induced C3H/Sm mammary tumors (lanes i and j) contained faint bands, suggesting a low degree of hypomethylation of viral sequences. A unique band at about 1.7 kb was found in the DNAs from mammary tumors induced by chemicals alone (Fig. 4, lanes i and j).

We attempted to determine whether different endogenous proviruses were hypomethylated in different C3H/Sm tumors. To approach this question, we selected 70S MMTV RNA on

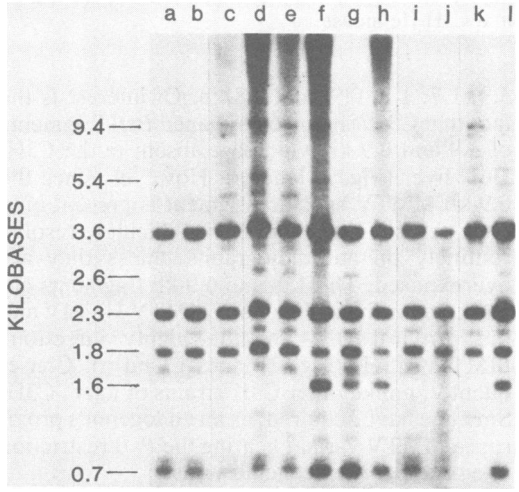

FIG. 3. MMTV-containing DNA fragments in *Msp*I-digested C3H/Sm tissues. Experimental conditions were as described in the legend to Fig. 1. Lane a, Normal C3H/Sm liver. Lane b, C3H/Sm virgin mammary gland. Lane c, Lactating C3H/Sm mammary gland. Lanes d and e, Spontaneous C3H/Sm mammary tumors. Lane f, Mammary tumor arising in a C3H/Sm mouse infected with MMTV (C3H). Lanes g and h, Mammary tumors arising in C3H/Sm mice infected with MMTV (C3H) and exposed to DMBA. Lane i, Mammary tumor arising in a DMBA-treated C3H/Sm mouse. Lane j, Mammary tumor arising in a urethane-treated C3H/Sm mouse. Lane k, Normal C3H/He liver. Lane l, Spontaneous mammary tumor arising in a C3H/He mouse.

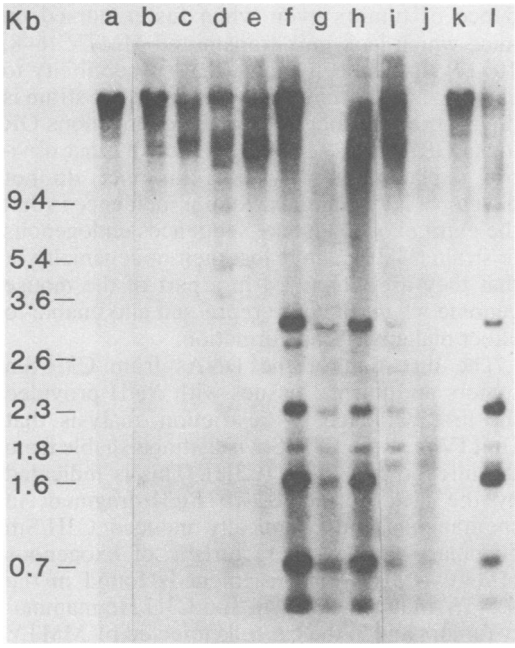

FIG. 4. MMTV-containing DNA fragments in *Hpa*II-digested C3H/Sm tissues. Experimental conditions were as described in the legend to Fig. 1. Lane designations are as listed in the legend to Fig. 3.

oligo(dT)-cellulose to isolate RNA enriched in fragments at the 3′ end. Since the 3′ end of the viral RNA is represented at each end of the integrated provirus in the form of long-terminal repeats, cDNA synthesized from oligo(dT)-selected MMTV RNA hybridizes best with fragments of cellular DNA containing virus/host DNA junctions. Assuming that proviruses are integrated at different sites, unique MMTV-containing fragments should appear if different proviruses are hypomethylated. When 3′ MMTV cDNA was hybridized to C3H/Sm tissues (Fig. 5), both spontaneously arising mammary tumors (Fig. 5, lanes d and e) contained hypomethylated bands different from those found in most other C3H/Sm tissues (indicated by arrows), suggesting the hypomethylation of specific endogenous proviruses in these tumors. In addition, these tumors, which are histologically different (one being a carcinoma [Fig. 5, lane d], the other being a fibroadenoma [Fig. 5, lane e]), also have hypomethylated sites which are different from each other.

