Organization and Expression of Mouse Mammary Tumor Virus Sequences in Normal and Neoplastic C3Hf/HeSed Mouse Tissues

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The organization and expression of germinally transmitted mouse mammary tumor virus (MMTV) proviruses in C3Hf/HeSed mouse tissues were examined. Digestion with the restriction enzymes *EcoRI*, *BamHI*, and *HindIII* and hybridization with cloned probes specific for the long terminal repeat and the 5' and 3' regions of the MMTV genome revealed three full-length (units Ib, II, and V) and two subgenomic (units I and IX) MMTV proviruses in C3Hf/HeSed mouse germ line DNA. The *EcoRI* fragments (15.0 and 5.7 kilobase pairs [kbp]) that contained unit Ib were previously described as separate, subgenomic MMTV proviruses. The methylated state of each full-length MMTV provirus was examined in DNA from C3Hf/HeSed mouse livers, spleens, mammary glands, and mammary tumors by digestion with *EcoRI* or *BamHI* in combination with the methyl-sensitive restriction enzymes *HhaI* or *HpaII*. Unit Ib contained *HhaI*- and *HpaII*-sensitive sites in spleen, mammary gland, and mammary tumor DNA but was completely methylated in liver DNA. Units II and V contained *HhaI*- and *HpaII*-sensitive sites in mammary gland and mammary tumor DNA, but the sites were extensively methylated in spleen and liver DNA. The *HhaI*-sensitive sites were mapped to the 5' end of the 5' and 3' long terminal repeats of each full-length MMTV provirus. C3Hf/HeSed mouse tissue RNA was examined for MMTV transcripts. Mammary glands contained MMTV RNA species of 9.0, 3.8, and 1.7 kb. Mammary tumors contained high levels of the 9.0- and 3.8-kb transcripts but lacked the 1.7-kb species. A very low level of the 3.8-kb MMTV transcript was present in spleens. Livers lacked detectable MMTV RNA. These results implicate mammary tissue as the site of unit V activation in the formation of MMTV virions.

Environmental agents, hormones, genes, and the milk-transmitted mouse mammary tumor virus (MMTV) are all factors that influence mammary tumorigenesis in laboratory mice (1). C3H/He mice are infected with the milk-transmitted MMTV and have a mammary tumor incidence of ca. 90% at 1 year of age (2).

C3Hf/He mice, which have been freed of the milk-transmitted MMTV, have a mammary tumor incidence of 22 to 52% at 2 years of age (14, 25). The development of late-occurring tumors in C3Hf/He mice is preceded by the appearance of MMTV virions in the milk of the mother (29). Van Nie and Verstraeten (29) have shown that the expression of MMTV particles in C3Hf/He mice is controlled by a single dominant gene, *Mtv-1*, and that virus production is correlated with an increased mammary tumor incidence. C3Hf/He mice contain several endogenous MMTV proviruses integrated in their germ line DNA (5, 13). Michalides et al. (21) demonstrated that *Mtv-1* is equivalent to the endogenous MMTV unit V.

The C3Hf/He mouse tissue in which unit V is activated to produce MMTV virions is unknown. Etkind and Sarkar (11) examined the methylated status of MMTV proviruses in DNA from C3Hf/He mouse tissues using the restriction enzymes *HpaII* and *MspI*. These studies revealed hypomethylated MMTV sequences in spleen and mammary tumor DNA but not in liver or mammary gland DNA. These results raised the possibility that the MMTV virions detected in the milk of C3Hf/He animals are of lymphoid origin (11).

We examined the organization and expression of endogenous MMTV sequences in both normal and neoplastic C3Hf/HeSed mouse tissues. Three full-length (units Ib, II, and V)

and two subgenomic (units I and IX) endogenous MMTV proviruses were detected in C3Hf/HeSed mouse DNA. MMTV unit Ib contained hypomethylated sites in spleen, mammary gland, and mammary tumor DNA but not in liver DNA. The endogenous MMTV units II and V contained hypomethylated sites in mammary gland and mammary tumor DNA but were extensively methylated in spleen and liver DNA. Mammary glands and mammary tumors contained those MMTV RNA transcripts necessary for the production of MMTV virions, whereas spleens and livers did not. Therefore, the MMTV virions that are detected in C3Hf/He mouse milk probably arise through an activation of the endogenous MMTV unit V in C3Hf/He mouse mammary glands.

MATERIALS AND METHODS

Animals. C3Hf/HeSed mice, a subline of C3Hf/He mice, were obtained from Robert S. Sedlacek of the Department of Radiation Medicine, Massachusetts General Hospital, Boston.

General methods. DNA and RNA isolation, agarose gel electrophoresis, nucleic acid transfer to nitrocellulose, and filter hybridization were performed as previously described (24).

MMTV molecular clones. John Majors kindly provided, cloned into pBR322, *PstI* restriction fragments of the C3H MMTV provirus, including the 1.4-kilobase-pair (kbp) long terminal repeat (LTR) region (clone 8-29), the 3.9-kbp *gag-pol* region (clone 2.5), and the 1.8-kbp *env* region (clone 8-21) (19). A 1.7-kbp *EcoRI* fragment (including 1 kbp of MMTV sequences) was removed from the *gag-pol* region clone, following the protocol of Maniatis et al. (20). The resulting plasmid (*gag*) contained 2.9 kbp of MMTV sequences that

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hybridized solely to 5' MMTV sequences upon *Eco*RI, *Hind*III, and *Bam*HI digestion (see Fig. 2).

Plasmid DNAs were radiolabeled by incubating 0.5 μ g of DNA in 50 mM Tris (pH 7.5)–10 mM $MgSO_4$ –50 μ g of bovine serum albumin per ml–100 μ M dATP–100 μ M, dGTP–100 μ M dTTP–2 μ M [α - ^{32} P]dCTP with 5 enzyme units of DNA polymerase I (Boehringer Mannheim) and 0.125 ng of DNase I in 50 μ l of total volume. After incubation at 16°C for 3 h, reactions were stopped by the addition of an equal volume of 20 mM Na_2EDTA –0.2% sodium dodecyl sulfate–2 μ g of sheared salmon sperm DNA per μ l. Radiolabeled DNA, 4×10^8 to 6×10^8 cpm/ μ g, was separated from unincorporated [α - ^{32} P]dCTP by gel filtration through Sephadex G-50 and concentrated by ethanol precipitation. Radiolabeled DNA was suspended in water and heated at 100°C for 5 min immediately before addition to hybridization solution. Hybridization solutions contained 2.5×10^6 cpm of each probe per ml.

RESULTS

Organization of MMTV endogenous units. The endogenous MMTV proviruses of the C3Hf/He strain have been characterized previously with the restriction enzyme *Eco*RI (6, 11, 13, 22). To analyze these endogenous MMTV sequences in more detail, we digested C3Hf/HeSed mouse DNA with *Eco*RI, *Bam*HI, *Hind*III, and combinations of the three enzymes in double digestions. After electrophoresis through agarose gels and transfer to nitrocellulose paper, the digested DNAs were hybridized separately to nick-translated cloned

MMTV probes specific for the LTR and 5' (*gag*) and 3' (*env*) regions of the MMTV genome (Fig. 1).

*Eco*RI, *Bam*HI, and *Hind*III restriction maps of the endogenous MMTV units Ib, II, and V are shown in Fig. 2. The 15.0- and 5.7-kbp MMTV-containing *Eco*RI fragments were previously described as separate, subgenomic MMTV proviruses designated MMTV units I and VII, respectively (27, 28). Data presented below, however, provide evidence that the 15.0-kbp *Eco*RI fragment that contains 5' MMTV sequences and the 5.7-kbp *Eco*RI fragment that contains 3' MMTV sequences are derived from a single full-length MMTV provirus we designate unit Ib. The endogenous MMTV units II and V are also full-length MMTV proviruses (5, 21).

The endogenous MMTV unit I is a subgenomic MMTV provirus that hybridizes with the LTR probe and weakly with the *env* probe (24). Unit I was contained within a 15.0-kbp *Eco*RI fragment, a 12.0-kbp *Bam*HI fragment, a 10.0-kbp *Eco*RI-*Bam*HI fragment, and a 7.0-kbp *Hind*III fragment that was resistant to *Eco*RI and *Bam*HI digestion (Fig. 1). MMTV unit IX, also a subgenomic MMTV provirus (27, 28), hybridized solely to the LTR probe (Fig. 1). Unit IX was contained within a 1.7-kbp *Eco*RI fragment that was resistant to *Bam*HI and *Hind*III digestion, a 7.5-kbp *Bam*HI fragment, a 6.5-kbp *Hind*III fragment, and a 4.5-kbp *Bam*HI-*Hind*III fragment (Fig. 1).