Although bands of 9.4 and 5.4 kb appeared in many of the *Msp*I digests of C3H/Sm tissues (whether hybridized with cDNA representing the entire MMTV genome [Fig. 3] or cDNA synthesized from the oligo(dT)-selected MMTV [data not shown]), they were hypomethylated only in one spontaneous mammary tumor (Fig. 5, lane e). Additional high-molecular-weight bands may represent a combination of hypo- and hypermethylated sites in the MMTVs of this tumor, resulting in bands which represent segments combining one or more of the low-molecular-weight fragments. This is possible because *Msp*I can cut at CmCGG but not at mCCGG, whereas *Hpa*II will cut mCCGG. Thus, a mixture of mCmCCC, mCCGG, CmCGG, and CCGG sequences at one or several sites could explain this observation. A similar phenomenon has been described by Maraud and co-workers (15).

The 3′ cDNA probe did not hybridize to the 1.7-kb fragment in two chemically induced C3H/Sm mammary tumors (Fig. 5, lanes i and j). However, this fragment was observed when the unselected MMTV cDNA probe was hybridized to DNA from the same mammary tumors (Fig. 4). Consequently, this 1.7-kb fragment, which is hypomethylated in chemically induced tumors, probably is derived from the middle of a specific endogenous provirus.

Hybridization with the 3′ cDNA MMTV probe also indicated a hypomethylated fragment in lactating C3H/Sm mammary gland DNA (see 3.6-kb fragment in Fig. 5, lane c) that was not observed in virgin mammary gland DNA (Fig. 5,

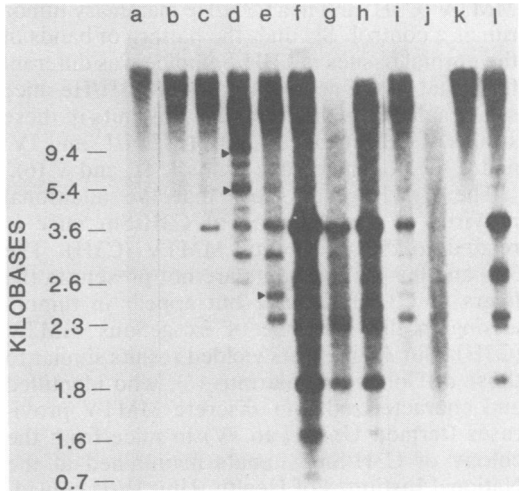

FIG. 5. MMTV-containing fragments in *Hpa*II-digested C3H/Sm DNA identified by hybridization to an MMTV cDNA probe enriched in sequences at the 3′ end of the MMTV genome. Experimental conditions were as described in the legend to Fig. 1, except that 1×10^5 cpm of MMTV cDNA per ml was hybridized. Lane designations are as listed in the legend to Fig. 3. The 70S MMTV RNA used to make MMTV cDNA was selected twice on oligo(dT)-cellulose as described in the text.

lane b). Thus, hormonal stimulation apparently leads to the hypomethylation of MMTV-specific sequences in mammary gland epithelium.

DISCUSSION

The C3H/Sm strain is unusual in that it lacks both the milk-transmitted MMTV and the high incidence of early arising mammary tumors seen in C3Hf mice. Spontaneous mammary tumors arise in about 1% of the mice of this substrain at an advanced age. We, therefore, sought to characterize the MMTV proviral content of their rare spontaneous tumors to determine whether they were associated with a rare infection by the MMTV (C3H) or by amplification of endogenous sequences.

We used six restriction enzymes (*Eco*RI, *Pst*I, *Bgl*II, *Sac*I, *Msp*I, and *Hpa*II) to characterize the MMTV proviral content of C3H/Sm mice and to look for amplification of these sequences in spontaneous, DMBA-induced, DMBA-hormone-induced, and urethane-hormone-induced mammary tumors. The results of the *Eco*RI analysis were interesting in three respects. First, they were indicative that no amplification of the endogenous proviruses had taken place in the spontaneous or chemically induced tumors, although amplification of MMTV DNA could be easily detected in the DNA from a C3H/Sm mammary tumor induced by infection with MMTV (C3H) and in a C3H/He mammary tumor run as a control. Second, the pattern of bands of the normal tissues of C3H/Sm mice was different from that of the normal tissues of C3H/He mice as a result of the different proviral units in these strains. C3H/Sm have Units I, II, III, and IV, and C3H/HeJ mice have Units I, II, and V (6).

The *Pst*I digests show that the additional provirus in the germline of C3H/Sm mice is related to the exogenous MMTV (C3H). The 3.9- and 0.9-kb fragments are not present in the livers of C3H/HeJ mice but appear in tumors arising in the presence of exogenous MMTV (C3H). Our *Pst*I digests yielded results similar to those of Cohen and Varmus (6), who identified and characterized four discrete MMTV proviruses (termed Units I to IV) in mice from the colony of C3H/Sm animals maintained at the National Institutes of Health. Unit IV is considered to be similar or identical to the exogenous virus since it contains the 3.9-, 1.7-, 1.3-, and 0.9-kb internal fragments of MMTV (C3H) proviral DNA. This raises the question of why the C3H/Sm mice have a low incidence of mammary tumors if they have incorporated the MMTV (C3H) virus into their germline. GR mice transmit a highly oncogenic MMTV in their breast milk and have also incorporated a copy of this virus into their germline and have a high incidence of tumors even when foster nursed on mice which lack milk-transmitted MMTV (6–8, 10, 17). It has been shown that susceptibility to early mammary tumorigenesis in the GR strain is due to the presence of a single endogenous GR locus GR-MTV-2 (1, 9, 16, 17). The most obvious explanations why C3H/Sm mice do not develop a high mammary tumor incidence is that the extra exogenous-like sequences endogenous to strain C3H/Sm have lost their oncogenicity or that they are integrated in a part of the mouse genome where they are repressed and unable to effect malignant transformation.

The digestion of the DNAs from C3H/Sm tumors and normal tissues with *Bgl*II provided the first evidence by restriction analysis that Unit IV in strain C3H/Sm is distinguishable from the infectious MMTV (C3H). This is indicated by the lack of the 4.3-kb *Bgl*II fragment in spontaneous and chemically induced C3H/Sm mammary tumors characteristic of exogenous MMTV (C3H). This fragment is found in the MMTV-induced C3H/Sm and C3H/He mammary tumors and in the cat cells infected by MMTV (C3H). The *Bgl*II data, like the *Eco*RI data, indicate that no amplification of the MMTV proviral content occurred in the spontaneous and chemically induced mammary tumors of C3H/Sm mice.

The absence of an 8.0-kb band in *Sac*I digests of C3H/Sm DNA is yet another distinction between the Unit IV provirus in these mice and the DNA of the exogenous MMTV (C3H). The *Sac*I data also are consistent with the *Eco*RI and *Bgl*II results in showing no additional bands in any of the tumors, with the exceptions of the C3H/Sm virus-induced tumor and the C3H/He tumor. Both of the MMTV (C3H)-induced mammary tumors contained a very prominent 8.0-kb band. Again, as with the *Eco*RI and *Bgl*II results, it will be noted that there are considerable differences in the patterns of bands resulting from the differences in their proviral units.

The apparent absence of amplification of DNA sequences related to MMTV in a series of spontaneous and chemically induced mammary tumors was confirmed with three different restriction enzymes. Each of the tumors examined were separate primary tumors. This observation is in contrast to the reports of others (7, 11, 18), who found amplification routinely in DNAs from tumors arising in animals infected with exogenous virus or expressing endogenous virus.

We have found no obvious MMTV provirus amplification in our relatively large experimental sample, except when exogenous MMTV (C3H) was present. It is possible, if a new MMTV band does not appear in an *Eco*RI digest, that the provirus might have been amplified in the originally transformed cell but that the tumor popu-

lation is not clonal. Although this could happen occasionally, it apparently has not been observed in previous studies (7, 12, 18) and, therefore, it is very unlikely to have occurred repeatedly in several different types of primary tumors, such as reported here. Further, when these animals were exogenously infected, we invariably found MMTV DNA amplification in their tumors. On the other hand, an amplified provirus might have been integrated into an *Eco*RI domain similar to that of the original endogenous proviruses. However, in that case, we would expect an increase in the intensity of hybridization of at least two bands in the *Eco*RI digest. This was not observed (Fig. 1).