Methylated status of endogenous MMTV sequences. The endogenous MMTV unit V has been demonstrated to be the source of the MMTV virions detected in C3Hf/He mouse

FIG. 1. Characterization of MMTV proviruses in C3Hf/HeSed mouse liver DNA. Samples (10 μ g) of C3Hf/HeSed mouse liver DNA were digested with *Bam*HI (lanes 1, 7, and 13), *Bam*HI and *Eco*RI (lanes 2, 8, and 14), *Eco*RI (lanes 3, 9, and 15), *Eco*RI and *Hind*III (lanes 4, 10, and 16), *Hind*III (lanes 5, 11, and 17), or *Hind*III and *Bam*HI (lanes 6, 12, and 18). After electrophoresis through 0.7% agarose gels and transfer to nitrocellulose sheets, the digested samples were hybridized with ^{32} P-labeled pBR322-MMTV probes representing the LTR region (lanes 1 through 6), the *gag* region (lanes 7 through 12), or the *env* region (lanes 13 through 18) of the MMTV genome. The nitrocellulose sheets were autoradiographed on Kodak XAR film with a Dupont Cronex Lightning-Plus intensifying screen for 21 days (lanes 1 through 6) or 7 days (lanes 7 through 18). The numbers in the left-hand margin refer (in kbp) to the fragment size of *Hind*III-digested λ DNA included as molecular weight markers.

FIG. 2. Restriction map of the full-length endogenous MMTV proviruses of C3Hf/HeSed mice. *Bam*HI (B), *Hind*III (H), and *Eco*RI (E) restriction endonuclease sites are shown for the endogenous MMTV units Ib, II, and V and their flanking cellular DNA. Thin lines represent cellular DNA, thick lines represent MMTV DNA, and open boxes represent proviral LTRs. Additionally, the cloned restriction fragments used as radiolabeled hybridization probes are shown with respect to their position within the MMTV genome.

milk (21). To determine the tissue in which MMTV unit V is activated to produce MMTV virions, we attempted to ascertain which tissue contains unit V in a transcriptionally active state. We chose to examine the methylated state of the endogenous MMTV proviruses because there appears to be

an inverse relationship between the level of transcription of a gene and the degree of methylation of cytosine in the sequence CpG within that gene (7, 26). The methylated state of MMTV sequences in DNA can be determined by digesting the DNA with the methyl-sensitive restriction enzymes

FIG. 3. Methylation analysis of C3Hf/HeSed mouse DNA. Samples (10 μ g) of DNA from C3Hf/HeSed mouse liver (lanes 1 through 6), spleen (lanes 7 through 12), and mammary gland (lanes 13 through 18) were digested with *Hha*I (lanes 1, 7, and 13), *Hha*I and *Eco*RI (lanes 2, 8, and 14), *Eco*RI (lanes 3, 9, and 15), *Eco*RI and *Hpa*II (lanes 4, 10, and 16), *Hpa*II (lanes 5, 11, and 17), or *Msp*I (lanes 6, 12, and 18). The digested samples were electrophoresed through 0.7% agarose gels, transferred to nitrocellulose, and hybridized with the ^{32} P-labeled MMTV *gag* probe. Autoradiography was for 7 days. Molecular weight markers are as described in the legend to Fig. 1.

FIG. 4. Methylation analysis of C3Hf/HeSed mouse DNA. Samples (10 μ g) of DNA from C3Hf/HeSed mouse liver (lanes 1 through 6), spleen (lanes 7 through 12), and mammary gland (lanes 13 through 18) were digested with *HhaI* (lanes 1, 7, and 13), *HhaI* and *EcoRI* (lanes 2, 8, and 14), *EcoRI* (lanes 3, 9, and 15), *EcoRI* and *HpaII* (lanes 4, 10, and 16), *HpaII* (lanes 5, 11, and 17), or *MspI* (lanes 6, 12, and 18). The digested samples were electrophoresed through 0.7% agarose gels, transferred to nitrocellulose, and hybridized with the ^{32} P-labeled MMTV *env* probe. Autoradiography was for 21 days. Molecular weight markers are as described in the legend to Fig. 1.

HhaI or *HpaII* and hybridizing the digested DNA with MMTV-specific probes (4, 10–12, 15). When *HhaI* or *HpaII* are used alone, however, it is difficult if not impossible to determine the methylated state of each MMTV provirus. In this study, DNA was digested with *EcoRI* or *BamHI* in combination with methyl-sensitive enzymes so that the sensitivity of each endogenous MMTV provirus to *HhaI* and *HpaII* could be determined.

The methylated state of the region containing the transcription-regulatory sequences of a gene provides the best indication of the transcriptional activity of that gene (3, 26). The transcription-regulatory region of MMTV is contained within the 5' LTR of the MMTV provirus (17). Therefore, the methylated state of the *EcoRI* and *BamHI* fragments containing the 5' portion of the endogenous MMTV units was first examined by hybridizing the digests with the 5'-specific *gag* probe. Fragments of 15.0, 8.3, and 6.1 kbp, representative of the 5' portion of the endogenous MMTV units Ib, II, and V, respectively, were detected in *EcoRI*-digested C3Hf/HeSed mouse DNA that was hybridized with the *gag* probe (Fig. 3, lanes 3, 9, and 15). In liver DNA, all *EcoRI* fragments containing 5' MMTV sequences were resistant to *HhaI* (lane 2) and *HpaII* (lane 4). *HhaI* (lane 1) and *HpaII* (lane 5) digestion of liver DNA produced MMTV-containing fragments of greater than 23 kbp. Liver DNA digested with *MspI* (lane 6), a methyl-insensitive isoschizomer of *HpaII*, produced distinct fragments of 2.0 and 1.7 kbp. Therefore, *MspI* recognition sites were present within the MMTV proviruses but were not digested by *HpaII*, the methyl-sensitive isoschizomer of *MspI*, because the recognition sites were methylated.

Similar methylation studies with spleen DNA from a 7-month-old animal in its third lactation are shown in Fig. 3

(lanes 7 through 12). The 15.0-kbp *EcoRI* fragment containing the 5' portion of unit Ib was completely sensitive to *HhaI* and *HpaII* digestion. The 8.3-kbp *EcoRI* fragment containing the 5' portion of unit II was slightly sensitive to *HhaI* digestion and resistant to *HpaII* digestion. The 6.1-kbp *EcoRI* fragment containing the 5' portion of unit V was resistant to *HpaII* digestion. It was difficult to determine the sensitivity of the 5' portion of unit V to *HhaI* digestion because the 5.8-kbp MMTV-containing fragment in the *EcoRI*-*HhaI* digests overlaps the 6.1-kbp *EcoRI* fragment of unit V. *HhaI* digestion produced an MMTV-containing fragment of 8.5 kbp. A broad MMTV-containing band of ca. 12 kbp was detected in *HpaII*-digested spleen DNA. The sensitivity of the endogenous MMTV proviruses to the methyl-sensitive restriction enzymes was the same in DNA from the spleens of younger animals (data not shown).

The results of methylation studies with DNA from the mammary gland of a 17-week-old animal in its first lactation are shown in Fig. 3 (lanes 13 through 18). The 15.0-kbp *EcoRI* fragment containing the 5' portion of unit Ib was sensitive to digestion by *HhaI* and *HpaII*. The 8.3-kbp *EcoRI* fragment containing the 5' portion of unit II was moderately sensitive to *HhaI* digestion and slightly sensitive to *HpaII* digestion. The 6.1-kbp *EcoRI* fragment containing the 5' portion of unit V was slightly sensitive to *HpaII* digestion, but the sensitivity of this fragment to *HhaI* was difficult to determine because of the 5.8-kbp *HhaI*-*EcoRI* fragment. An 8.5-kbp *HhaI* fragment and a 12.5-kbp *HpaII* fragment were detected in mammary gland DNA. The endogenous MMTV proviruses demonstrated the same sensitivity to the methyl-sensitive enzymes in DNA from mammary glands of older, multiparous animals (data not shown).