The above suggests that the mechanism(s) involved in the development of these tumors may differ from that of virus-induced mammary tumorigenesis where amplification of viral sequences is clearly evident. In addition, the absence of MMTV DNA amplification is consistent with the absence of MMTV virions and antigens in chemically induced and spontaneous C3H/Sm tumors (23, 24) and with the conclusion that viral and chemical oncogens may follow separate pathways in the malignant transformation of mammary epithelium.

It has been suggested that the methylation of cytosine to form 5-methylcytosine might play a role in the regulation of transcription of certain genes (13, 20). Although recent evidence indicates that methylation does not affect transcription of genes in the sea urchin (2), the work of Cohen (3) argues strongly for a correlation of hypomethylation of MMTV proviral DNA with the active transcription of these sequences in mouse mammary tumors.

Cohen's studies were conducted on tumors induced by infectious virus and, therefore, cannot clearly discriminate between the extent of methylation of the endogenous provirus compared with that of the acquired, infectious viral DNA. In the absence of infectious virus, we found specific patterns of hypomethylation which appeared to vary with respect to tumor type or carcinogenic stimulus or both. For example, different restriction patterns were found for the hypomethylated regions representative of each of the two spontaneous C3H/Sm mammary tumors examined. Each of these tumors exemplified a separate and specific histopathological class of mammary tumor. One was an adenocarcinoma; the other was a fibroadenoma.

Using the unselected MMTV cDNA probe, we saw a new (1.7 kb) fragment in tumors induced by chemicals in the absence of virus infection (Fig. 4). Unlike tumors induced by MMTV, which arise from alveolar cells, tumors induced by chemicals in C3H/Sm mice are ductal in origin. Therefore, it is conceivable that, in tumors arising from ductal lesions, a different provirus may be transcribed (or be close to a different area of active transcription). When MMTV was also present, however, this fragment appeared to be absent. This could be explained by the fact that this particular tumor may have arisen from a hyperplastic alveolar nodule, rather than from a chemically induced ductal lesion. Both lesions are found in mammary glands when simultaneously exposed to MMTV and DMBA (23).

One of the DMBA plus MMTV (C3H)-induced tumors was found to be positive for MMTV antigens (by immunoperoxidase), whereas the other DMBA plus MMTV (C3H) tumor was not. Both DNAs contained a 1.6-kb hypomethylated restriction fragment unique to DNAs from tissues infected with exogenous MMTV (C3H) (Fig. 3 and 4). A block which affects viral protein synthesis may therefore occur in some tumors induced by chemical treatment plus MMTV.

In the C3H/Sm tumors examined in this report, several of the MMTV proviral sequences became hypomethylated, suggesting transcriptional activity. In contrast, our earlier reports (23, 25) demonstrated that MMTV proviral expression, as determined by the presence of radioimmunologically detectable MMTV gp52 and p28, was negligible or absent in spontaneous, DMBA-, or urethane-induced C3H/Sm mammary tumors. Similar immunological results were obtained with normal C3H/Sm mammary tissues. In all of these tissues, MMTV RNA was readily detectable by hybridization with MMTV cDNA, which was consistent with our hypomethylation data. The final extent of protection of the cDNA probe indicated that all MMTV genome sequences were present in the total RNA (22). Further analysis showed that MMTV RNA was present in the cytoplasm of C3H/Sm mammary tissues, but that these sequences were representative of only approximately half of the MMTV cDNA, whereas nuclear RNA was able to protect the cDNA probe completely (23a).

With respect to MMTV proviral sequences in the C3H/Sm tumor we studied, there were several patterns of hypomethylation. These patterns may be dependent on tumor cell type or etiological factors. In spontaneous tumors, different tumor types appeared to have different methylation patterns. Chemically induced tumors had a pattern which may be reflective of a nonspecific hypomethylation, as well as having a characteristic 1.7-kb band. In the presence of exogenous virus, a unique pattern was consistently observed, despite the addition of other carcinogenic factors.

It remains to be determined whether the same

MMTV sequences are characteristically found in hypomethylated regions of different types of C3H/Sm mammary tumors.

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