The nitrocellulose filters shown in Fig. 3 were treated to

FIG. 5. *HhaI* sensitivity of MMTV sequences in C3Hf/HeSed mouse DNA. Samples (10 μ g) of DNA from C3Hf/HeSed mouse liver (lanes 1 and 2), spleen (lanes 3 and 4), and mammary gland (lanes 5 and 6) were digested with *Bam*HI (lanes 1, 3, and 5) or *Bam*HI and *HhaI* (lanes 2, 4, and 6). The digested samples were electrophoresed through 0.7% agarose gels, transferred to nitrocellulose, and hybridized with the ^{32} P-labeled MMTV *gag* probe. Autoradiography was for 4 days. Molecular weight markers are as described in the legend to Fig. 1.

remove the *gag* probe (24) and rehybridized with the *env* probe (Fig. 4). Fragments of 6.5, 5.7, and 4.5 kbp, representative of the 3' portions of the endogenous MMTV units II, Ib, and V, respectively, were detected with the *env* probe in *Eco*RI-digested C3Hf/HeSed mouse DNA (Fig. 4, lanes 3, 9, and 15). Additionally, in *Eco*RI-digested DNA a faint band of 15 kbp containing the subgenomic MMTV unit I was detected with the *env* probe as previously reported (24). In liver DNA, the *Eco*RI fragments containing the 3' portion of each full-length endogenous MMTV provirus were resistant to *HhaI* and *Hpa*II digestion (Fig. 4, lanes 2 through 4). In spleen DNA, the 5.7-kbp MMTV-containing *Eco*RI fragment was completely sensitive to *HhaI* and *Hpa*II digestion. The 6.5-kbp *Eco*RI fragment containing the 3' portion of unit II was slightly sensitive to *HhaI* digestion and resistant to *Hpa*II digestion. The 4.5-kbp *Eco*RI fragment containing the 3' portion of unit V was resistant to digestion by both methyl-sensitive enzymes (Fig. 4, lanes 8 through 10). In mammary gland DNA, the *Eco*RI fragments containing the 3' portions of each full-length endogenous MMTV provirus were at least partially sensitive to the methyl-sensitive enzymes (Fig. 4, lanes 14 through 16). Therefore, the *Eco*RI fragments containing the 3' portions of the full-length MMTV proviruses showed the same sensitivity to the methyl-sensitive enzymes as did the *Eco*RI fragments containing the 5' portions of each MMTV provirus in C3Hf/HeSed mouse liver, spleen, and mammary gland DNA.

Double digestions with *Bam*HI and *HhaI* were performed to determine the sensitivity of the 5' portion of unit V to *HhaI* digestion (Fig. 5). The 5'-specific *gag* probe detected three fragments in *Bam*HI-digested C3Hf/HeSed mouse DNA: a 23.0-kbp fragment that contains the 5' portion of unit Ib; a 12.0-kbp fragment that contains the 5' portion of unit II; and a 8.5-kbp fragment that contains the 5' portion of unit V. The 8.5-kbp *Bam*HI fragment containing the 5'

portion of unit V was hypomethylated in mammary gland DNA but was extensively methylated in both liver and spleen DNA. The *Bam*HI fragments containing the 5' portions of units Ib and II demonstrated the same sensitivity to *HhaI* digestion as the *Eco*RI fragments containing the 5' portions of these MMTV proviruses in liver, spleen, and mammary gland DNA.

An 8.5-kbp *HhaI* fragment was detected in spleen and mammary gland DNA that hybridized with the *gag* (Fig. 3, lanes 7 and 13) and *env* (Fig. 4, lanes 7 and 13) probes. Additionally, *HhaI-Eco*RI fragments of 5.8 and 2.7 kbp were observed that hybridized with the *gag* (Fig. 3, lanes 8 and 14) and *env* (Fig. 4, lanes 8 and 14) probes, respectively. These data map the *HhaI*-sensitive sites to the 5' region of the 5' and the 3' LTRs in each endogenous MMTV provirus.

The methylated state of MMTV sequences in DNA from a spontaneous C3Hf/HeSed mouse mammary tumor was examined to determine if the endogenous MMTV proviruses demonstrated similar sensitivity to *HhaI* and *Hpa*II digestion in mammary tumor DNA as in mammary gland DNA (Fig. 6). *Eco*RI, *Hind*III, and *Bam*HI digestions of this mammary tumor DNA, hybridized with the LTR, *gag*, and *env* probes, revealed MMTV sequences in addition to those of the normal endogenous MMTV units (data not shown). The germinally transmitted MMTV sequences had approximately the same sensitivity to the methyl-sensitive enzymes in mammary tumor DNA (Fig. 6) as in mammary gland DNA (Fig. 3). However, intense low-molecular-weight MMTV-containing fragments appeared in both *HhaI*- and *Hpa*II-

FIG. 6. Methylation analysis of C3Hf/HeSed mouse mammary tumor DNA. Samples (10 μ g) of DNA from a spontaneous C3Hf/HeSed mouse mammary tumor were digested with *HhaI* (lane 1), *HhaI* and *Bam*HI (lane 2), *Bam*HI (lane 3), *Bam*HI and *Hpa*II (lane 4), *Hpa*II (lane 5), or *Msp*I (lane 6). The digested samples were electrophoresed through 0.7% agarose gels, transferred to nitrocellulose, and hybridized with the ^{32} P-labeled MMTV *gag* probe. Autoradiography was for 4 days. Molecular weight markers are as described in the legend to Fig. 1.

TABLE 1. *HhaI* and *HpaII* sensitivity of the full-length MMTV proviruses endogenous to C3Hf/HeSed mice^a

MMTV provirus	Sensitivity of provirus from:							
	Liver		Spleen		Mammary gland		Mammary tumor	
	<i>HhaI</i>	<i>HpaII</i>	<i>HhaI</i>	<i>HpaII</i>	<i>HhaI</i>	<i>HpaII</i>	<i>HhaI</i>	<i>HpaII</i>
Unit Ib	0	0	++	++	++	+	++	++
Unit II	0	0	+	0	++	+	++	+
Unit V	0	0	0	0	++	+	++	+

^a The sensitivity of MMTV-containing *EcoRI* and *BamHI* restriction fragments to *HhaI* and *HpaII* was determined by scanning the autoradiograms shown in Fig. 3, 4, 5, and 6 with an LKB densitometer. The sensitivity, which indicates the reduction in intensity of the MMTV-containing *EcoRI* or *BamHI* restriction fragments (or both) in double digestions with the methyl-sensitive restriction enzymes, is expressed as 0 to 33% (0), 34 to 66% (+), or 67 to 100% (++) .

digested mammary tumor DNA that were not detected in similarly digested DNA of other tissues. These fragments were most likely derived from internal *HhaI*-sensitive sites within the nongerminally acquired MMTV provirus(es). Cohen (4) and others (10–12) detected similar fragments in the DNA of mammary tumors induced by the milk-transmitted MMTV. Therefore, somatically acquired MMTV proviruses appeared to be extensively hypomethylated throughout their genome; whereas germinally transmitted MMTV proviruses had selective demethylation within the LTR regions.

The results of these methylation experiments are summarized in Table 1. The endogenous MMTV unit Ib contained hypomethylated sites in spleen, mammary gland, and mammary tumor DNA but not in liver DNA. The endogenous MMTV unit II was completely methylated in liver DNA, extensively methylated in spleen DNA, and moderately hypomethylated in mammary gland and mammary tumor DNA. Finally, the endogenous MMTV unit V, which has been implicated in the production of MMTV virions in C3Hf/He mice, contained *HhaI*- and *HpaII*-sensitive sites in mammary gland and mammary tumor DNA but was extensively methylated in liver and spleen DNA.

MMTV RNA Expression. Transcription of the MMTV provirus potentially produces three MMTV RNA species: a 9.0-kb MMTV RNA transcript that, in addition to serving as genome for progeny virions, encodes the *gag* and *gag-pol* polypeptide precursors; a 3.8-kb MMTV RNA species that is a spliced transcript that encodes the *env* polypeptides precursors; and a 1.7-kb MMTV RNA transcript that contains predominantly sequences derived from the LTR region of the MMTV (30, 31). Although a long open reading frame is present within the LTR region of the MMTV (8, 9, 16), no protein product of the 1.7-kb MMTV RNA transcript has been identified.

Northern blots were performed to determine if the hypomethylated sites detected in the endogenous MMTV proviruses reflected transcriptional activity (Fig. 7). Polyadenylated [poly(A)⁺] RNA from the MMTV-producing mammary tumor cell line 34I-101 (23) contains the 9.0- and 3.8-kb MMTV transcripts (24) (Fig. 7, lane 1). BALB/c mouse mammary glands, which do not produce MMTV virions (29), contain only the 1.7-kb MMTV RNA species (30, 31) (Fig. 7, lane 2). RNA from C3Hf/HeSed mouse livers contained no detectable MMTV transcripts (data not shown). After long autoradiographic exposure, a faint band at 3.8 kb appeared in poly(A)⁺ RNA from spleens (Fig. 7, lane 3). Mammary gland poly(A)⁺ RNA contained all three MMTV transcripts (Fig. 7, lane 4). High levels of the 9.0- and 3.8-kb MMTV

transcripts were detected in mammary tumor poly(A)⁺ RNA (Fig. 7, lanes 5 and 6). Therefore, C3Hf/HeSed mouse mammary glands and mammary tumors contained the MMTV RNA transcripts necessary for the production of MMTV virions, whereas spleens did not contain the genomic MMTV RNA transcript, and livers lacked detectable MMTV RNA.

DISCUSSION

By separately hybridizing *EcoRI*-, *HindIII*-, and *BamHI*-digested C3Hf/HeSed mouse DNA with cloned probes specific for the LTR and the 5' and 3' regions of the MMTV genome, we have more clearly characterized the endogenous MMTV proviruses of this strain. The full-length MMTV units Ib, II, and V, in addition to the subgenomic MMTV units I and IX, are transmitted through C3Hf/HeSed mouse germ line DNA.

EcoRI digestion of C3Hf/HeSed mouse DNA produced, in addition to the MMTV-containing fragments of units I, II, V, and IX, a 15.0-kbp fragment containing 5' MMTV sequences and a 5.7-kbp fragment containing 3' MMTV sequences. Previously, the 15.0- and 5.7-kbp MMTV-containing *EcoRI* fragments have been described as separate subgenomic MMTV proviruses designated units I and VII, respectively (27, 28). Several lines of evidence indicate, however, that these fragments are derived from a single full-length MMTV provirus (unit Ib). First, *HhaI* digestion of spleen DNA produced a fragment of 8.5 kbp that hybridized with both the *gag* and *env* probes. This fragment was cleaved by *EcoRI* to produce a 5.8-kbp fragment that hybridized to the *gag* probe and a 2.7-kbp fragment that hybridized to the *env* probe.

FIG. 7. Molecular sizing of C3Hf/HeSed mouse tissue poly(A)⁺ RNA. Poly(A)⁺ RNA was isolated from cell lines or tissues, fractionated by size in denaturing 1.1 M formaldehyde-agarose (1.2%) gels, and transferred to nitrocellulose filters. Lanes 1 and 2 contained standards for MMTV RNA transcripts. Lane 1 contained 0.5 µg of poly(A)⁺ RNA from the C3H mouse mammary tumor cell line 34I-101. Lane 2 contained 10 µg of poly(A)⁺ RNA from lactating BALB/c mouse mammary glands. Lanes 3 and 4 contained ca. 4 µg of poly(A)⁺ RNA from C3Hf/HeSed mouse spleens and lactating mammary glands, respectively. Lanes 5 and 6 contained 1.0 µg of mammary tumor poly(A)⁺ RNA from two C3Hf/HeSed retired breeder mice. Each hybridization was with a mixture of the ³²P-labeled MMTV LTR, *gag*, and *env* probes. Autoradiography was for 40 h (lanes 1 and 2), 240 h (lanes 3 and 4), and 24 h (lanes 5 and 6). The numbers in the left-hand margin refer to the size, in kb, of the MMTV transcripts.

Therefore, the 8.5 kbp *HhaI* fragment is produced by digestion in the 5' and 3' LTRs of a full-length MMTV provirus. In spleen DNA, the *EcoRI* fragments containing the MMTV units II and V were not extensively digested by *HhaI*. Consequently, the 8.5-kbp MMTV-containing fragment detected in *HhaI*-digested spleen DNA cannot be derived exclusively from units II and V. The 15.0- and 5.7-kbp MMTV-containing *EcoRI* fragments were completely sensitive to *HhaI* digestion in spleen DNA. Therefore, the 15.0-kbp *EcoRI* fragment that contains 5' MMTV sequences and the 5.7-kbp *EcoRI* fragment that contains 3' MMTV sequences are derived from a single full-length MMTV provirus (unit Ib) that is sensitive to *HhaI* digestion in spleen DNA.

The MMTV sequences that are contained in the 15.0- and 5.7-kbp *EcoRI* fragments of unit Ib are detected in *BamHI* fragments of 23, 4.4, and 1.3 kbp (Fig. 1 and 2). MacInnes et al. (18) have reported similar-sized MMTV-containing *EcoRI* and *BamHI* fragments in DNA of DBA mice. The 16.7- and 5.8-kbp MMTV-containing *EcoRI* fragments cosegregate in crosses between the DBA strain and mice that lack these fragments (18). The MMTV provirus contained in the 16.7- and 5.8-kbp *EcoRI* fragments in DNA of DBA mice (18), a parent of the C3H strain, is probably equivalent to unit Ib (Fig. 2) of C3Hf/HeSed mice.

The endogenous MMTV unit V (*Mtv-1*) is activated in C3Hf/He mice to produce MMTV virions that are detected in the milk from normal mammary glands and in mammary tumors (21, 29). Additionally, the appearance of these MMTV virions in the milk of C3Hf/He mothers is associated with the moderate mammary tumor incidence observed in these mice (29). Nevertheless, the C3Hf/He tissue in which MMTV unit V is activated to produce MMTV virions is unknown. Etkind and Sarkar (11) examined the methylated status of the endogenous MMTV proviruses in the DNA of C3Hf/He mice by digesting C3Hf/He mouse DNA with *HpaII* and *MspI*. They did not detect hypomethylated MMTV sequences in liver or mammary gland DNA, but hypomethylated MMTV sequences were detected in spleen DNA. Therefore, Etkind and Sarkar (11) postulated that the MMTV virions in C3Hf/He mouse milk could be of lymphoid origin.

We examined the methylated status of each full-length endogenous MMTV provirus by digesting C3Hf/HeSed mouse DNA with *EcoRI* or *BamHI* in combination with *HhaI* or *HpaII*. These data demonstrate hypomethylated sites within the LTR regions of the endogenous MMTV unit V (*Mtv-1*) in mammary gland and mammary tumor DNA that are extensively methylated in liver and spleen DNA. These studies also suggest that the hypomethylated MMTV sequences detected by Etkind and Sarkar (11) in spleen DNA are derived from the endogenous MMTV unit Ib (Table 1).

We also examined RNA from C3Hf/HeSed mouse tissues for MMTV transcripts. Mammary gland and mammary tumor RNA contained the MMTV transcripts necessary for the production of MMTV virions. Spleens contained very low levels of the spliced envelope MMTV RNA transcript, and livers lacked detectable MMTV RNA. The hypomethylation of MMTV unit V in mammary glands (Table 1), the accumulation of the MMTV *gag-pol* and *env* RNA transcripts in mammary glands (Fig. 7), and the evidence that MMTV unit V is responsible for the virions detected in mammary glands (21) are all consistent with the hypothesis that unit V is activated in C3Hf/He mouse mammary glands.

Two C3Hf/HeSed mouse mammary tumors contained the genomic-size MMTV RNA transcript and the spliced enve-

lope MMTV RNA species at ca. 20- to 30-fold-higher levels than observed in C3Hf/HeSed mouse mammary glands. Nevertheless, the germinally transmitted MMTV proviruses displayed approximately the same sensitivity to the methyl-sensitive enzymes in mammary tumor DNA as in mammary gland DNA. The somatically acquired MMTV sequences detected in the mammary tumor DNA were extensively hypomethylated. These data suggest that the high levels of MMTV RNA observed in mammary tumors may be due to transcription of the somatically acquired MMTV provirus(es). The endogenous MMTV unit V may at first serve as the site for the majority of MMTV transcription; however, as MMTV virions reintegrate hypomethylated MMTV proviruses into the DNA of mammary tissue, the majority of MMTV transcription may be from the newly integrated MMTV proviruses.

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